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Proteome Analysis of Vernix Caseosa

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ABSTRACT: Vernix caseosa (vernix) is a white creamy substance covering the skin of the fetus during the last trimester of pregnancy. The function of vernix has long been debated but no consensus has been reached. We here report a proteome analysis of vernix using two-dimensional gel electrophoresis, matrix-assisted laser desorption/ionization mass spectrometry and liquid chromatography coupled to tandem mass spectrometry. We have identified 41 proteins, of which 25 are novel to vernix. Notably, 39% of the identified vernix proteins are components of innate immunity, and 29% have direct antimicrobial properties. These results form a substantial contribution to the knowledge of vernix composition and demonstrate that antimicrobial protection of the fetus and the newborn child is a major and important function of vernix. **(*Pediatr Res* 60: 430–434, 2006)**

A white creamy substance designated Vernix caseosa (latin for "Cheese-like varnish") covers the skin of the fetus during the last trimester of pregnancy. The human is the only species known to produce this substance, and its function has been debated for decades. Many protective functions have been proposed, such as antimicrobial protection, heat insulation, moisturization and protection of the skin from macerating effects of the amniotic fluid. Other functions have also been suggested, such as hormonal effects, anti-inflammatory effects, nutritive functions, and facilitation of passage through the birth canal (1). Furthermore, vernix has been suggested to constitute a mechanical obstruction to bacterial passage (2). Recently, it was reported that vernix exhibits a skin cleansing function (3).

Early studies on whether vernix possesses direct antimicrobial activities yielded contradictory results (1,2), most likely due to use of different antimicrobial assays. However, by preparation of a protein extract of vernix and identification of individual components we have proved that vernix contains potent antimicrobial polypeptides (4,5). Members of the antimicrobial peptide families α-defensins (Human Neutrophil Peptide, (HNP) 1–3) and cathelicidins (LL-37) have been identified in vernix, in addition to a number of antimicrobial proteins, *i.e.*, psoriasin, secretory leukocyte protease inhibitor (SLPI) and calprotectin (calgranulin A and B) (4–6).

In association with the discussion on the antimicrobial properties of vernix, there has been a debate on whether vernix should be left on the skin after birth (7,8). Skin colonization of neonates was compared after leaving vernix on the skin and after bathing (removing vernix) the newborn. That study resulted in no difference in microbial colonization (8). However, the bathing reduced heat loss, and made the newborns calm, quiet and comfortable (8). The amount of vernix on the skin correlates with gestational age (9,10). Preterm infants lacking vernix have a higher rate of nosocomial and community acquired infections (10).

The composition of vernix is water (81%), lipids (9%), and proteins (10%) (11). Research on vernix has mainly focused on the lipid fraction (12–15), while the vernix proteins have long been neglected. However, in a recent study 18 vernix proteins were identified (5), but a comprehensive proteome analysis has to our knowledge not been performed of this material. We have therefore investigated the vernix proteome by 2-D (two-dimensional) gel electrophoresis, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), identifying many proteins novel to vernix. This now brings important insight into the role of vernix caseosa.

MATERIALS AND METHODS

Collection of vernix caseosa. Vernix caseosa was collected from the skin of newborns shortly after delivery, and before washing the neonates. The vernix samples were placed in sterile plastic containers and were immediately frozen and stored at -20°C until analyzed. All newborns were without prenatal or perinatal complications, and without clinical signs of infection. The study was reported to the Data Protection Authority and the sample collection was approved by the parents.

Peptide/protein extraction. The vernix samples were extracted as recently described (5). Briefly, the samples were homogenized in 60% acetonitrile containing 1% (vol/vol) aqueous trifluoroacetic acid (TFA), and extracted during shaking overnight at 4°C. After centrifugation of the extracts at 10,000 g, the supernatants were lyophilised. The lyophilised material was dissolved in 0.1% (TFA) and loaded onto OASIS HLB cartridges (Waters). Bound proteins were eluted with 80% acetonitrile in 0.1% TFA and the eluates were lyophilized.

