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Detection of dermcidin-derived peptides in sweat by ProteinChip® Technology

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Abstract

Recently, a novel antimicrobial peptide DCD-1, derived from the *Dermcidin* (DCD) gene and secreted by sweat glands, has been described by Schittek et al. [*Nat. Immunol.* 2 (2001) 1133.]. Here we describe the application of the surface-enhanced laser desorption/ionisation (SELDI) technology for the detection of DCD-1 and other dermcidin-derived peptides directly from microlitre amounts of human sweat. The advantages of the technique are as follows: (a) it can be carried out with ease and rapidity; (b) multiple samples can be processed simultaneously; (c) prior purification is not required; and (d) only a limited sample volume is necessary for both protein profiling and semiquantitation. Profiling of human sweat from various donors revealed that in addition to DCD-1, other DCD-derived peptide species were also present in significant quantities. Four of five identified peptides were DCD-1 related, while the fifth corresponded to a portion of the DCD protein outside the DCD-1 core. This provides clues as to how the novel protein is processed to its active form, though further work remains to elucidate this fully. Thus, we have demonstrated the applicability of such technology to the detection of DCD-1 and for the protein profiling of sweat in general. Such studies could reveal valuable new biomarkers for diagnosis and treatment of skin and sweat gland disorders.

Keywords: SELDI; Antimicrobial peptides; Sweat; Dermcidin; Mass spectrometry

1. Introduction

Dermcidin was identified for the first time as a gene specifically expressed in sweat glands, coding for a 110 amino acid protein (DCD) that is proteolytically processed to form a 47 amino acid peptide found in secreted sweat and encompassing positions 63–109 of the preprocessed product. This peptide was named

Abbreviations: RP-HPLC, reversed phase high-performance liquid chromatography; TFA, trifluoroacetic acid; MALDI, matrix-assisted laser desorption/ionisation; TOF, time of flight; SELDI, surface-enhanced laser desorption/ionisation; Da, daltons; *m/z*, mass-to-charge ratio; FMOC, 9-fluorenylmethyloxycarbonyl.

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DCD-1 and was shown to exhibit various antimicrobial activities. It displayed dose- and time-dependent toxicity against organisms such as *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, as well as the fungi *Candida albicans*, at pH and salt conditions characteristic of human sweat (Schittek et al., 2001). Thus, the dermcidin peptide probably plays a key role in the innate immune response of the skin. DCD shares no homology with any known antimicrobial peptides described to date and, as yet, nothing is known of its mode of processing or interindividual variation in the amount of peptide processed.

By comparison with the analysis of bodily fluids such as blood serum/plasma or urine, the analysis of sweat for its protein constituents has not received much attention. Relatively large volumes of sweat sample are needed, usually requiring thermal (mainly saunas) or exercise induction methods in order to obtain enough sample to detect proteins. Subsequent analyses have relied mostly upon immunoassays, immunoblotting or 2D gel electrophoresis for their identification and characterisation (Marshall, 1984; Saito, 1994; Imayama et al., 1995). Studies have revealed associations between proteins present and disease states. For example, levels of allergen-specific IgE antibodies and the severity of dermatitis (Jung et al., 1996) were correlated in sweat analyses, as was the absence of a 60-kDa protein in the sweat of a cystic fibrosis patient (Penneys et al., 1984). Functional studies of proteins in sweat have also been reported: biologically active interleukin 1 (IL-1) was shown to be present in sweat, with a differential distribution of IL-1 alpha and beta at different sample locations on the body (Didierjean et al., 1990). Also, interleukin 8 has been shown to be produced by the human sweat gland epithelium and is postulated to act as a chemoattractant for neutrophils in sweat (Jones et al., 1995).

