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Research Article

Ascaris nematodes from pig and human make three antibacterial peptides: isolation of cecropin P1 and two ASABF peptides

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Abstract. Organisms co-habiting with bacteria have developed efficient bactericidal agents to control their microbe-rich environment. The *Ascaris* nematode lives in its final development stages in the gut of its host and is believed to feed on bacteria. *Ascaris suum* survive in pig intestine while *A. lumbricoides* is the principal species in humans. Here we show that *A. suum* and *A. lumbricoides* both produce linear (cecropin P1) and cysteine-rich (ASABF) peptides with activity against either Gram-negative or Gram-positive bacteria, respectively. Thus nematodes rely in part on a peptide-based antibacterial system for digestion of bacteria, which may also confer protection against infection. Cecropin P1 was previously isolated from pig intestine but we can now conclude that was due to contaminating nematodes.

Key words. Antibacterial peptide; nematode; *Ascaris*; cecropin P1; mass spectrometry; HPLC.

Cecropins were the first antibacterial peptides to be isolated from the animal world and their activity was clearly linked to the immunity of the *Cecropia* pupae from which they were isolated [1]. The structures of the cDNA and the gene for a cecropin from the same insect were described 4 and 6 years later, respectively [2, 3]. However, parallel work in *Drosophila* showed greater progress, and the analysis of the cecropin genes in the fly [4] was finished a year before that with the cecropin locus in the moth [5].

In 1989, we isolated cecropin P1 from pig small intestine [6]. The size of this peptide was somewhat smaller than the insect cecropins (31 residues compared to 35–39 for peptides from moths and flies). At that time, isolating a cDNA clone for cecropin P1 was the obvious next step. However, despite intense efforts by different scientists, we failed to obtain any clone related to the peptide. The small size and the abundance of amino acids with four or six codons were considered a possible explanation for the failure.

While searching the human genome (GenBank), Dan Hultmark found an EST clone from *Ascaris suum* (accession number AW165880), which showed one reading frame with a sequence almost identical to cecropin P1. Recalling that *Drosophila* contains two pseudogenes for cecropins [4], we decided to investigate whether the nematode, rather than the pig, produces cecropin P1. This study has now been accomplished and in addition we show that *A. lumbricoides* produces the same cecropin. Applying the same extraction and HPLC methods, we were unable to demonstrate any cecropin in a 'nematode-free pig'. Kato et al. [7] have described *A. suum* antibacterial factor (ASABF), an antibacterial peptide isolated from *A. suum* with potent activity against *Stapyloccus aureus* but only low activity against *Escherichia coli* [7]. From *A. lumbricoides* we have now isolated two related

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peptides with activity against *S. aureus*, which have sequences in accordance with those reported for ASABF-β and ASABF-γ [8, 9].

Without cultural interference, most large animals likely live in co-existence with one or more nematodes. *Ascaris* nematodes, common in humans and pigs, were the subject of much research in the period 1960–1985. However, in retrospective, the enthusiasm for *Caenorhabditis elegans* research, a wave which kept growing during the 1970s, seems, as a side effect, to have terminated all work on *Ascaris*.

The fact that *Ascaris* nematodes produce potent antibacterial peptides make them interesting form both evolutionary and ecological perspectives. This would also apply to *Homo sapiens*, which could take advantage of the nematode peptides for protection against potentially harmful bacteria.

Materials and methods

Peptide extraction, HPLC purification and analysis of purified components

A. suum was obtained from Scan Foods AB in Uppsala, Sweden, a few hours after the death of the pigs. *A. lumbricoides* was obtained from the Karolinska Hospital, Stockholm, Sweden. They were spontaneously delivered from the rectum of children and diagnosed at the Parasitology Laboratory of the Karolinska Hospital.