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Abbreviations: LC-MS/MS, liquid chromatography tandem mass spectrometry; **MALDI,** matrix-assisted laser desorption/ionization; **PLUNC** palate lung nasal epithelial clone; **RNase 7,** ribonuclease 7; **2-D gel,** two dimensional gel

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2-D gel electrophoresis. The protein content of samples was determined by the Bradford assay (16). Sample loads of 350–450 μg protein were applied onto 17-cm Bio-Rad strips of pI 3-10 (Non Linear) *via* active in-gel rehydration. The sample (in 9 M urea, 65 mM DTT, 0.5% Igepal CA 630, 1.5% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5% ampholyte 3-10, 35 mM Tris, and complete proteinase inhibitor mix (Roche) was diluted with rehydration buffer (7 M urea, 2 M thiourea, 4% wt/vol CHAPS, 0.5% Igepal CA 630, 0.5% ampholyte 3–10 and 2.8 mg/mL DTT) to a final volume of 300 μL. The isoelectric focusing was carried out on a PROTEAN IEF (Bio-Rad) at 20°C as follows: 12 h at 50 V (active rehydration), 0–500 V for 1 h, 500 V for 1 h, 500–2000 V for 2 h, 2000–8000 V for 1 h and finally 8000 V for 3.5 h. All gradients were linear. After isoelectric focusing the strips were incubated for 15 min with 2% DTT in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS and a trace of bromphenol blue), followed by 15 min with 2.5% iodoacetamide in the same buffer. For the second dimension, proteins were separated in 15% polyacrylamide gels (1.5 × 260 × 200 mm) in an Ettan™ DALTsix system (Amersham). Electrophoresis was carried out at a constant current, 15 mA/gel for 1 h and thereafter 40 mA/gel until the bromophenol blue dye front was one cm from the gel bottom.

Coomassie staining. The gels were fixed over night with 2% phosphoric acid in 30% ethanol, washed for 3 × 20 min in 2% phosphoric acid, and equilibrated for 30 min in equilibration buffer (2% phosphoric acid/5% aluminium sulphate/10% ethanol). The gels were then stained for 2 d in 0.01% Coomassie brilliant blue G-250 in equilibration buffer, and destained in water.

In-gel digestion and protein fingerprinting. The gel spots were manually excised and digested with trypsin using a MassPREP robotic protein handling system (Micromass/Waters), employing a protocol described (17). Tryptic fragments were analyzed by MALDI-MS (Voyager DE-Pro, Applied Biosystems), using α-cyano-4-hydroxycinnamic acid as matrix (5 mg/mL in methanol:acetonitrile 1:1) and mixed 1:1 (vol/vol) with the sample. Database searches were carried out utilizing the ProteinProspector MS-Fit program (http://prospector.ucsf.edu/).

LC-MS/MS. Tryptic digests were analyzed by liquid chromatography tandem mass spectrometry using Waters CapLC and Q-Tof Ultima API instruments. Before LC-separation, digests were desalted using an LC-Packings Nano-Precolumn Cartridge (300 μm ID × 1 mm; 5% Acetonitril/0.1% formic acid; 20 μL/min). A Waters Atlantis C_{18} column (3 μm, 100 Å, 75 μm ID × 15 cm) was used for LC separation and the peptides were eluted with a linear gradient of 14–50% acetonitrile in 0.1% formic acid for 20 min at 200 nL/min. Peptides were introduced into the mass spectrometer using a Pico Tip sprayer and data-dependent acquisition was used over a mass range of 300-2000 Da (*m/z*). Data analysis was performed using ProteinLynx Global SERVER 2.1 software (PLGS 2.1, Waters) and MassLynx peptide sequence software (version 4.0, Waters). Data sets were analyzed using the NCBI BLAST and Mascot search engines.

RESULTS

Vernix caseosa was collected from newborns directly after birth. The vernix proteins were extracted and separated by 2-D gel electrophoresis using 350–450 μg vernix protein per gel. Due to low amounts of protein from each neonate, and high inter-individual variation (5) (data not shown), material from several (five to nine) neonates were pooled for each preparative gel. Two gels were used for protein identification which had matching protein patterns, including areas of high background. Using a clean up system (2-D Clean Up kit, Amersham), did not reduce the background (data not shown). To better visualize the protein spots, the gels were combined using the 2-D analysis software PDQuest (Bio-Rad) to make a graphic picture (Fig. 1).

