Cathepsin D is present in human eccrine sweat and involved in the postsecretory processing of the antimicrobial peptide DCD-1L*  

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Running title: Processing of DCD-1L by cathepsin D in sweat  

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The protein pattern of healthy human eccrine sweat was investigated and 10 major proteins were detected from which apolipoprotein D, lipophilin B and cathepsin D (CatD) were identified for the first time in human eccrine sweat. We focused in our studies on the function of the aspartate protease CatD in sweat. In vitro digestion experiments using a specific fluorescent CatD substrate showed that CatD is enzymatically active in human sweat. To identify potential substrates of CatD in human eccrine sweat, LL-37 and DCD-1L, two antimicrobial peptides present in sweat, were digested in vitro with purified CatD. LL-37 was not significantly digested by CatD whereas DCD-1L was cleaved between 44L and 45D and between 29L and 30E almost completely. The DCD-1L derived peptides generated in vitro by CatD were also found in vivo in human sweat as determined by surface-enhanced laser desorption/ionization (SELDI) mass spectrometry. Furthermore, besides the CatD processed peptides we identified additionally DCD-1L derived peptides which are generated upon cleavage with a 1,10-phenanthroline sensitive carboxypeptidase and an endopeptase. Taken together proteolytic processing generates 12 DCD-1L derived peptides. To elucidate the functional significance of postsecretory processing the antimicrobial activity of three CatD processed DCD-1L peptides was tested. Whereas two of these peptides showed no activity against gram-positive and gram-negative bacteria, one DCD-1L derived peptide showed an even higher activity against E. coli than DCD-1L. Functional analysis indicated that proteolytic processing of DCD-1L by CatD in human sweat modulates the innate immune defense of human skin.  

Human skin serves as a first line of defense against potential pathogens by building a mechanical barrier. In addition to physical mechanisms, the epithelia of mammalian skin produce antimicrobial peptides such as cathelicidins (1,2) and β-defensins (3,4) or small proteins, such as the recently described 11 kDa S100 protein psoriasin (5). Cathelicidins and β-defensins share properties concerning their biosynthesis as proforms, which are subsequently processed into mature peptides (1,6,7). During inflammatory conditions such as wound healing (8) or psoriasis (9) the expression, processing and secretion of the antimicrobial peptides LL-37 (10) and β-defensin 1 and 2 (4) are increased. As part of the innate defense of human skin the antimicrobial peptide dermcidin (DCD) is constitutively expressed in eccrine sweat glands, secreted into sweat (11) and present on the skin surface at an average concentration of 1 to 10 µg/ml (12). In human sweat the 110 amino acid dermcidin-proform is first processed to the 48 amino acid peptide DCD-1L. Subsequently DCD-1L is further C-terminally processed by a yet
unidentified carboxypeptidase (13,14).
The sole human cathelicidin hCAP-18 is extracellularly processed by proteinase 3 (15) to an active 37 amino acid peptide named LL-37 which is found in sweat at an average concentration of 0.013 µM (16). After secretion LL-37 undergoes further enzymatic processing by an unidentified serine protease yielding 3 peptides which enhances the antimicrobial activity of LL-37 itself (17).

Beside these antimicrobial peptides and proteins the surface of human skin provides other proteins such as human serum albumin (18), cytokeratin I (19), Zn-α-2-glycoprotein (20), prolactin-inducible protein (21) and cystatin A (22,23). Additionally certain proteases have been described in human sweat such as gelatinolytic and caseinolytic proteases (24), cathepsin B- and H-like cysteine proteases (25), human stratum corneum chymotryptic enzyme (26) and tissue kallikrein and kininase II (27). In general, the physiological function of these proteins and proteases as well as their significance for the immunity of the skin and their interaction with other sweat components, especially with antimicrobial peptides, still remains to be determined.

This contribution presents an overview of the major proteins in human eccrine sweat involving 10 dominant proteins. 7 out of these proteins have already been described in sweat glands, in sweat or to be secreted by keratinocytes. For the first time apolipoprotein D, lipophilin B and CatD (EC 3.4.23.5) were detected and identified in human eccrine sweat.

Furthermore our data show, that CatD takes part in the postsecretory processing of the antimicrobial peptide DCD-1L. During these in vitro and in vivo studies we observed two other yet unidentified protease activities: a 1,10-phenanthroline sensitive carboxypeptidase and an endoprotease which in combination with CatD are responsible for the degradation of DCD-1L in sweat yielding 12 DCD-1L derived peptides. The antimicrobial activity of three CatD processed DCD-1L peptides was tested. Whereas two of these peptides showed no activity against gram-positive and gram-negative bacteria, one DCD-peptide showed a higher activity against these microorganisms. These data indicate that by postsecretory proteolytic processing the antimicrobial activity of DCD-1L is modulated and therefore the immune defense on the skin surface.