The nematodes were cut into 1–2 cm pieces, snap-frozen in liquid nitrogen and ground to powder. The frozen tissue was extracted for 10 min (repeated vortexing for 2 min) with ice-cold 60% aqueous acetonitrile containing 1% trifluoroacetic acid (TFA) as described elsewhere [10] with minor alterations. The extracts were centrifuged at 17,000 g for 30 min. Supernatants were freeze-dried, redissolved in 10% acetonitrile with 0.1% TFA and cleared by centrifugation. Starting from 0.9 g tissue, the extract (1.8 ml) had a protein concentration of 6 and 9 mg/ml for *A. suum* and *A. lumbricoides*, respectively. A crude subfractionation was carried out using Sep-Pak tC18 Plus columns (Waters). The Sep-Pak fractions were characterized by reverse-phase (RP) HPLC. The amounts of material applied to HPLC corresponded to 0.3 g of tissue. Elution was performed with 0.18% TFA in water (solvent A) and a gradient of 0.15% TFA in acetonitrile (solvent B) on a 0.46 × 25 cm Vydac C18 column (Vydac, 218TP54: The Separation Group, Hesperia, Calif.) with a flow rate of 0.8 ml/min. Initial conditions were 10% B for 10 min, then gradients from 10 to 25% in 5 min, from 25 to 65% in 70 min and finally from 65 to 80% in 5 min. Fractions of 0.8 ml were collected and lyophilized. The molecular sieving column for HPLC was YMC-Pack (0.46 × 25 cm; YMC Europe, Schermbeck/Weselerwald, Germany) in equilibrium with 38% acetonitrile and 0.1% TFA. Chromatographic fractions were freeze-dried and redissolved in water.

Sequence analyses

N-terminal sequence analyses were performed by Edman degradation in Procise cLC or HT instruments (PE Applied Biosystems, Foster City, Calif.). Matrix–assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF) analysis was carried out using a Reflex III (Bruker Daltronics, Bremen, Germany). One microliter of each fraction was mixed with 1 µl matrix (α-cyano-4 hydroxycinnamic acid) solution in 60% acetonitrile/0.1% TFA. The spectra were acquired in the linear or the reflectron mode. Calibration was performed using external calibration.

Enzymatic digestion

Peptide samples were digested for 3 h at room temperature in 10 µl 50 mM ammonium bicarbonate with 1 µg 'V8 protease' (Glu-C: Boehringer Mannheim, Mannheim, Germany).

Bacterial strains and assays for antibacterial activity

Antibacterial activity was recorded with an inhibition zone assay. Briefly, thin plates were poured containing Luria-Bertani broth, 1% agarose and approximately 3×10^5 log-phase bacteria (*E. coli*, strain D21 or *S. aureus*, Cowan 1 from our own collection). Small wells were punched in the assay plates (3 mm in diameter) and loaded with 3 µl of sample. After overnight incubation at 30 °C, the diameter of inhibition zones was recorded. Cecropin units were read from a standard curve obtained with synthetic cecropin P1 and one unit corresponds to the activity of one nanogram cecropin P1. The unit used for activity against *S. aureus* was based on a standard curve made with purified ASABF.

Results

Purification of cecropin P1 from *A. suum* and *A. lumbricoides*

Based on the elution profile of synthetic cecropin P1 from our C18 column (between 39–40% acetonitrile), the crude nematode extract was eluted from the Sep-Pak columns into three fractions as shown in table 1. The antibacterial activities were estimated from standard curves with cecropin P1 on *E. coli* and ASABF-β and ASABF-γ on *S. aureus*. Both ASABF peptides from *A. suum* eluted at 25–30% acetonitrile and overloading the Sep-Pak may explain the 51% recovery in fraction 2 from *A. lumbricoides* material compared to 87% *A. suum*.

Starting from Sep-Pak fraction 2 from *A. suum*, two consecutive HPLC separations gave a fraction with anti-*E. coli* activity. Edman degradation of this material gave a

