

ARTICLES

Antimicrobial Polypeptides of Human Vernix Caseosa and Amniotic Fluid: Implications for Newborn Innate Defense

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ABSTRACT

Antimicrobial peptides/proteins are widespread in nature and play a critical role in host defense. To investigate whether these components contribute to surface protection of newborns at birth, we have characterized antimicrobial polypeptides in *vernix caseosa* (vernix) and amniotic fluid (AF). Concentrated peptide/protein extracts were obtained from 11 samples of vernix and six samples of AF and analyzed for antimicrobial activity using an inhibition zone assay. Proteins/peptides in all vernix extracts exhibited strong antibacterial activity against *Bacillus megaterium* (strain Bm11), in addition to antifungal activity against *Candida albicans*, whereas AF-derived proteins/peptides showed only the former activity. Fractions obtained after separation by reverse-phase HPLC exhibited antibacterial activity, with the most pronounced activity in a fraction containing α -defensins (HNPI-3). The presence of HNPI-3 was proved by dot blot analysis and confirmed by mass spectrometry. Lysozyme and

ubiquitin were identified by sequence analysis in two fractions with antibacterial activity. Fractions of vernix and AF were also positive for LL-37 with dot blot and Western blot analyses, and one fraction apparently contained an extended form of LL-37. Interestingly, psoriasin, a calcium-binding protein that is up-regulated in psoriatic skin and was found recently to exhibit antimicrobial activity, was characterized in the vernix extract. The presence of all of these antimicrobial polypeptides in vernix suggests that they are important for surface defense and may have an active biologic role against microbial invasion at birth. (*Pediatr Res* 53: 211–216, 2003)

Abbreviations

AF, amniotic fluid
TFA, trifluoroacetic acid
Pepsin: EC 3.4.23.1

Vernix caseosa (vernix) is a lipid-rich substance that covers the skin of the fetus and is present also on the skin of newborns. Several reports on biochemical characterization of vernix have focused mainly on its lipid components (1–4), and there is evidence indicating that vernix contains a combination of lipids derived from sebaceous sources and stratum corneum.

However, the integral composition and the exact physiologic role of vernix is still unknown. Regarding antimicrobial activity, vernix as a natural biofilm may constitute a mechanical obstruction to bacterial passage (4, 5). Vernix reflects intimate maternal and fetal interactions, and during the third trimester, vernix on the skin surface detaches into the amniotic fluid (AF) under the putative influence of pulmonary surfactant (6). Therefore, vernix-like AF may contain antimicrobial factors (7–12). Neonatal bacterial infections are a serious cause of morbidity and mortality among newborns. Because the newborn adaptive immunity is immature, antimicrobial peptides as effectors of innate immunity may have a critical role for newborn defense reactions.

The widespread appearance of naturally occurring antibacterial proteins and peptides, including defensins and cathelicidins, has been established (13, 14), but their abundance, dis-

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tribution, and *in vitro/vivo* activity have not been fully elucidated. Defensins, the major group of bactericidal peptides in humans, form two structurally distinct groups: α -defensins, mainly found in neutrophils (15, 16) and Paneth cells of the small intestine (17, 18), and β -defensins, mainly synthesized by epithelial cells (19–22). LL-37, a 37-residue antimicrobial peptide, is the only human cathelicidin located in neutrophils (23), lymphocytes (24), and various epithelial cells (25–27).

We previously detected two human antimicrobial polypeptides in vernix, LL-37 and lysozyme (28), and Baker *et al.* (29) have shown that human vernix is active against *Staphylococcus aureus* and *Klebsiella*. Our aim in the present study was to characterize peptides and proteins in vernix and AF with emphasis on antimicrobial activity. We have found that both vernix of the newborn skin and AF contain several polypeptides with antimicrobial activity, *i.e.* α -defensins (HNP1–3), lysozyme, LL-37, and ubiquitin, of which α -defensins (HNP1–3) seem to be the major contributor of this antimicrobial activity. Some of these characterized antimicrobial polypeptides have also been identified in AF. In addition, psoriasin, a calcium-binding protein with chemotactic activity (30, 31), which is highly up-regulated in psoriatic skin and recently has been found to exhibit antimicrobial activity (32), has been isolated from the vernix material.