MATERIALS AND METHODS

Collection and preparation of sweat - Human sweat samples from several healthy donors were collected from the surface of the face, neck or chest during physical exercise or in a hot environment as previously described (12). For some experiments sweat samples were pooled (pooled sweat). SDS-PAGE analysis was performed with 100 µl sweat. Before gel electrophoresis sweat samples were dialyzed (48 h) using a 1 kDa membrane Tube-O-Dialyzer™ (Geno Technology, St. Louis, USA) to remove small interfering substances such as salts. The resulting 150 µl sample were concentrated to dryness using a centrifugal vacuum concentrator (Savant SpeedVac® SPD111, Thermo Quest, Egelsbach, Germany) and then resuspended in 20 µl water. Western blot analyses and in vitro digestion experiments were performed with pure sweat, centrifuged at 1500 g (Biofuge pico, Kendro, Osterode, Germany). The obtained supernatants were stored at -80 °C until use.

In-gel tryptic digestion and mass analysis of tryptic fingerprint - Excised gel bands were diced to approximately 1 mm³ and washed once for 15 min with water before being resuspended in 40 µl 50% (v/v) acetonitrile (ACN)/water and incubating for further 15 min. Gel pieces were then subsequently shrunk with 40 µl 100% ACN and 40 µl of 100 mM (NH₄)₂CO₃. Afterwards gel pieces were incubated for 15 min with a 1:1 solution of ACN and 100 mM (NH₄)₂CO₃ and then vacuum-centrifuged to complete dryness. 40 µl of a trypsin solution (10 ng/ml) in digestion buffer (5 mM CaCl₂ and 50 mM (NH₄)₂CO₃) were added and incubated for 45 min. The trypsin solution was removed and gel pieces were incubated for 16 h at 37 °C in 50 µl digestion buffer. Tryptic peptides were eluted from the gel pieces by covering them with 0.1% (v/v) TFA in water and sonicated for 30 min. The extraction step was repeated successively with 30% (v/v) ACN/water and with 60% (v/v) ACN/water. The obtained supernatants were pooled and vacuum centrifuged to 50% of the original volume to remove TFA and ACN. After adding 10 µl formic acid, samples were measured by MALDI-Re-TOF (time of flight) MS on a Voyager DE-STR (Applied Biosystems, Foster City, CA, USA). Peptide fingerprints were exported for database matching, and subsequently identified using the Mascot program (28) available through www.matrixscience.com. In certain cases the results of tryptic fingerprint analysis were not
definite, thus the samples were additionally analyzed by automated LC-ESI-MS/MS using an Esquire 3000 plus ion-trap mass spectrometer (Bruker-Daltonics, Bremen, Germany). The obtained MS/MS data were also analyzed using Mascot sequence query for probability based peptide identification.

**Solid-phase peptide synthesis** - Peptide sequences were: Amca-EEKPISFFRLGK (CatD substrate), SSLLEKLDGAKAVGLGKLG KDAVEDLESVGKAHVVDKVLDLSVL (DCD-1L), ESVGKGAHVVDVLDS (EVS-17), LLGDFFRKSKEKIGKEFKRIVQIKDF LRNLVPRTES (LL-37), LEKGLDGAKAVGLGKLGKDAVE (LEK-24), SSLLEKGLGDA KKAVGLGLGKLKDAVEDL (SSL-29) and SSLLEKGLDGAKAVGLGKLGKDAVEKGLDGAKAVGLGKLGKDA (SSL-25). Peptides were synthesized using standard Fmoc/tBu chemistry (29) and synthesis was performed on the multiple peptide synthesizer Syro II (MultiSynTech, Witten, Germany) on a 0.025 mmol scale using a six fold molar excess of Fmoc amino acids (MultiSynTech, Witten, Germany) on TCP-resin (PepChem, Reutlingen, Germany). All other reagents and solvents for peptide synthesis were purchased from Merck KGaA (Darmstadt, Germany).

In situ activation was performed using TBTU (6 eq.) and HOBt (1 eq.) followed by the addition of N-methylmorpholine (12 eq.) in DMF. After completion of the automated synthesis, the resin bound peptides were Fmoc-deprotected using 60% (v/v) piperidine in DMF twice for 15 min and washed subsequently with DMF, isopropanol and diethyl ether. To release the peptides from the resin and to remove the side chain protecting groups the following solution was used: 95% (v/v) TFA containing 3% (v/v) thioanisol, 3% (w/v) phenol and 2% (v/v) ethanediol. The peptides then were precipitated in diethyl ether, dried and dissolved in 80% (v/v) tert-Butanol in water followed by lyophilisation. Crude peptides were purified using preparative reversed-phase high-performance liquid chromatography (RP-HPLC) and identity of the peptides was confirmed using electrospray ionization mass spectrometry (ESI-MS). Peptide purities were determined via analytical RP-HPLC and proved to be higher than 97 %. The peptides were stored at 4 °C until use.

**In vitro digestion experiments** - For the determination of CatD activity the synthetic substrate Amca-EEKPISFFRLGK was assayed according to our previously described protocol (30) using RP-HPLC with fluorescence detection. The reaction was performed in CatD buffer (50 mM Glycine/HCl buffer pH 3.5) at 37 °C for 60 min with a final substrate concentration of 0.3 µM. For all in vitro digestion experiments involving sweat samples pooled human eccrine sweat was used.