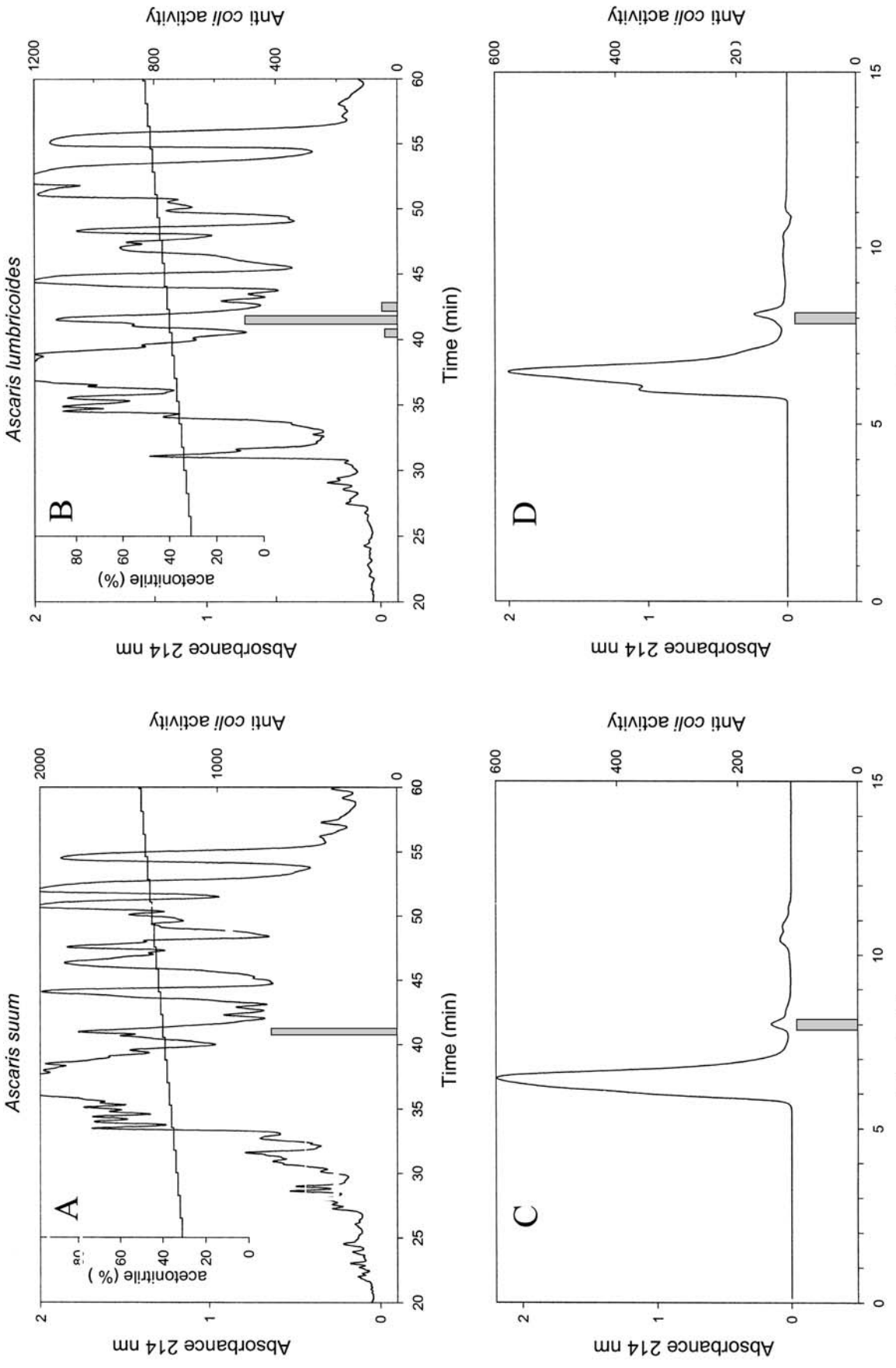

Figure 1. Steps 2 and 3 in the isolation of cecropin P1. (*A, B*) Step 2, RP HPLC fractionation of Sep-Pak fraction 2 from *A. suum* and *A lumbricoides*. Full line, UV absorption at 214 nm; hatched bars, antibacterial activity against *E. coli*. (*C, D*) Molecular-sieving HPLC of the fractions with antibacterial activity isolated after step 2. Full line, UV absorption at 214 nm; hatched bars, antibacterial activity against *E. coli*.

Table 1. Antibacterial activity in fractions eluted from Sep-Pak columns.