Most vernix proteins are of low molecular size (5), and are consequently located to the lower region of the 2-D gel (Fig. 1). However, the 2-D gel reveals that many large proteins are also present in the vernix extract. In total, we detected approximately 350 spots in each gel. Of these, 117 distinct protein spots were excised from the two gels and proteins were in-gel digested with trypsin. Using MALDI mass spectrometry and LC-MS/MS, 13 proteins were identified with MALDI, 16 with LC-MS/MS and 12 with a combination of the two methods (Table 1). Thereby, 41 proteins were identified in a total of 78 spots. Of these proteins, 16 (39%) are involved in innate immunity and 12 (29%) have been demonstrated to have direct antimicrobial activities.

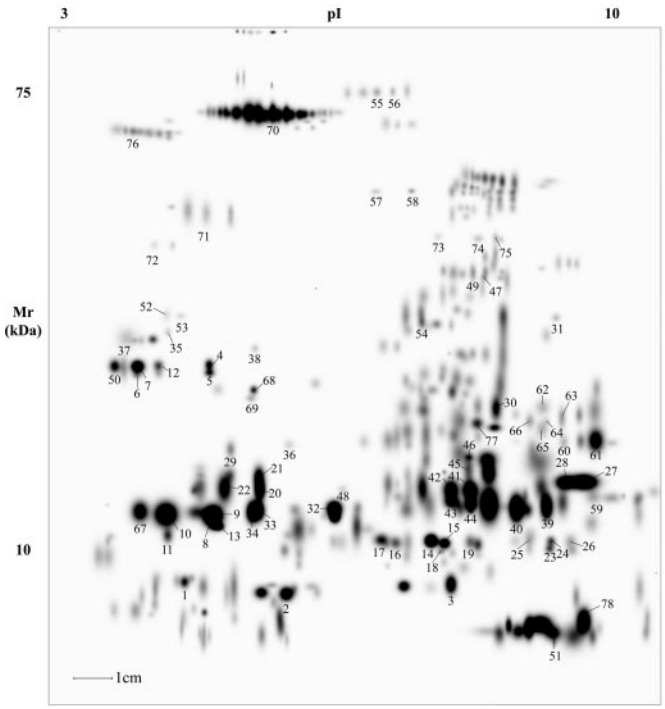

Figure 1. Graphic picture of proteins in Vernix caseosa separated on 2-D gel electrophoresis. Numbers indicate identified spots and are the same as those listed in (Table 1).

DISCUSSION

In this study we have analyzed proteins in vernix caseosa with 2-D gel electrophoresis. The proteins identified are given in Table 1, and include 25 proteins novel to vernix. This is a substantial addition to the knowledge of the composition of vernix. Notably, several of the proteins are components of the innate immune system, compatible with a protective role of vernix against infection.

Vernix caseosa is a complex material. It has a high lipid content making the material difficult to work with. Consistently, a high background was seen in particular areas of the gels. It was probably derived from a high lipid content and an abundance of particular proteins including Hb, profilaggrin and their fragments giving a trailing effect. This would be supported by the fact that we found these proteins over a large area of the gel (data not shown). To visualize the spots we therefore used the software PDQuest to produce a computerized image were the spots are clearly visible (Fig. 1).

Essentially all clearly visible spots were excised, and of the 117 protein spots analyzed, 41 proteins were identified in 78 spots. Many of the non-analyzed spots are concluded to be modified versions of the identified proteins. Typical sets of spots with multiple pIs but the same mass are noticed, representing differently modified proteins. Some were confirmed to

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Table 1. *Proteins identified in vernix*