**In vitro** digests of synthetic LL-37 and DCD-1L with CatD (Sigma, Taufkirchen, Germany) were performed in 50 mM Glycine/HCl buffer pH 3.5 and in sweat buffer pH 5.5 (40 mM NaCl, 10 mM KCl, 1 mM CaCl2, 1 mM MgCl2 and 1 mM NaH2PO4) (14). DCD-1L (24.9 µM) and LL-37 (26.7 µM) were incubated with 0.6 µM CatD for 8 h at 37 °C. The reaction was stopped by addition of 25 µl stop solution (95% (v/v) ACN, 1% (v/v) TFA in water). Peptide fragments were separated via analytical RP-HPLC using a 125 × 2 mm Nucleosil 100 C8 column (Wicom, Heppenheim, Germany) with the following solvent system: (A) 0.055% (v/v) TFA (trifluoroacetic acid) in H2O and (B) 0.05% (v/v) TFA in ACN/H2O (4:1, (v/v)). The column was eluted with a 5% to 80% gradient of solvent B for 40 min. UV-detection was carried out at 214 nm (UV detector SPD-10AV, Shimadzu, Duisburg, Germany). Manually collected fractions were subsequently analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and Edman degradation on an automated 494A Procise peptide sequencer (Applied Biosystems, Darmstadt, Germany). The in vitro inhibition experiments were analyzed in the same way using a RF-10AXL fluorescence detector (Shimadzu, Duisburg, Germany) by measuring the emission at 450 nm following excitation at 350 nm. Aminopeptidase activity was measured using the substrate H-Leu-AMC (Bachem AG, Bubendorf, Switzerland) (15 µM) according to the manufacturer’s protocol and 25 µl pooled sweat to a final volume of 200 µl in PBS. Fluorescence signal was continuously measured using the microplate reader Spectra Fluor (Nunc, Wiesbaden, Germany) at an emission wavelength of 465 nm with excitation at 360 nm. **MALDI-MS** - 0.5 µl of each HPLC-fraction was mixed with 0.5 µl of 2,5-dihydroxyacetophenon matrix (20 mg of 2,5-dihydroxyacetophenon, 5 mg of ammonium citrate in 1 ml of isopropyl alcohol/H2O (4:1, (v/v)) and applied on a gold target for MALDI-MS using a MALDI time-of-flight system (G2025A, Hewlett-Packard, Waldbronn, Germany). Signals were generated by accumulating up to 50-100 laser shots in the single shot mode.
SDS-PAGE and Western blot analysis - The sweat samples were run on a 15% Bis-Tris (Bis-(2-hydroxyethyl)-glycin) separating gel with Tris-glycine running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3) and stained with Simply Blue™ Safe Stain (Invitrogen, Karlsruhe, Germany). Gel bands were excised and stored at -18 °C until digestion with trypsin. Western blot analysis was performed using a 10% Bis-Tris gel (Invitrogen, Karlsruhe, Germany) with MES ((2-(N-morpholino)ethanesulfonic acid) running buffer according to the manufacturer’s instructions. Proteins then were transferred to a PVDF (polyvinylidene difluoride) membrane (Amersham Biosciences, Freiburg, Germany) in a Novex mini-trans blot-apparatus (Invitrogen, Karlsruhe, Germany). The membrane was blocked for 16 h at 4 °C in Tris buffered saline with Tween 20 (TBST, 0.15 M NaCl, 10 mM Trizma® Base, 0.05% (v/v) Tween 20, pH 8.0) containing 10% (v/v) Roti® Block (Roth, Karlsruhe, Germany). Rabbit anti-human CatD (Sigma, Taufkirchen, Germany) was diluted 1:10,000 in TBST containing 10% (v/v) Roti® Block. After 2 h incubation membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immuno Research, West Grove, USA) (1:10,000) for 1 h. Western blots were then developed according to the ECL protocol of Amersham Biosciences.

SELDI Protein Chip® Technology - Sweat samples were analyzed by surface-enhanced laser desorption/ionization (SELDI) mass spectrometry (31) on reversed-phase (H4) chips (Ciphergen Biosystems, Fremont, USA) which were equilibrated three times for 5 min in ACN/water (1:1, (v/v)). 1 µl human sweat was diluted into 2 µl binding buffer (50 mM sodium phosphate pH 6.5) and the chromatographic arrays were incubated in a humid chamber for 30 min. Then chips were washed three times for 5 min with binding buffer followed by a final water wash to remove interfering substances such as salts. After drying a saturated solution of sinapinic acid in ACN/H2O (1:1, (v/v)) supplemented with 0.5% (v/v) TFA was added and peptide masses were read from the array surface using a ProteinChip Reader (Ciphergen Biosystems, Fremont, USA). The instrument was externally calibrated using two different synthetic peptides (DCD-1L and DCD-1) and bovine insulin. Internal calibration was performed by adding 100 fmol of porcine dynorphin A, adrenocorticotropic hormone (1-24) and bovine insulin. The data obtained were analyzed using the ProteinChip Software (Version 3.0).