Fraction number	Elution in acetonitrile (%)	Anti-*E. coli*		Anti- *S. aureus*	
		A. suum	*A. lumbricoides*	*A. suum*	*A. lumbricoides*
1	10–25	15%	20%	12%	48%
2	25–52	78%	77%	87%	51%
3	52–66	< 7%	< 3%	< 1%	< 1%

sequence SWLxKxAKKLxN(S)A(K)xRIxE. This sequence was overshadowed by a primary sequence in part identical to ABA-1 allergen (MEHYLKTYLSWLT) elongated N-terminally by GESPQxH. The mass of the minor component was 3339.4 Da, while ABA-1 is a high-molecular-weight protein of 1095 amino acid residues [11]. The mass difference between cecropin P1 and ABA-1 provided the rationale for a more efficient purification procedure.

The final three-step purification for the isolation of cecropin P1 from *A. suum* and *A. lumbricoides* was the Sep-Pak fractionation followed by an RP and a size-exclusion-based HPLC. The two last steps are shown in figure 1. The minor symmetrical peak in the last chromatograms (fig. 1C, D) contained the anti-*E. coli* activity and showed a major component with a mass of 3339.6 Da. This is in reasonable agreement with 3339.9, the theoretical value for cecropin P1. The peptide from *A. lumbricoides* and standard synthetic cecropin P1 were cleaved with *S. aureus* V8 protease. The resulting mass spectrum (fig. 2) showed very similar profiles. The theoretical masses (monoisotopic) of the expected fragments are 1290.53, 1032.16 and 1052.24 Da. These masses were found in both protease-digested samples (fig. 2C, D) but the N-terminal fragment (1290.53) was frequently oxidized to give peaks of 1322.21 (synthetic peptide) and 1322.58 (*A. lumbricoides* peptide). Most likely, the tryptophan in position two is the target for the addition of two oxygens (+32 Da). The high-quality mass data also show that the cecropins from both nematodes have free carboxyl termini and thus differ from insect cecropins, which are amidated.

Identification of ASABF peptides from *A. lumbricoides*

Gross activity of crudely separated proteins (Sep-Pak fractions 1–3) showed in addition to anti-*E. coli* activity, high anti-*S. aureus* activity in the first Sep-Pak fraction. The components responsible for this activity were isolated through a series of HPLC chromatograph steps (fig. 3) and subjected to Edman degradation.

One sequence (AL 39:17 in fig. 4) was determined by 41 Edman steps and found to be derived from the N-terminal part of ASABF-β (fig. 4). ABSAF-β is a predicted 7-kDa antibacterial protein from *A. suum* [8]. Most of the sequence gaps are at places where ASABF-β has cysteines which are not expected to be recovered under the non-reducing conditions used. MALDI-TOF data showed the ASABF-like peptide (AL 39:17) to have a mass of 7055.61 Da. Assuming that ASABF peptides have four disulfides, the mass found is consistent with a C-terminally truncated ASABF-β, lacking the predicted last dipeptide GR (an amidation signal). An amidated ASABF peptide with the sequence found would have a theoretical mass of 7054.18 Da compared to 7055.18 Da for a peptide with a free COOH group. Since the latter is in better agreement with our observed mass, we conclude that the AL 39:17 peptide has a free C-terminal carboxyl group.

A second fraction (AL 38:15, fig. 3) was purified and subsequent Edman degradation showed an amino acid sequence in part identical to ASABF-γ [9]. The mass of this peptide was 6150.77 Da, suggesting that the peptide has four internal disulfide bonds and a truncated C terminus (fig. 4; theoretical mass 6150.01 Da).

Both ASABF peptides showed potent activity toward *S. aureus* (lethal concentration = 0.05 µM for both), but we could not detect any activity against *E. coli*.

Discussion

The origin of cecropin P1

About one hundred years ago, most humans probably carried the nematode *A. lumbricoides*. Today the estimate is that about one-quarter of the Earth's population still carry this worm. A closely related nematode, *A. suum*, has pig as its normal host. Medical and veterinary scientists have as a rule considered *Ascaris* as a parasite, but mild infestations do not give clinical symptoms in their hosts.