The recent trend to develop high-throughput, sensitive methods for the analysis of proteins in mixtures has led to the development of technologies such as surface-enhanced laser desorption/ionisation (SELDI), embodied commercially in the ProteinChip® System (Ciphergen Biosystems, Fremont, CA), which combines activated or chromatographic surfaces (ProteinChip Arrays) with solid-state time of flight (TOF) mass spectrometry. SELDI was first introduced by Hutchens and Yip (1993), and its applications are described in recent reviews by Merchant and Weinberger (2000) and Fung et al. (2001). The technology facilitates a rapid, sensitive and reproducible 'protein profile' of complex protein mixtures by mass and has been successfully applied to the definition of candidate disease biomarkers, for example, in prostate (Wright et al., 1999), breast (Watkins et al., 2001) and renal cancers (von Eggeling et al., 2001). Recently, the antimicrobial β-defensins were detected as protein constituents of human saliva (Diamond et al., 2001) using SELDI, demonstrating the application of the technology for direct analysis of functionally significant proteins in bodily fluids. For the purpose of this study, SELDI technology was used to develop a fast and easy method that can be used to profile many different human sweat samples simultaneously for their content of DCD-1, with a view to transferring the process to detecting proteins relating to skin disorders. Here, we demonstrate that SELDI technology can be applied to defining DCD-derived peptides as the dominant peptide constituents of human sweat, as well as direct semiquantitation of DCD-1 from an initial sample volume as low as 1 μl.

2. Materials and methods

2.1. Collection of sweat

Sweat (5–100 μl) was collected from healthy donors with the use of a 1.5-ml polypropylene cup applied to the surface of the face, neck or chest after different methods of induction (physical exercise or sauna). From one donor, a larger volume of sweat (15 ml) was collected in a 50-ml polypropylene tube. This was subsequently subjected to RP-HPLC fractionation and Edman sequencing. This sample was the same as that used by Schittek et al. (2001) for the first identification of DCD-1.

2.2. RP-HPLC fractionation of sweat with subsequent Edman sequencing

Fractionation of sweat was performed as described recently (Schittek et al., 2001). Briefly, 10 ml of pooled sweat from the single donor was lyophilized overnight and the material redissolved in 500 μl of 10% acetic acid. After centrifugation, 100 μl of the

supernatant was applied to RP-HPLC on a Nucleosil C18 column (150 × 4 mm) with 5-μm particles and a flow rate of 1 ml/min. Solvent A was 0.055% aqueous trifluoroacetic acid (TFA); Solvent B was 80% acetonitrile in 0.050% aqueous TFA. A linear gradient of 0% B to 60% B over 40 min was used. The resulting elution peaks were concentrated in a vacuum concentrator to give a volume of ~ 15 μl. Single fractions were analysed by automated Edman degradation with an Applied Biosystems 494 protein sequencer (Applied Biosystems-MDS Sciex, Concord, Canada).

2.3. SELDI analysis of sweat

The sweat samples were analysed on three different ProteinChip Arrays: A strong anion exchange (SAX2), a weak cation exchange (WCX2) and a reversed phase (H4) chip (Ciphergen Biosystems) were used. In the first step, the ProteinChip Arrays were equilibrated for 5-min periods with two binding buffers (SAX2, PBS buffer pH 7.2 and WCX2, 50 mM sodium acetate pH 4.0; H4, 50 mM sodium phosphate pH 6.5). Human sweat (1 μl) was diluted into 4 μl binding buffer, and the chromatographic arrays were incubated in a humid chamber for 30 min. After incubation, the chips were subjected to a 3 × 5 min wash with binding buffer. As a final step, a further wash with water was performed to remove interfering substances such as salts. After drying, a saturated solution of sinapinic acid in 50% acetonitrile (v/v), 0.5% trifluoracetic acid was added. Protein and peptide masses were read directly from the array surfaces with a ProteinChip Reader (Ciphergen Biosystems). The instrument was either externally calibrated using two different synthetic dermcidin peptides (DCD-1 and DCD positions 66–109) and bovine insulin on a separate calibration chip with the corresponding position for each spot, or internally by adding 100 fmol of porcine dynorphin A (209–225; MW 2147.5u), ACTH (MW 2933.5u) and bovine insulin (MW 5733.6u). For the preparation of the H4 array, an alternative faster protocol was also employed in which 1 μl of sweat was added to 2 μl 50 mM sodium phosphate, pH 6.5. The array surface was pretreated with 3 μl of 50% (v/v) acetonitrile/water for 1 min with 50% (v/v) acetonitrile/water, dried and 1 μl of the diluted sweat solution was added to the H4 array. Shortly after drying, a water wash was performed on the spot followed by the addition of matrix solution. For the semiquantitation of DCD-1 in the sweat sample described by Schittek et al. and for the standard curve, 100 fmol of bovine insulin was added to each spot and the peak intensities of insulin were used for normalisation. These experiments were done in triplicate and the average for each concentration was used. The data were analysed using the ProteinChip Software (Version 3.0).