Antimicrobial assays - Antimicrobial activities of synthetic peptides were tested by the department of dermatology using the colony-forming units (CFU) assay as previously described (32). *Escherichia coli* (ATCC 25922) or *Staphylococcus aureus* (ATCC 25923) single cell colonies were cultured overnight, subcultured and grown to mid-exponential growth phase prior to the antimicrobial assay. Cells were washed twice with 10 mM Na-phosphate buffer pH 7.0 containing 10 mM NaCl. Bacterial concentration was estimated photometrical at 600 nm. Absorbance of 1.0 corresponded to 8.56*10^8 cells of E. coli and to 1.97*10^8 cells of S. aureus per ml. After dilution to a concentration of 10^6 CFU/ml in 30 mM Na-phosphate buffer pH 7.0 containing 30 mM NaCl, 10µl of the dilutions were incubated at 37°C for 2h with 20µl various peptide concentrations in water. After incubation cells were diluted 1:100 in 10 mM Na-phosphate buffer pH 7.0 containing 10 mM NaCl. Then 90 µl of the diluted bacterial suspension were plated in triplicates on blood agar. The plates were incubated overnight at 37 °C and the number of colonies were counted. Antimicrobial activity is expressed as percentage of cell death (PCD):

\[
PCD = \left(1 - \frac{CFU_{sample}}{CFU_{control}}\right) \times 100
\]

where CFU_{control} is the number of colony-forming units without peptide incubation and CFU_{sample} is the number of colony-forming units of the appropriate sample.

RESULTS

Identification of dominant proteins in human eccrine sweat

In order to identify dominant proteins in human eccrine sweat standard proteomic techniques were applied. In a first step female and male human eccrine sweat samples were separated by SDS-PAGE and proteins were stained using Simply Blue™ Safe Stain (Fig. 1). Major protein bands were then excised and digested in-gel with trypsin. Resulting tryptic peptides were analyzed by MALDI-Re-TOF-MS and LC-ESI-MS/MS, respectively. Altogether 10 dominant proteins
were identified by peptide mass fingerprinting and are listed in Table I. We identified human serum albumin (band at 65 kDa) and the antimicrobial peptide DCD-1L (bands at 4 kDa) which both have already been described in sweat (18,12). Cytokeratin 1 (19; 53 kDa) as well as Zn-α-2-glycoprotein (20; 38 kDa) and cystatin A (22,23; 12 kDa) have been described to be present in the duct and secretory coil of sweat glands. Myal et al. showed that the prolactin-inducible protein (15 kDa) is present in sweat glands (21). Psoriasin has been recently identified in keratinocytes (5). Since psoriasin is secreted by keratinocytes in large amounts onto the skin surface it is not surprising that we identified this protein also in human sweat. Besides these proteins already described to be present in sweat glands or sweat we identified for the first time in eccrine sweat apolipoprotein D, lipophilin B and CatD.

The active β-chain of cathepsin D is present in human eccrine sweat

We identified for the first time the β-chain of the aspartate protease CatD in human eccrine sweat. The results obtained with tryptic fingerprint analysis were proven by Western blot analysis of male (two individuals), female (two individuals) and pooled human eccrine sweat. Purified human CatD was used as a positive control. Both forms, the active β-chain of CatD (31 kDa) as well as the proform (56 kDa) could be detected in eccrine sweat, the latter in lower amounts (Fig. 2). We did not observe any significant differences between male and female sweat. Next, we investigated if the β-chain of CatD is enzymatically active in sweat by performing in vitro digestion experiments using a specific fluorescent CatD substrate as we reported previously (30). The substrate was incubated for 30 min with purified CatD or pooled sweat, respectively. Digestion products were then separated using analytical RP-HPLC with fluorescence detection (Fig. 3). CatD cleaves the substrate Amca-EEKPSFFRLGK specifically between the two phenylalanine residues yielding the two peptides Amca-EEKPSF and FRLGK. Only the first peptide can be detected using fluorescence detection. The amount of generated fluorescent Amca-EEKPSF is proportional to the CatD activity. Figure 3A shows the undigested CatD substrate with a retention time of 21.7 min. Figure 3B shows the digestion product Amca-EEKPSF eluting at 20.6 min after digestion with purified CatD. In Figure 3C the same sample was measured in presence of the metalloprotease inhibitor 1,10-phenanthroline (peak at 11.6 min). The peak areas of Amca-EEKPSF in chromatogram B and C are comparable indicating that the inhibitor 1,10-phenanthroline has no influence on CatD activity.

After incubation of the substrate with pooled human eccrine sweat Amca-EEKPSF was generated according to the specificity of CatD (Fig. 3D). This cleavage site could be completely inhibited with the aspartate protease inhibitor pepstatin A (Fig. 3E), but not with iodoacetamide and E-64 (inhibitors for cysteine proteases, Fig. 3F and Fig. 3G), leupeptin and PMSF (inhibitors for serine proteases, Fig. 3H and Fig. 3I) or EDTA and 1,10-phenanthroline (inhibitors for metalloproteases, Fig. 3J and Fig. 3K). These data show, that digestion between the phenylalanine residues is caused by CatD and consequently that CatD is present in its enzymatically active form in human eccrine sweat.