Ascaris nematodes are believed to live on bacteria (like *C. elegans*) but nothing is published about the ways bacteria are digested. Certain bacteria induce a strong antibacterial defense system [12]. Against this background, it is relevant that we now have purified and identified three peptide antibiotics produced by *A. lumbricoides*.

From the present work, both *A. suum* and *A. lumbricoides* clearly do produce cecropin P1 (fig. 1), purified from an extract by a three-step procedure. The identity of cecropin P1 was ascertained by partial sequencing, and by a mass analysis followed by digestion with a Glu-specific enzyme. Compared to *A. suum* worms, there was about twice as much cecropin P1 in the nematode from a hu-

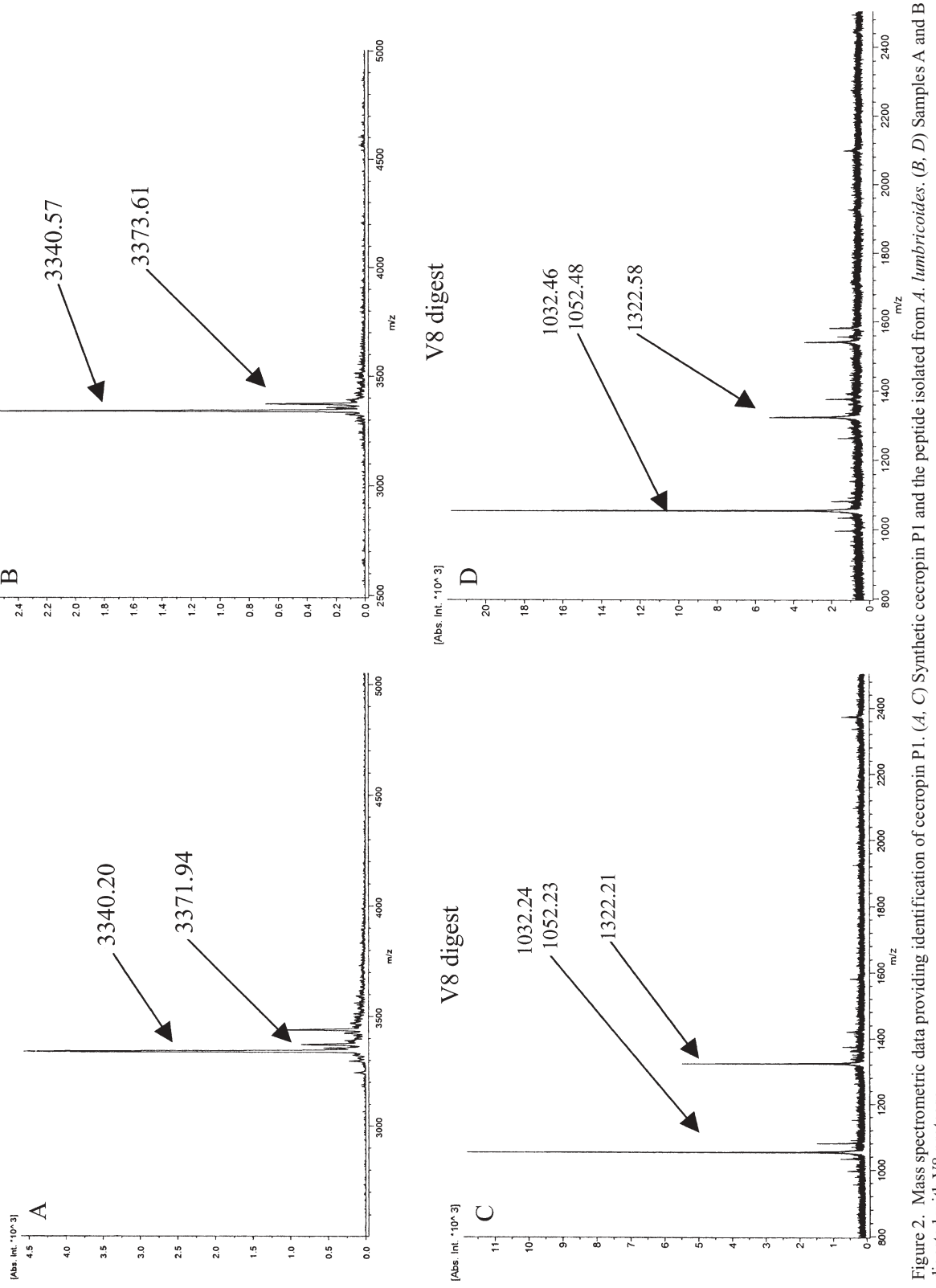

Figure 2. Mass spectrometric data providing identification of cecropin P1. (*A, C*) Synthetic cecropin P1 and the peptide isolated from *A. lumbricoides*. (*B, D*) Samples A and B digested with V8 protease.