METHODS

Vernix and AF collection. Eleven samples of vernix from healthy infants who were born at term and six samples of AF from healthy mothers were obtained at elective cesarean section at Karolinska Hospital after institutional board and parents' approval. Samples were free of blood and meconium and were kept at -70°C until examination.

Extraction and chromatography. Vernix (1 g) was homogenized and extracted overnight at 4°C in 60% acetonitrile containing 1% trifluoroacetic acid (TFA). After centrifugation at 2000 rpm for 15 min at 4°C , the supernatants were further centrifuged at 8000 rpm for 5 min and lyophilized. For enriching proteins/peptides, the lyophilized material was dissolved in 0.1% TFA and applied onto OASIS (Waters, Milford, MA, U.S.A.) columns activated in acetonitrile and equilibrated in 0.1% TFA. The columns were then washed with 0.1% TFA, and the bound material was eluted with 80% acetonitrile in 0.1% TFA and lyophilized. These protein/peptide extracts were the starting material for analyses. Collected AF (40 mL) was adjusted with concentrated TFA to a final concentration of 0.1% TFA and applied onto OASIS columns for enrichment of proteins/peptides in the same manner as for the vernix extracts.

An ÄKTA purifier system (Amersham Pharmacia Biotech, Uppsala, Sweden) was used in HPLC steps, and the column effluent was monitored at 214 and 280 nm. Reverse-phase HPLC was performed on two different Vydac C_{18} columns, 4.6×250 mm and 2.1×150 mm (Separations Group, Hesperia, CA, U.S.A.) equilibrated in 0.1% TFA, at a flow rate of 1 mL/min and 0.2 mL/min, respectively. A gradient of 0–15%, 15–60%, and 60–100% acetonitrile for 4, 45, and 12 min was used for the larger column, and a flatter gradient of 0–15%, 15–40%, and 40–100% acetonitrile for 13, 64, and 26 min was

used for the smaller column. Some of the fractions from the reverse-phase HPLC step were further purified on a Superdex Peptide PC 3.2/30 size exclusion column (3.2×300 mm; Amersham Pharmacia Biotech) in 0.1% aqueous TFA with 20% acetonitrile at a flow rate of 0.8 mL/min.

Antibacterial and antifungal assay. Thin plates (1 mm) were made of 1% agarose in Luria Bertani broth containing approximately 6×10^4 cells/mL of one of the two test bacteria, *Bacillus megaterium*, strain Bm11, or *Escherichia coli*, strain D21. In Luria Bertani broth, 170 mM NaCl is included and for the test of D21 Luria Bertani was supplemented with medium E, a physiologic salt medium developed for *E. coli* (33, 34). The agarose plates for the antifungal assay were in YM broth (Bacto; DIFCO Laboratories, Detroit, MI, U.S.A.), seeded with 6×10^4 cells/mL *Candida albicans* (ATCC 14053). For detection of lysozyme, a zone clearing on plates with cell walls from *Micrococcus luteus* (1 mg/mL) was used. Small wells (3 mm) were punched in the plates, and 3 μL of samples dissolved in 0.1% TFA were loaded in each well. As a positive control, synthetic LL-37 (9 μg) was used for Bm11 and D21 and Nystatin (7.5 ng) for *Candida*. After an overnight incubation at 30°C , the diameters of the inhibition zones were recorded.

Dot blot and Western blot analyses. LL-37 and α -defensin immunoreactivities in lyophilized protein/peptide extracts and chromatographic fractions were determined with dot blot and Western blot analyses using mouse monoclonal antibodies specific for LL-37 and HNP1–3 (Biogenesis Ltd, Poole, U.K.), respectively. The antibody for LL-37 was produced in our laboratory by injection of LL-37 (100 μg), mixed with Freund's complete adjuvant, intramuscularly into a mouse and twice repeated, after which monoclonal antibodies were obtained using conventional hybridoma technology.