A 1,10-phenanthroline sensitive Carboxypeptidase is present in human eccrine sweat

The CatD-substrate is additionally cleaved between serine and phenylalanine when incubated with sweat yielding Amca-EEKPS which has a retention time of 16.2 min. This finding indicates that another protease is present in sweat. Its activity could be completely and specifically inhibited with 1,10-phenanthroline (Fig. 3K) showing that the protease is presumably a Zn2+-metalloprotease as the common metalloprotease inhibitor EDTA had no effect on the activity (Fig. 3J). Surprisingly pepstatin A inhibited both, CatD and the observed cleavage between serine and phenylalanine (Fig. 3E). This finding show, that the 1,10-phenanthroline sensitive protease cannot cleave the intact substrate until CatD has digested the substrate. This suggests that the carboxypeptidase could not cleave the basic amino acid lysine but the hydrophobic amino acid phenylalanine. To verify this hypothesis the substrate was incubated longer in order to achieve additional digestion products which are not generated within 30 min. After 1 h over 95% of the substrate was cleaved and digestion products were then identified using UV-detection and...
The intact CatD substrate (lysine at its C-terminus) and Amca-EEKP are not C-terminally trimmed showing that the 1,10-phenanthroline sensitive protease is a carboxypeptidase which releases C-terminal amino acids such as phenylalanine, serine and isoleucine but not lysine and proline. Our results show that the β-chain of CatD as well as a 1,10-phenanthroline sensitive metalloprotease with carboxypeptidase activity are present in their active forms in human eccrine sweat.

### In vitro digestion of the antimicrobial peptides LL-37 and DCD-1L with cathepsin D

Next we investigated whether CatD is able to cleave peptides present in sweat. Therefore we incubated the antimicrobial peptides DCD-1L and LL-37 with purified CatD and digestion products were then analyzed using RP-HPLC (Fig. 4) followed by MALDI-MS of the single fractions (Tab. 2). These in vitro digestion experiments were performed in CatD-buffer (Fig. 4A) and in sweat buffer (Fig. 4B). There were no differences for CatD activity detectable between these buffer systems. In both buffer systems CatD could be completely inhibited with pepstatin A showing that the detected digestion products are exclusively generated by CatD.

DCD-1L (28.66 min) was cleaved by CatD dominantly between 44L and 45D yielding SSL-44 (25.58 min) and the tetrapeptide DSVL (9.11 min) and to a lower amount between 29L and 30E leading to the peptides SSL-29 (22.96 min) and the corresponding peptides ESV-19 (22.50 min) and ESV-15 (16.48 min), respectively. These cleavage sites agree well with the specificity of CatD, which preferably cleaves proteins and peptides with leucine or an aromatic amino acid residue in P1 position (33).

LL-37 (31.18 min) was also digested with purified CatD but only weak cleavage was seen between 5F and 6F yielding FRK-32 (28.16 min) and between 27F and 28L yielding LRN-10 (13.08 min) and LLG-27 (25.36 min). Taken together, DCD-1L proved to be a good substrate for CatD (over 60 % degradation) whereas LL-37 is barely digested by CatD (less than 20 % degradation) under the same experimental conditions. These results agree well with recently published data, stating that LL-37 is processed by a serine protease and not by an aspartate protease like CatD (17).

Since CatD shows almost no reaction with LL-37 further studies concerning the function of CatD in human eccrine sweat focused on DCD-1L. The CatD generated DCD-1L derived peptides as well as intact DCD-1L are further processed by the above described 1,10-phenanthroline sensitive carboxypeptidase causing a successive release of C-terminal amino acids. As a consequence of these in vitro results, in vivo investigations were made to find out if one or more of these predicted peptides upon cleavage with CatD and/or the carboxypeptidase are present in human eccrine sweat.

### In vivo detection of DCD-1L derived peptides in human eccrine sweat

The following DCD-1L derived peptides were found after in vitro digestion of DCD-1L with CatD: SSL-29, SSL-44, ESV-19, ESV 15 and DSVL (Tab. 2). These DCD-1L derived peptides could be further processed by the carboxypeptidase and lack one or more C-terminal amino acids leading to a bigger set of predicted DCD-1L derived peptides. Now we investigated if one or more of these DCD-1L peptides are present in human eccrine sweat. For their detection, sweat samples of 18 healthy individuals were applied to SELDI Protein Chip® Technology using reversed-phase (H4) chips. Figure 5 shows a representative SELDI-spectrum of sweat in the mass range between 2000 Da and 5000 Da. The expected and found molecular masses are assigned to the different processed DCD-1L derived peptides seen in the SELDI-spectrum and are listed in Table 3. Altogether we identified 12 DCD-1L derived peptides in sweat in agreement with recently published data (14). The DCD-1L peptides in sweat agree well with our in vitro prediction for the processing of DCD-1L. In addition, peptides lacking of the N-terminal tripeptide SSL have been identified. Further enzymatically specification of postsecretory processing was achieved by kinetic measurements.
Temporal order of the postsecretory processing