Spot	Protein	Accession number	Mr (kDa)/pI	MALDI-MS coverage (%)	LC-MS/MS sequences
1	**Ubiquitin**	P62988	8.6/6.6	35	12-27
2					12-27, 43-48, 55-63
3				56	
4	Cystatin A*	P01040	11.0/5.4		11-22, 45-56
5					45-56, 72-89
6				70	
7				77	
8				84	
9				77	
10				64	
11				53	23-30, 38-44, 45-56, 64-68
12					11-22, 45-56
13				45	
14	**Calgranulin A**	P05109	10.8/6.5	58	
15				50	
12					37-47
16				46	
17				40	
18				20	
19				40	
20	**Calgranulin B**	P06702	13.2/5.7	85	
21				61	
22				82	
23	Caspase 14**	P31944	27.7/5.4	26	203-213, 225-235
24				31	
25				15	
26				16	
27	**Lysozyme**	P61626	16.5/9.4	29	
28				56	
29	Transthyretin	P02766	15.9/5.5	64	
30	Glycosylasparaginase†	P20933	37.2/5.9		266-277
31	**NGAL/lipocalin2**	P80188	22.6/9.0		36-50
32	**Psoriasin**	P31151	11.3/6.3	74	
33				85	
34				76	
35	PLUNC	Q9NP55	26.7/5.4	34	129-138, 139-152
36					95-109, 139-152
37				34	
38				34	
39	Hem α	P69905	15.1/8.7	74	
40				34	
41	Hem β	P68871	15.9/6.8	65	
42				82	
43				82	
44				88	
45				82	
46				40	
47	Hem γ-1 chain	P69891	16.0/6.7		134-145
48	Hem γ-2 chain	P69892	16.0/6.7		19-32, 134-145
49	Pulmonary surfactant-associated protein B	P07988	42.1/5.3	28	225-236
50					253-264
51					253-264
52	Apolipoprotein A-1	P02647	30.8/5.6	33	
53				28	
54	Proteasome subunit beta type 2	P49721	22.8/6.5		20-29, 63-68
55	Serotransferrin	P02787	77.0/6.8	35	
56				24	
57	Arginase 1	P05089	34.7/6.7	36	
58				45	173-180, 181-191, 211-222, 256-267
59	**Histone H2A**	P02261	14.0/10.9		83-89
59	**Histone H2B**	P62807	13.8/10.3	38	101-109
26					59-74

(*continued*)

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Table 1. *Continued*

Spot	Protein	Accession number	Mr (kDa)/pI	MALDI-MS coverage (%)	LC-MS/MSsequences
60	Histone H3	P68431	15.3/11.1		58-64
59 26	Histone H4	P62805	11.2/11.4		61-68 61-68
60	RNase 4	P34096	16.8/9.3		83-93
61	**RNase 7**	Q9H1E1	17.5/9.7	62	
62	**SLPI**	P03973	14.3/9.1		72-83
63 64 65 66	**hCAP18/LL-37**	P49913	19.3/9.5	30 34 18 20	 12-49, 50-57, 89-100, 108-118, 119-127 132-140
67	Thioredoxin	P10599	11.6/4.8		9-21
5	Cytokeratin 10	P13645	59.5/5.1	19	166-177, 246-256
12	Cytokeratin 9	P35527	62.0/5.1		170-183
5 12	Cytokeratin 1	P04264	65.9/8.2		258-267, 444-455 46-65, 186-197, 444-455
68 69	Super oxid dismutase	P00441	15.8/5.70	42 22	10-23, 80-91
70	Serum albumin	P02768	69.4/5.92	56	
50	SCCA 1/2	P29508/ P48594	1:44.6/6.4 2:44.9/5.9		11-20
71	Zn-alpha 2-glycoprotein	P25311	33.9/5.6	51	
72	IGFBP-1	P08833	27.9/5.11	23	38-53, 201-208
73 74 75	Annexin A1	P04083	38.6/6.6	33 44 51	
76	Alpha 1-antitrypsin	P01009	46.7/5.4	34	
77	Epididymal secretory protein	P61916	16.6/7.6		36-51
78	**Mucin 7**‡	NP_689504	39.2/9.0		74-86

Bold type indicates proteins reported to exhibit antimicrobial activity.

* Cystatin A tryptic fragments were identified both with and without the first methionine. An amino acid exchange of Q to R was also observed in spot 4.

** All tryptic fragments of caspase 14 were located in the C-terminal region.

† The sequenced peptide in glycosylasparaginase was located in chain β with a Mr/pI of 15.1 kDa/7.6.

‡ The mucin 7 fragment was identified in four different forms with amino acid exchanges at positions 79 (N to D), 80 (N to D), 84 (F to L) and 86 (N to K).

contain the same protein, *e.g.* serotransferrin (spots 55 and 56 in Fig. 1), and others are well known to produce such patterns, *e.g.* albumin (18). In addition, some proteins were localized to several spots with both different masses and pIs. Cystatin A, for example, was identified in ten spots with different localizations (spots 4–13 in Table 1 and Fig. 1), corresponding to post-translational modifications, complex formations or polymorphisms. For Cystatin A, we could show by MS/MS that at least one of the modifications was an amino acid exchange (Table 1, spot 4). Furthermore, in spot 78 we could identify a fragment of Mucin 7 in four different forms with multiple amino acid exchanges (Table 1). This likely reflects polymorphisms since the material originated from several neonates.