In Western blot analysis, proteins/peptides were separated by discontinuous SDS-PAGE using 10–20% Tricine Ready Gels (Invitrogen, Carlsbad, CA, U.S.A.) and further blotted onto polyvinylidene difluoride membranes by electrophoretic transfer according to the instructions from the manufacturer. In the dot blot analysis, 1/12 (1 μL) of each chromatographic fraction was spotted onto a Hybond C Super membrane (Amersham Pharmacia Biotech). For HNP1–3, the membrane was fixed with 0.05% glutaraldehyde in PBS; this step was excluded for LL-37. The membranes in both Western and dot blot analyses were blocked in PBS containing 5% fat-free milk. The concentrations of monoclonal antibodies for LL-37 and HNP1–3 were 0.35 $\mu\text{g}/\text{mL}$ and 0.2 $\mu\text{g}/\text{mL}$ in PBS with 5% fat-free milk.

The second antibody was an antimouse IgG conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). An ECL Western blot detection system (Amersham Pharmacia Biotech) was used to visualize the results.

Mass spectrometry and amino acid sequence analysis. Chromatographic fractions analyzed by MALDI MS (Voyager DE-PRO; PE-Applied Biosystems, Foster City, CA, U.S.A.) used α -cyano-4-hydroxycinnamic acid as matrix (saturated in 70% acetonitrile) mixed 1:1 (vol/vol) with the sample. Edman degradation of peptides was performed with a Prosice cLC sequencer (PE-Applied Biosystems).

SDS-PAGE. HPLC fractions were analyzed for content of proteins/peptides by SDS-PAGE using 10–20% Tricine Ready Gels. After electrophoresis, the protein/peptide bands were visualized with SilverXpress Silver Staining Kit (Invitrogen).

Pepsin degradation. If bactericidal activity was derived from protein/peptide components, the fractions were subjected to enzyme degradation with pepsin. After lyophilization, the samples were dissolved in 10 μ l of 5% formic acid and 1 μ l of pepsin (2 mg/mL; Sigma Chemical Co., St. Louis, MO, U.S.A.) in 5% formic acid was added. After incubation at 37°C for 5 h, the samples were lyophilized and assayed for antibacterial activity, as described above.

RESULTS

Antimicrobial activity. From 1 g crude vernix, 0.5–1 mg lyophilized protein/peptide extract was obtained and from 40 mL of AF; the yield was 2–3 mg proteins/peptides. The proteins/peptides were then dissolved in 0.1% TFA to a final concentration of 10 μ g/ μ L, from which 30 μ g was assayed for antimicrobial activity against *B. megaterium* Bm11, *E. coli* D21, and *C. albicans*. All vernix samples exhibited antimicrobial activity against Bm11 and *Candida*. The inhibition zones ranged in diameter from 11.2 to 15.0 mm and from 5.1 to 6.7 mm, respectively. All samples of AF exhibited antibacterial activity against Bm11 (inhibition diameters from 9.8 to 12.1 mm) but not against *C. albicans*. Both vernix- and AF-derived material failed to inhibit the growth of *E. coli* D21.

Isolation and characterization of polypeptides with antibacterial activity. Lyophilized protein/peptide extracts from vernix or AF (0.5–1 mg) were fractionated by reverse-phase HPLC. After lyophilization, all fractions were dissolved in 12 μ l of 0.1% TFA and analyzed for antibacterial activity against Bm11. The chromatographic profiles are shown in Fig. 1, with horizontal arrows indicating fractions giving zone diameters >5 mm. The chromatographic patterns of vernix and AF are different, but α -defensins (HNP1–3), lysozyme, and LL-37 were identified in corresponding fractions between the two sources. In vernix, lysozyme was identified by sequence analysis in fraction 32–33 (see Table 1), whereas in AF, lysozyme was detected by the zone clearance assay using *Micrococcus luteus* cell wall fragments. In fraction 33 of vernix material, the chemotactic protein psoriasin was identified by sequence analysis (see Table 1), and in fraction 27, two main polypeptides were detected by SDS-PAGE and subsequent silver staining. This fraction was further subjected to size exclusion chromatography, showing that the lower molecular weight component exhibited antibacterial activity (Fig. 2A). This component was identified as ubiquitin by sequence analysis (see Table 1) after an additional reverse-phase step (Fig. 2B).