Sweat was analyzed at various time points after secretion using SELDI-MS to elucidate the temporal order of the postsecretory processing steps. Initially the 48 amino acid peptide DCD-1L is processed from its C-terminus into the peptide DCD-1 agreeing with our in vitro results that a carboxypeptidase is active in human sweat. This initial processing step starts immediately after secretion of DCD-1L into sweat and therefore is experimentally difficult to detect. Since harvesting measurable amounts of sweat takes time the initial processing steps are barely detectable. To solve this problem synthetic DCD-1L was spiked to sweat so that the initial cleavage steps could be analyzed in detail. The resulting enzyme-substrate ratio decelerated the reaction so that longer incubation was needed. After cleavage of C-terminal leucine the peptide DCD-1 (4705.3 Da) is further C-terminally processed into SSL-46 (4606.2 Da). This processing step occurs simultaneously to the N-terminal cleavage of SSL since the peptide LEK-44 (4418.0 Da) arises at about the same time (Fig. 6).

Figure 7 shows an example for the temporal order of one particular sweat sample whereas the initial processing step into DCD-1 is not apparent for the above-described reason so the time point “0 min” indicates the time point when the sweat sample was thawed and set at 37 °C to start the kinetic measurement.

After the carboxypeptidase has processed DCD-1L into DCD-1 the next processing step is further C-terminal trimming in parallel with N-terminal cleavage of the tripeptide SSL by an endoprotease. This processing step generates the peptides LEK-45, LEK-44, LEK-43, LEK-42 and LEK-41 (Fig. 7, first 4 spectra). Cleavage between 44L and 45D can be performed by CatD as well as by subsequent cleavage of four C-terminal amino acids by the carboxypeptidase. We wanted to find out which of the two proteases is mainly responsible for this processing step. Therefore we synthesized the peptide ESV-17 which represents the C-terminus of DCD-1L lacking the last C-terminal amino acids leucine and valine. ESV-17 showed no reaction with purified CatD in vitro (data not shown) indicating that the two amino acids are essential for the cleavage by CatD. This finding shows that the C-terminus of DCD-1L is dominantly processed by the carboxypeptidase. In a third processing step the previously generated peptides are cleaved between 29L and 30E by CatD obtaining the peptides LEK-26 and SSL-29 (Fig. 7, last 2 spectra). The two peptides can be generated from all present DCD-1L derived peptides in sweat differing only in their N-terminal sequence.

The final processing step consists of two reactions which occur in parallel. One reaction is the successive cleavage of the C-terminal amino acids leucine and aspartic acid of LEK-26 yielding LEK-24 which is stable against further proteolytic breakdown. The other reaction is the conversion of SSL-29 into SSL-25. Since the intermediate peptides SSL-28, SSL-27 and SSL-26 could not be detected it remains unclear whether this proteolytic degradation is also caused by the 1,10-phenanthroline sensitive carboxypeptidase or by another endoprotease. The peptide SSL-25 undergoes no further enzymatic degradation.

Single processing steps are summarized in Figure 8 considering data of various sweat samples and not only those representatively shown in Figure 6 and Figure 7, respectively. First DCD-1L is converted to DCD-1 by the 1,10-phenanthroline sensitive carboxypeptidase (Fig. 8A). In a next step DCD-1 undergoes further C-terminal trimming simultaneously to the cleavage of the N-terminal tripeptide SSL by an endoprotease (Fig. 8B). Since no aminopeptidase activity was detectable using the specific substrate H-Leu-AMC (data not shown) this processing step is caused by an endoprotease and not by an aminopeptidase. The third processing step is the cleavage between 29L and 30E by CatD obtaining the peptides LEK-26 and SSL-29 which are further processed to the proteolytical stable peptides LEK-24 and SSL-25 (Fig. 8C).

Antimicrobial activity of the DCD-1L derived peptides

Antimicrobial peptides such as LL-37 and DCD-1L play an important part in establishing a skin defense barrier against microbes and should be considered as an integral part of the innate immune system (10,12,17). Therefore the effect of the postsecretory processing on the antimicrobial activity of DCD-1L and DCD-1L derived peptides was investigated. The above-described postsecretory processing leads to the proteolytical stable peptides LEK-24 and SSL-25 which differ from all other DCD-1L derived peptides since they are cationic (for pI
values see Table 3, last column). Therefore further studies focused on these peptides. In addition, the peptide SSL-29 was chosen since recently published data showed that SSL-29 is contained in 89% of sweat samples analyzed (14). This indicates that SSL-29 is a quite stable intermediate in postsecretory processing.

In order to elucidate the biological function of the postsecretory processing by CatD, the peptides SSL-29, SSL-25 and LEK-24 were tested for their antimicrobial activity against *E. coli* (Fig. 9A) and *Staph. aureus* (Fig. 9B) and compared to the activity of DCD-1L.