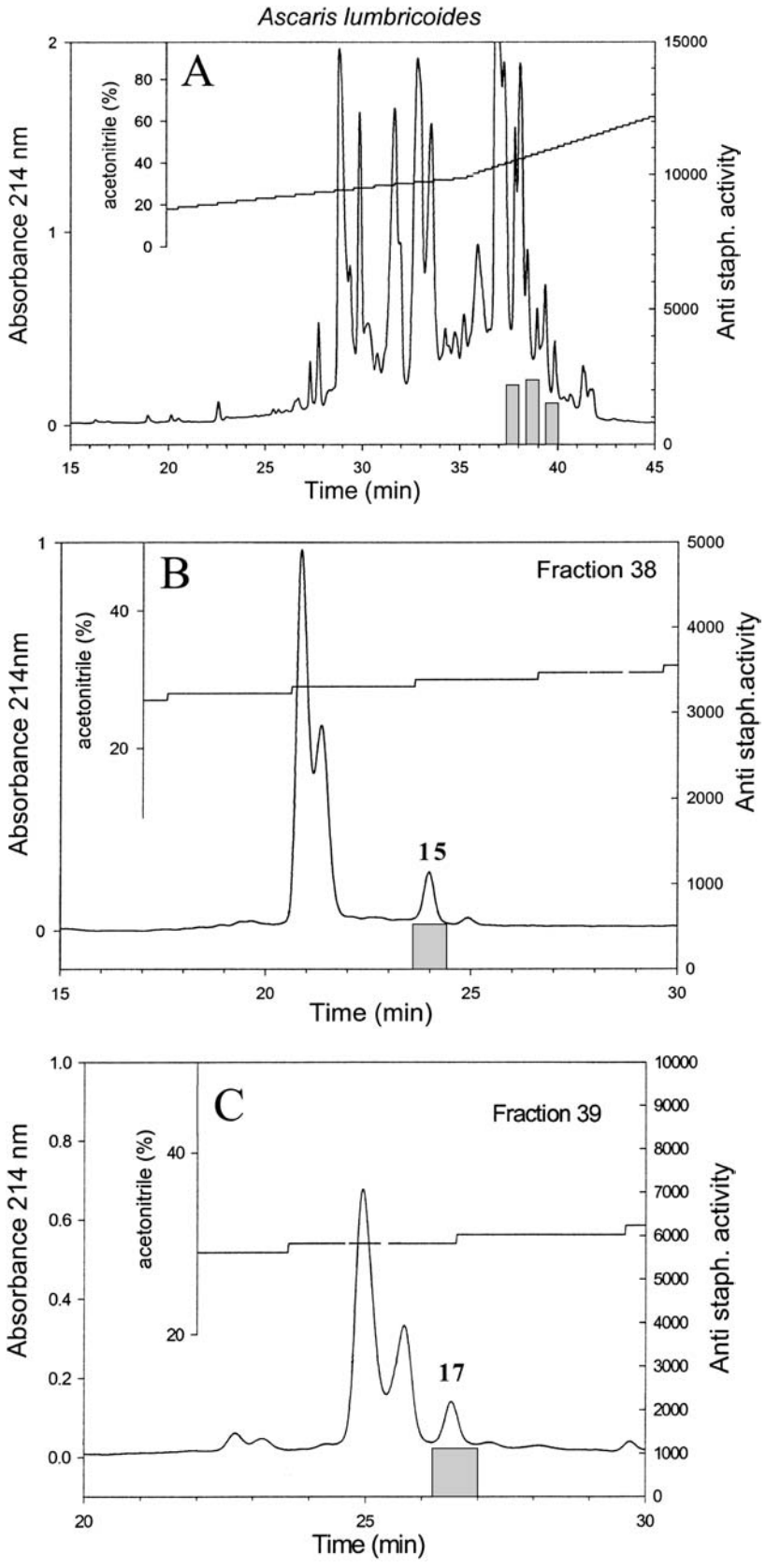

Figure 3. Steps 2 and 3 in the isolation of ASABF from *A. lumbricoides.* (*A*) Step 2, RP HPLC of Sep-Pak fraction 1 from *A. lumbricoides.* Full line, UV absorption at 214 nm; hatched bars, antibacterial activity against *S. aureus*. (*B, C*) Rechromatography of fractions 38 and 39 from *A* with a flat gradient.

A

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Cecropin P1              SWLSKTAKKL ENSAKKRISE GIAIAIQGGP R
AW165880    LIYLLVQTAES SWLSKTAKKL ENSAKKRISE GIAIAIKGGS RRRRSVGEED AIPSHIEVNK FFLRKPAKEH I
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B

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AL 39:17            IDFSXXARM DVPGLNKVXQ GLXISSXKYQ NXXTGHXEKR . . . . . .
ASABFβ             AIDFSSCARM DVPGLNKVAQ GLCISSCKYQ NCGTGHCEKR GGRPTCVCGR CGTGGGEWPS VPMPKGGKKg r
AL 38:15            IDFSTXARM DVPGLSKVAQ G. . . . . .
ASABFγ             AIDFSTCARM DVPGLSKVAQ GSCISSCKFQ NCGTGHCEKR GGRPTCVCSR CGSGGGSWPS Vgk
ASABFα             AVDFSSCARM DVPGLSKVAQ GLCISSCKFQ NCGTGHCEKR GGRPTCVCDR CGRGGGEWPS VPMPKGRSSR Grrhs
ASABFδ             GIDFSTCARM DVPGLSKVAR GLCINSCKFQ . . . . . .
ABF-1                 EASCARM DVPVMGRIAQ GLCTSSCTAQ KCMTGICKKV DSHPTCFCGG CSNANDVSLD TLISQLPHN
ABF-2              DIDFSTCARM DVPILKKAAQ GLCITSCSMQ NCGTGSCKKR SGRPTCVYRC ANGGGDIPLG ALIKRG
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Figure 4. Cecropin P1 and ASABF peptides isolated from *A. lumbricoides*. (*A*) Sequence of cecropin P1 and the deduced sequence of EST clone AW165880. (*B*) AL 39:17 and AL 38:15 denote partial sequences from isolated peptides. ASABF$\delta\gamma\beta$ are