Identification of α -defensins. Dot blot analysis for α -defensins (HNP1–3) was performed on HPLC fractions. A positive signal was detected in fraction 21, both in vernix and in AF material (Fig. 3A). Further analysis by MALDI MS of this material gave mass values of 3371, 3441, and 3486 for vernix and 3371, 3442, and 3490 for AF, corresponding to the mass values of HNP1–3, respectively (Fig. 3B). The most pro-

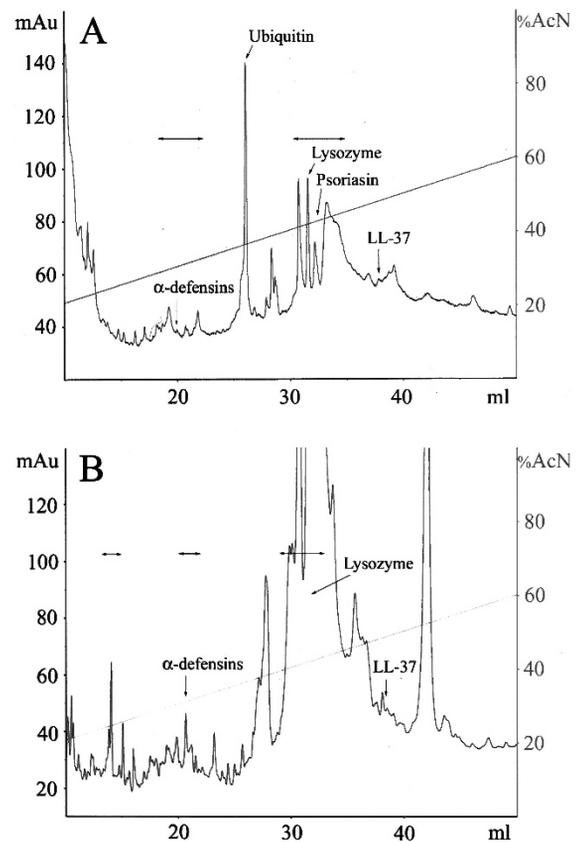


Figure 1. Reverse-phase chromatography of lyophilized crude material (1 mg) from vernix (A) and AF (B). The distribution of the antibacterial activity (zones >5 mm) is depicted in the chromatographic profile by double-headed arrows. The identified polypeptides are labeled α -defensins (HNP1–3), lysozyme, LL-37, ubiquitin, and psoriasin. Absorbance at 214 nm is indicated on the left y axis and % acetonitrile (AcN) on the right.

Table 1. Polypeptides identified in extract of vernix after separation by reverse-phase HPLC

Fraction No.	CH ₃ CN %	Sequence determined	Mass value	Components identified
21	30	Not determined	3371 3441 3486	Defensin HNP1 Defensin HNP2 Defensin HNP3
27	38	MQIFVKLTITGK TITL	8530	Ubiquitin
32–33	43–44	KVFERXELART LKRL	14745	Lysozyme
33	44	SNTQAERSIIGM IDM	11371	Psoriasin
39	50	Not determined	Not determined	LL-37

nounced bactericidal activity against Bm11 (inhibition zone diameter: 7.5mm) was detected in this fraction, supporting the presence of α -defensins (HNP1–3). In addition, the activity diminished after pepsin degradation, confirming that the bactericidal activity is based on polypeptides.