DCD-1L is highly active against *E. coli* (LC50 = 6.2 μM) and *Staph. aureus* (LC50 = 2.1 μM). The peptides LEK-24 and SSL-29 were found to have lost the antimicrobial activity against these microorganisms. The peptide SSL-25, the most cationic peptide (pI = 9.4) among the DCD-1L derived peptides shows a twofold higher activity against *E. coli* (LC50 = 2.9 μM) and a slightly lower activity against *Staph. aureus* (LC50 = 2.9 μM) compared to DCD-1L.

**DISCUSSION**

Altogether we identified 10 dominant proteins in human eccrine sweat from which 6 proteins (human serum albumin, cytokeratin I, Zn-α-2-glycoprotein, prolactin-inducible protein, cystatin A, DCD-1L) have already been described in sweat or in sweat glands. Psoriasin was recently identified in keratinocytes as a secretory antimicrobial protein (5) indicating, that sweat also contains proteins secreted by keratinocytes. The proteins apolipoprotein D, lipophilin B and CatD were identified for the first time in human eccrine sweat. Their origin, from sweat glands or keratinocytes still remains to be determined. The presented sweat protein pattern summarizes the dominant proteins in human eccrine sweat and thus provides useful information for further investigations concerning skin diseases such as atopic dermatitis or psoriasis in which some of the proteins are involved e.g. psoriasin genes are markedly upregulated in psoriatic versus normal skin whereas the expression of cytokeratin I gene is, in contrast, high in normal skin and decreased in psoriatic skin (34). Regarding apolipoprotein D it has been suggested as a new prognostic factor for cutaneous malignant melanoma (35).

The presence of proteases in sweat and their function is barely understood. There are only a few proteases described until now, such as the serine proteases tissue kallikrein and kininase II (27) and the human stratum corneum chymotryptic enzyme (26). Beside these serine proteases Yokozeki et al. (25) described cysteine proteases in general to be present in sweat but without further specifications. Screening studies revealed that sweat also contains several gelatinolytic and caseinolytic proteases (24). However, the functional significance of these proteases in sweat is almost completely unknown.

Recently published data show, that a yet unidentified serine protease is responsible for the postsecretory processing of LL-37 in sweat (17) revealing one possible physiological function of sweat proteases. In consideration of these results we show that DCD-1L is postsecretory processed into 12 DCD-1L derived peptides. Additionally the temporal order of the postsecretory processing was investigated in more detail. The initial step is the cleavage of the C-terminal amino acid leucine by a 1,10-phenanthroline sensitive carboxypeptidase which has not been described in sweat until now. The data show, that the 1,10-phenanthroline sensitive carboxypeptidase resembles carboxypeptidase A (EC 3.4.17.1) concerning their specificity to release C-terminal amino acids such as Leu, Val, Phe, Ile, and Ser but little or no action with Arg, Lys, Glu, Asp or Pro (36). Furthermore the sweat carboxypeptidase is inhibited with 1,10-phenanthroline but not with EDTA indicating that it is presumably a Zn2+-metalloprotease.

The second processing step is the cleavage between L3 and L4 by an unidentified endoprotease which occurs simultaneously to further C-terminal trimming steps. Afterwards DCD-1L derived peptides are cleaved by CatD between L29 and L30 leading to the peptides SSL-29 and LEK-26 which are further processed yielding the proteolytic stable peptides LEK-24 and SSL-25.

These peptides and the peptide SSL-29 were tested for their antimicrobial activity to show the physiological significance of the postsecretory processing. The peptide SSL-25 is highly active against *E. coli* and *Staph. aureus* whereas the cleavage products LEK-24 and SSL-29 loose their antimicrobial activity.

The biological role of the postsecretory processing of the antimicrobial peptide DCD-1L is to modulate the innate immune response of human skin by the generation of a set of shortened peptides which differ in their antibacterial activity. CatD, a 1,10-phenanthroline sensitive carboxypeptidase and at
least one endoprotease are involved in the postsecretory processing of DCD-1L and therefore modulate the immune defense on the skin surface. It has been recently shown that the postsecretory processing is of physiological relevance, since the amount of several DCD-1L derived peptides in sweat of patients with atopic dermatitis is significantly reduced (14). The described postsecretory immune modulation on the skin surface may smoothen the way for new clinical approaches in treatment of skin diseases such as psoriasis since CatD is involved in the etiopathology of this inflammatory disease (37).

REFERENCES


FOOTNOTES

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1 The abbreviations used are: ACN, acetonitrile; CatD, cathepsin D; Da, daltons; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; pI, isoelectric point; PMSF, phenylmethanesulfonyl fluoride; RP-HPLC, reversed-phase high-performance liquid chromatography; SELDI-MS, surface-enhanced laser desorption/ionization mass spectrometry; TFA, trifluoroacetic acid

FIGURE LEGENDS

Table 1. Proteins identified in human eccrine sweat as determined by in-gel tryptic digestion followed by mass spectrometry analysis. In certain cases (*) data were additionally obtained by automated LC-ESI-MS/MS analysis. All MS data were analyzed using Mascot Sequence Query at www.matrixscience.de for probability based peptide identification.