Origins of the vernix proteins are multiple. First, there is a close contact of vernix with the amniotic fluid, and therefore an exchange of proteins between vernix and the amniotic fluid is possible. Second, the amniotic fluid is also in contact with the fetal lungs and vernix can therefore contain lung proteins. Third, since the vernix material analyzed here was obtained from neonates delivered by vaginal deliveries there is a risk of blood contamination, consistent with the abundance of hemoglobins detected. Finally, many vernix proteins have dermal origins. Many of the identified proteins have been implicated in innate immunity.

One protein now detected in vernix is LL-37 (spots 63–66). We have previously demonstrated its presence, but only by highly sensitive immunologic methods (4). Here, we report that spots of LL-37 are detected by direct Coomassie staining after 2-D gel electrophoresis of vernix extract. This demonstrates the presence of high levels of LL-37 in vernix, more than previously considered. A contradictory result was reported recently, where LL-37 could not be detected in vernix extract (6), most likely depending on extraction in PBS instead of 60% acetonitrile with 1% TFA. Furthermore, fragments were here identified from both the cathelin and the processing regions, indicating that at least one of the identified LL-37 spots contains the unprocessed precursor and not only the cathelin region. Interestingly, apolipoprotein A-1 (spots 52–53), the major LL-37-binding protein in blood (19) was also identified in vernix.

Palate lung nasal epithelial clone (PLUNC, also named LUNX and SPURT) is a protein expressed in the upper airways (20). This indicates that the PLUNC detected in vernix probably originates from the airways of the fetus. It

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was here found in multiple spots (spots 35–38), demonstrating the presence of several PLUNC isoforms in vernix. PLUNC isoform formation has previously been described in human nasal lavage fluid and modifications such as truncation, glycosylation, phosphorylation and de-amidation were proposed (21). PLUNC displays sequence homology and predicted structure similarity to the LPS binding proteins LBP and Bactericidal/Permeability Increasing protein (BPI) (22) and has been shown to be able to bind LPS (21). In rats, PLUNC is up-regulated in nasal respiratory epithelium upon olfactory neuronal injury, suggesting that PLUNC can provide protection to infection after injury (23). Because of these properties the family of PLUNC proteins has been proposed to have host defense functions (22,24) and to work as sensors of Gram-negative bacteria in the oral cavity (24).

Neutrophil gelatinase-associated lipocalin (NGAL, also named human neutrophil lipocalin, uterocalin) is a bacteriostatic protein that interferes with the iron acquisition in bacteria through specific binding to bacterial chatecolate-type ferric siderophores (25). It is mainly expressed in neutrophils and tissues exposed to microbes such as trachea, uterus and colon (26). Hence, NGAL detected in vernix (spot 31) can originate from the maternal uterus and the neonatal respiratory tract or skin.

Ribonuclease 7 (RNase 7) was first isolated from stratum corneum of the skin and is an antimicrobial ribonuclease (27). RNase 7 is constitutively expressed in epithelial tissues including skin, respiratory tract, and uterus (27) but also in other tissues such as liver and kidney (28). It has also been shown to be induced in keratinocytes by certain bacteria (27). RNase 7 exhibits a broad spectrum of antimicrobial activity against many pathogens including vancomycin-resistant *Enterococcus faecium* (27). Our finding of RNase 7 in vernix (spot 61) suggests that it also is expressed in fetal skin thereby enhancing its role in innate immunity. Interestingly, another member of the ribonuclease A superfamily, RNase 4, was also identified in vernix (spot 60).

Annexins (spots 73–75) are a family of proteins that bind to phospholipids and carbohydrates in the presence of calcium ions. Annexin I has been shown to bind surface molecules of both Gram-positive and Gram-negative bacteria, *i.e.*, lipoteichoic acids (29) and Lipid A (30). It has been shown to reduce neutrophil and monocyte infiltration in animal models (31–33), and to suppress the attachment of *Staphylococcus aureus* to macrophages (29), activities that give Annexin I a role in inflammation.

In conclusion, our proteome analysis defines many proteins novel to vernix, with over one third related to innate immunity. This constitutes a substantial improvement to our knowledge of vernix and supports the conclusion that antimicrobial protection of the fetus is a major function of vernix.

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