postulated mature peptide based on *A. suum* mRNA sequences in the NCBI database (note that ASABFδ might not be a complete sequence). ASABFα is identical to ASABF described by Kato el al [7]. ABF-1, -2 are sequences from *C. elegans* [21]. Bold and underlined letters show differences from ASABFβ. … indicate undetermined C-terminal residues. The lowercase letters in the N-terminal position of ASABF$\alpha\gamma\beta$ indicate N-terminal processing as determined by Kato and Komasuto [7] and by us.

man. This could be due to the fact that the human nematode left a healthy host during normal life conditions, while the porcine ones were received an hour or more after slaughtering of the host.

The present investigation was initiated by Dan Hulmark´s finding of the EST clone AW165880 from *A. suum* [13]. This clone was submitted by Mark Blaxter, Universiy of Edinburgh, to PubMed in 1999. The clone AW165880 contains information for cecropin P1 but with two amino acid replacements and neither start nor stop codon. The translated clone AW165880 shows two amino acid substitutions (fig. 4) that would have altered the mass by 10 Da. However, recent work by Mark Blaxter's group (http://www.nematodes.org/) has generated several cecropin P1 sequences from *A. suum*, *A. lumbricoides* and *Toxocara canis* suggesting that single amino acid exchanges can be a consequence of either sequencing error or sample origin. The deduced sequence for the cecropin precusor from *A. suum* shows a higher variability in the amino acids in the sequence preceding and following cecropin P1. Additional studies are needed to clarify the exact nature of the proform.