Identification of LL-37. The presence of LL-37 was analyzed by dot blot analysis, and positive signals for LL-37 were found in fraction 38–39 and 41–43 for both vernix and AF. These results were confirmed by Western blot analysis, which also revealed that the peptide in fraction 38–39 was slightly

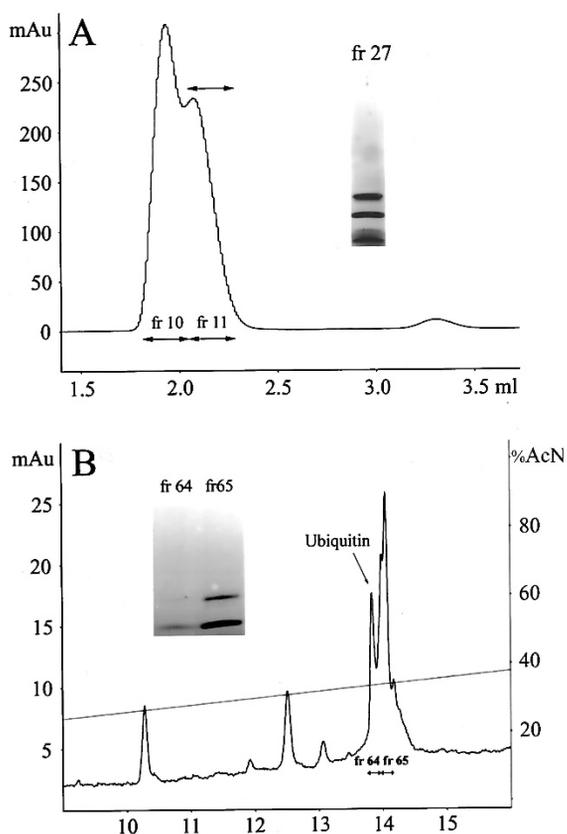


Figure 2. Isolation of ubiquitin from vernix material. (A) Fraction 27 from the reverse-phase HPLC of vernix was separated on a size exclusion column of Superdex peptide PC 3.2/30. Material in fraction 27, which was loaded on the Superdex column, contained at least two main polypeptides visualized on SDS-PAGE staining with silver (insert A). The fraction containing a polypeptide with the lower molecular weight indicated by the arrow showed antibacterial activity against Bm11. (B) Fractions 10 and 11 in A were rechromatographed on a smaller reverse-phase column. One main protein/peptide band was visualized in fraction 64 by SDS-PAGE stained with silver (insert B) and identified as ubiquitin by sequence analysis. Absorbance at 214 nm is indicated on the left y axis and % AcN on the right.

larger in size than the synthetic LL-37 (Fig. 4), indicating the presence of yet another processing of the human cathelicidin LL-37. However, in fraction 41–43, a band corresponding in size to the synthetic LL-37 was indeed detected.

DISCUSSION

Vernix contains up to 80% water, and the main components therein are lipids (35), but little is known about its protein/peptide components. In the present study, antimicrobial activity was demonstrated in vernix and AF. α -Defensins (HNPI-3), LL-37, and lysozyme were characterized among the active components. In addition, psoriasin and ubiquitin were identified in fractions derived from vernix that exhibited antibacterial activity. Our study shows that α -defensins (HNPI-3) are the main antimicrobial components in vernix, indicating a role for α -defensins (HNPI-3) in surface defense of the newborns. The fraction containing α -defensins (HNPI-3) exhibited the highest activity of all chromatographic fractions tested. This activity was confirmed to be peptide based because the activity was abolished after enzyme degradation by pepsin. In addition,

material in the corresponding fraction of AF exhibited pronounced antibacterial activity (Fig. 1B). Vernix contains the desquamation of fetal corneocytes as the putative result of sebaceous gland secretion during the last trimester of pregnancy (36). α -Defensins in vernix may be derived from the fetal skin through secretion and most likely not from neutrophils, because the samples analyzed are free from blood. AF consists of fetal pulmonary components and fetal desquamated cells; therefore, α -defensins in AF may originate from the fetal lung or the fetal skin.

In Western blot analysis using monoclonal antisera against LL-37, we identified an extended form of the peptide that may indicate an additional processing site. Proteinase 3 has been shown to liberate the active LL-37 peptide from the cathelin propeptide extracellularly, where the precursor protein originates from neutrophils (37).