Table 2. Identified peptides after *in vitro* digestion of DCD-1L and LL-37 with cathepsin D as determined by MALDI-MS and Edman microsequencing. Retention times allude to those in figure 5.

Table 3. DCD-1L derived peptides present in human eccrine sweat and their theoretical isoelectric points (pI’s). Asterisks (*) indicate the DCD-1L derived peptides generated upon cleavage with cathepsin D.

Fig. 1. 15% SDS-PAGE of 100 µl desalted and concentrated female and male sweat samples. Proteins were stained using Simply Blue™ Safe Stain.

Fig. 2. Western blot analysis of 16 µl female (f1 and f2), male (m1 and m2) and pooled (p) human eccrine sweat. Polyclonal rabbit anti-human CatD was used as primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG as secondary antibody. Purified human cathepsin D (human liver) served as control and shows a major band at 31 kDa (enzymatically active β-chain) and weaker bands at 50 kDa representing several proforms.
The CatD substrate Amca-EKPIFFRLGK was incubated with CatD (B), without CatD (A), with CatD and the aspartate protease inhibitor pepstatin A (C) and with CatD and 1,10-phenanthroline. The substrate further was incubated with pooled sweat (D) and with pooled sweat in the presence of pepstatin A (E), iodacetamide (F), E-64 (G), leupeptin (H), PMSF (I), EDTA (J) and 1,10-phenanthroline (K). Digestion products were then separated using RP-HPLC with fluorescence detection ($\lambda_{ex}=350$ nm; $\lambda_{em}=450$ nm).

In vitro digestion of synthetic DCD-1L and LL-37 with CatD in 50 mM Gly/HCl buffer pH 3.5 (A) and in sweat buffer pH 5.5 (B). Digestion products were separated using RP-HPLC with UV-detection at 214 nm. Peaks then were collected and peptides were analyzed using MALDI-MS and Edman microsequencing (see Table 2).

In vivo detection of DCD-1L derived peptides in human eccrine sweat using SELDI protein chip® technology. 1 µl sweat was diluted into 2 µl binding buffer (50 mM sodium phosphate pH 6.5) and applied to a reversed phase (H4) chip surface for 30 min in a humid chamber. After washing three times with binding buffer and a final water wash, peptide masses then were read directly from the array surface using a ProteinChip reader.

50 µl pooled sweat was spiked with 12 µg synthetic DCD-1L and analyzed by SELDI protein chip® technology after 1 h (upper lane) and 15 h (lower lane) at 37 °C.

SELDI spectra of sweat after the indicated incubation times at 37 °C. Mass labels refer to the DCD-1L derived peptides listed in table 3.

Temporal order of the postsecretory processing of DCD-1L in sweat. The initial processing step is the cleavage of the C-terminal amino acid leucine by a 1,10-phenanthroline sensitive carboxypeptidase (A). The second processing step is the cleavage between $^3$L and $^4$L by an unidentified endoprotease simultaneously to further C-terminal trimming steps (B). The generated DCD-1L derived peptides are finally digested by CatD yielding LEK-26 and SSL-29 which are processed to LEK-24 and SSL-25, respectively (C).

The antimicrobial activity of DCD-1L and DCD-1L derived peptides SSL-29, LEK-24 and SSL-25 evaluated by colony-forming units assay against *E. coli* (A) and *Staph. aureus* (B).
Figure 2

<table>
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<th>f1</th>
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<th>f2</th>
<th>m2</th>
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</table>

CatD proforms
CatD β-chain
Figure 3

Fluorescence intensity (arbitrary units)

Time (min)

11.6 min: 1,10-phenanthroline
21.7 min: Amca-EEKPISFFRLGK
20.6 min: Amca-EEKPISF
16.2 min: Amca-EEKPIS

Amca-EEKPISFFRLGK
+ 1 µg cathepsin D
+ 1 µg cathepsin D
+ 10 mM 1,10-phenanthroline
+ pooled sweat
+ 10 µM pepstatin A
+ 10 mM iodacetamide
+ 10 µM E-64
+ 12.6 µM leupeptin
+ 1 mM PMSF
+ 10 mM EDTA
+ 10 mM 1,10-phenanthroline
Figure 4

A) DCD-1L

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B) LL-37

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<td>+ CatD + pepstatin A</td>
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</table>
Figure 5
Figure 6

The figure shows mass spectrometry data with two panels, one labeled 1 h and the other labeled 15 h. The x-axis represents m/z values ranging from 4000 to 5000, and the y-axis represents relative intensity from 0 to 100.

Key peaks are highlighted at m/z values: 4416.4 m+H, 4706.8 m+H, and 4819.4 m+H. The data shows a decrease in intensity over time, especially noticeable in the 15 h panel.
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<td>48</td>
<td>4819</td>
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*protein scores greater than 63 are significant (p<0.05)
Table 2

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n.f. = not found
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Cathepsin D is present in human eccrine sweat and involved in the postsecretory processing of DCD-1L


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