The material used for the original isolation of cecropin P1 was derived from the late Viktor Mutts fractionation schedule for hormones that started from one metric ton of frozen small pig intestine obtained from a slaughterhouse [6]. Here we could not obtained any cecropin P1 from the small intestine of a nematode-free pig (bred for studies of allergy). In retrospective, this would suggest that the slaughterhouse material used in 1988–1989 also included the nematode *A. suum*. Thus, the cecropin P1 isolated in 1988 was most likely not produced by the pig small intestine but originated from fifth-instar nematodes which had been living in the pig intestine. In agreement with this are the contemporary immunohistochemical results, which showed the presence of cecropin P1 in pig intestine in distinct locations [14].

Antibacterial peptides produced by *A. lumbricoides*

Six years ago, the antibacterial peptide ASABF was isolated from the body fluid of *A. suum* and the authors stressed that it was the sole antibacterial peptide of the organism [7]. After additional cloning work, this statement was modified to a family of ASABF-like molecules [8]. However, it was not clear if the corresponding peptides had been isolated.

Here we show that *A. lumbricoides* does in fact produce a minimum of three antibacterial peptides, namely cecropin P1 (fig. 4) and two related ASABF peptides, β and γ (fig. 4). The identity of the second and third peptides was ascertained by mass values in combination with two partial amino acid sequences. They are derived from sequences reported by Kato in PubMed for peptides as ASABF-β [8] and ASABF-γ [9]. For ASABF-β and ASABF-γ we obtained with MALDI-TOF a mass of 7055.61 and 6150.77 Da, respectively. These values are in close agreement with the theoretical values of 7055.18 and 6150.01, assuming in each peptide four disulfide bridges and C-terminal processings (removal of GR and GK, potential amidation signals).

The antibacterial activity of cecropin P1 is chiefly directed against Gram-negative bacteria [6], while the specificity of the ASABF peptides are chiefly directed against Gram-positive organisms [7]. Thus, the two peptides together provide *A. lumbricoides* with a broad-spectrum protection against the intestinal flora. For Kato's putative peptides ASABF-β and ASABF-γ, the same protection would exist for *A. suum*.

Are *A. suum* and *A. lumbricoides* one and the same species?

The debate whether porcine and human *Ascaris* are one or two species is a long-standing one [15] and a recent study using two-dimensional electrophoresis found only six proteins to differ [16]. Here, we have shown that both

nematodes produce cecropin P1 and from *A. lumbricoides* we have isolated the two peptides ASABF-β and ASABF-γ, previously postulated to be present in *A. suum* [8, 9]. We also found the RP HPLC profile to be almost identical (fig. 1 A, B), which means that the gross protein composition of the two nematodes must be quite similar. Assuming that the genomes are identical, one could still expect gene expression to be influenced by the host.

The pig was one of the earliest animals domesticated by our ancestors [17] and the normal route of infection is by soil (containing eggs or first- and second-instar larvae). One of our nematode donors was slightly underweight, but in Sweden, people with *A. lumbricoides* are generally free of symptoms. In fact, the nematode could be beneficial for its host because it offers an effective protection against a broad range of bacteria. A similar type of argument was recently used to suggest a beneficial role also for *Helicobacter pylori*, found to produce cecropin-like peptides [18]. Most certainly, both organisms have in the past been normal inhabitants of the gastrointestinal system of *H. sapiens*.

The earliest microfossils of eukariotic cells are estimated to be at most 1.5 billion years old and atmospheric oxygen may also be of the same age. Insects may have evolved some 550 million years ago. All known insect cecropins have an amidated C terminal [19]. Nematodes are likely much older evolutionarily than insects. If C-terminal amidations evolved at the same time as insects, the nematode peptides as well as the cecropin-like peptides from *Helicobacter* should have free C-terminal carboxyl groups. Thus the amidation signals -GR and -GK that are present in the nematodes may serve as sites for proteolytic mechanisms which are more primitive than the amidation used by insects and mammals. Amidation, which requires both vitamin C and molecular oxygen [20], is probably much younger than the proteolysis used by the nematodes.

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