Psoriasin was identified in a fraction also containing lysozyme, and therefore we could not conclude that psoriasin also exhibits antimicrobial activity. However, Schröder et al. (32) found psoriasin to exhibit antibacterial activity, indicating that psoriasin also contributes to the antimicrobial activity in vernix. Psoriasin was discovered as a calcium-binding protein with a molecular weight of 11.4 kD, belonging to the S100 family, and was first described in psoriatic keratinocytes (30). It has been reported that recombinant human psoriasin induces T-lymphocyte and neutrophil migration (31). Similar chemotactic activity has also been demonstrated for human α -defensins (HNPI-2) and LL-37 (24, 38). Furthermore, the bovine homologue to psoriasin has been found to be abundant in AF (39). The fraction with ubiquitin as the main component exhibits limited activity, and its contribution to antibacterial defense is not clear. Ubiquitin is a small protein of 76 residues that adopts a stable, compact, globular conformation, with four β -sheet strands and a single α -helix, and has a key role in the ubiquitin-mediated protein degradation system (40). There is limited knowledge on the selection of proteins for degradation by ubiquitin and also whether ubiquitin can be involved in the degradation of abnormal exogenous protein, including microbial proteins. Here we have shown that ubiquitin is able to kill bacteria, indicating yet another function for this protein. Lysozyme, a ubiquitous microbicidal protein localized in many tissues, is recognized to work synergistically with other components, e.g. LL-37 and lactoferrin (26, 41). It is evident that lysozyme also contributes to the antibacterial activity of vernix and AF because we have demonstrated strong bactericidal activity in a fraction containing lysozyme. *In vitro* antibacterial polypeptides have by themselves a broad spectrum of activities; however, *in vivo*, the effects may be further increased most likely as a result of synergy (42, 43). Interestingly, crude vernix extract showed inhibitory activity against *C. albicans* but with further separation the activity diminished, indicating synergistic antifungal components. Bactericidal activity against *E. coli* D21 was not detected in vernix and AF extracts, despite the presence of LL-37 and α -defensins (HNPI-3), which both are able to kill Gram-negative bacteria (15, 23). A possible explanation is that the critical concentration of these peptides were not reached in the assay against D21. Others have detected antibacterial activity against *E. coli* in material

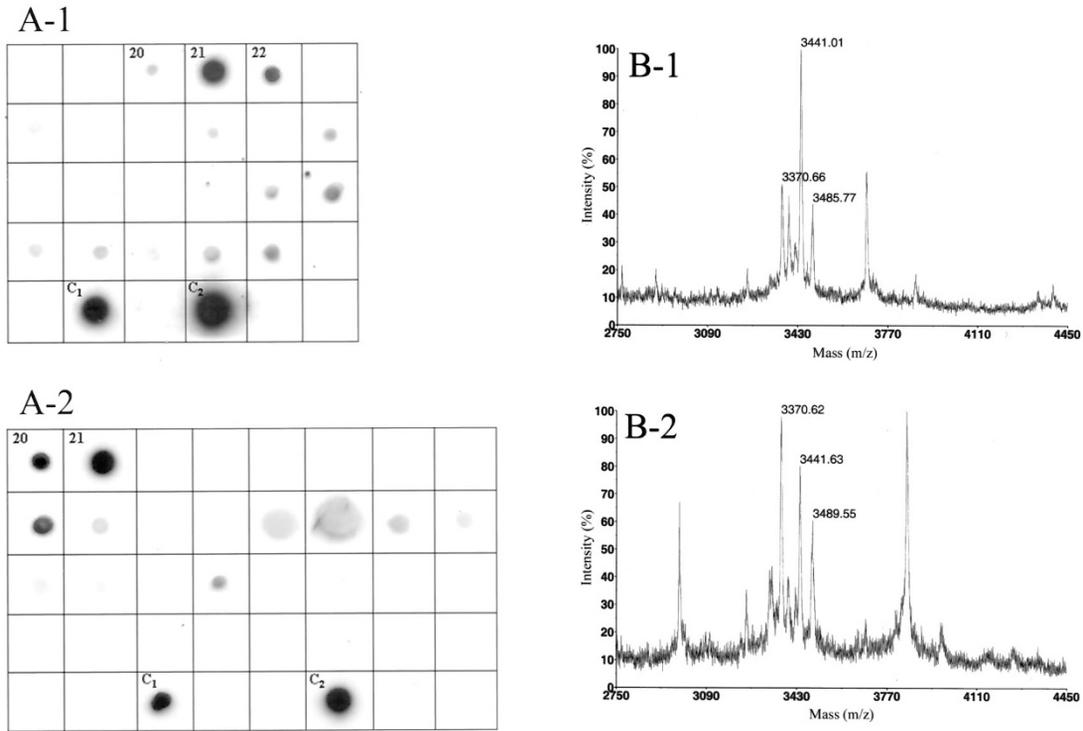


Figure 3. Detection of α -defensins (HNP1–3) with dot blot (A) and mass spectrometry (B). All fractions from the reverse-phase column with lyophilized crude material of vernix (A-1) and AF (A-2) were dissolved in 12 μ L of 0.1% TFA; thereof 1 μ L was spotted onto a nitrocellulose membrane. After fixation with 0.05% glutaraldehyde and blocking with 5% fat-free milk in PBS of the membrane, immunoreactivity was recorded with the specific antisera for HNP1–3 together with a second antisera conjugated with horseradish peroxidase. Visualization was with an ECL system. Strong positive fractions in the dot blot analysis of both vernix (Fr. 21) and AF (Fr. 21) were analyzed further by MALDI MS (1 μ L), giving mass values (vernix, B-1; AF, B-2) in agreement with the theoretical molecular weight of HNP1–3 (3371, 3442, and 3492).

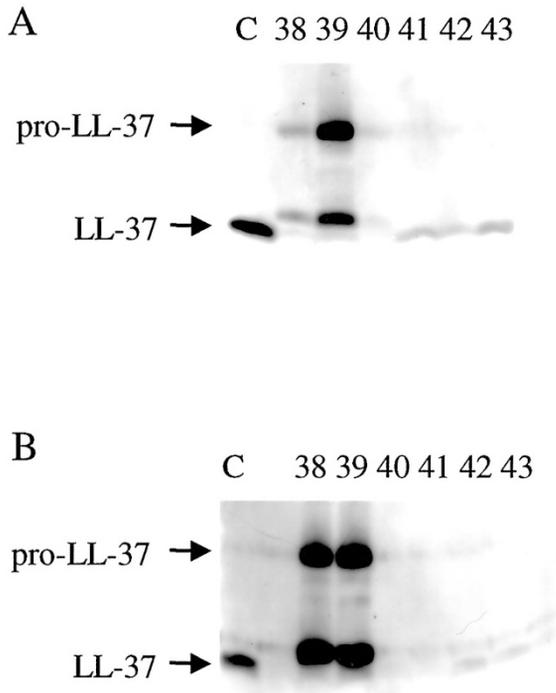


Figure 4. Western blot analyses of LL-37 on fractions derived from vernix (A) and AF (B). HPLC fractions that were positive in dot blot analysis were further analyzed by Western blot. Lane 1, synthetic LL-37 of 0.5 ng, indicated as C for control; other lanes, chromatographic fractions as indicated. The high molecular weight bands correspond to pro-LL-37.

derived from AF (8–10), results that we could not reproduce even after increasing the concentration of crude lyophilized material up to 100 μ g/ μ L.

In summary, antibacterial polypeptides are a part of the antibacterial defense in vernix, and AF and some of these polypeptides may also interact as chemotactic factors in the fetal and neonatal skin immunity. In addition, antimicrobial polypeptides present in vernix may contribute to strengthen the skin barrier against microbial invasion. Because preterm infants often lack vernix, this may be one reason for their increased susceptibility to invasive bacterial infections. In vernix, some lipids may play an important role in the protection against microbes, maybe in synergy with peptides/proteins. Our results suggest that a defense adaptation of the naked newborn includes production of antimicrobial components for active surface defense.

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