2.4. Peptide synthesis

The DCD-1 peptide (amino acids 63–109 of dermcidin) and the synthetic peptide encompassing positions 66–109 of dermcidin were synthesized by the FMOC solid phase strategy on a MilliGen 9050 continuous flow synthesizer (Millipore, Bedford, MA). After RP-HPLC purification, the peptides were lyophilized and resolved in 50 mM phosphate buffer pH 6.5 to a concentration of 1 μg/μl. Serial dilutions were made to achieve the concentration range used within the calibration curve.

3. Results

3.1. Analysis of sweat using SAX2, WCX2 and H4 ProteinChip Arrays

Based on the biochemical properties of DCD-1, it should be possible to detect the peptide on different ProteinChip Arrays. Therefore, to test for binding sensitivity and ease of preparation, the same sweat sample as used for the initial identification of DCD-1 by Schittek et al. (2001) was applied to three different ProteinChip Arrays. DCD-1 (theoretical average mass 4705.3 Da) was easily detectable on all three surfaces (strong anion exchange [SAX2]; weak cation exchange [WCX2]; reversed phase [H4]) and as the dominant peptide even when using a sweat sample volume as small as 1 μl (Fig. 1A–C). In the mass range up to 10 kDa, all three surfaces demonstrated similar peak patterns with the most abundant signals observed between 4 and 6 kDa. Differences in the binding properties of the surfaces were observed as expected as shown, for example, by the presence of a peak at *m/z* 2869.7 only on the WCX2 (Fig. 1B). For further studies, the H4 chip was

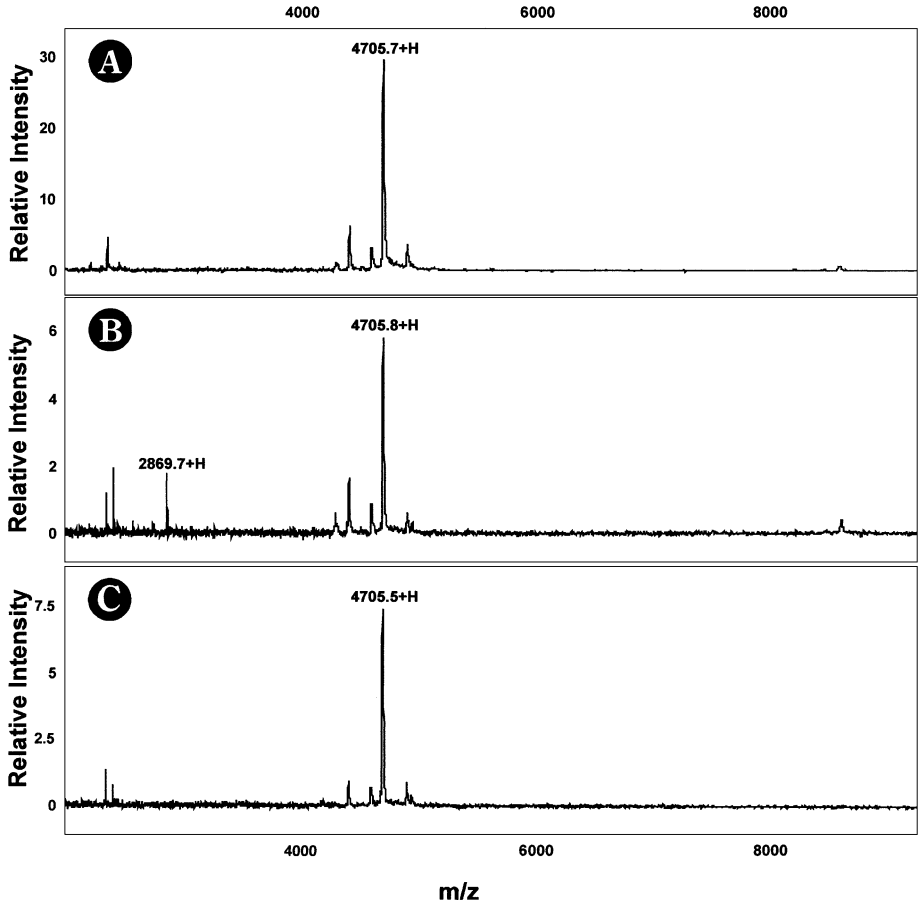


Fig. 1. Analysis of sweat on different ProteinChip Arrays : 1 μl of the sweat from one donor was diluted into 4 μl of binding buffer. This solution was applied to different chip surfaces. (A) Strong anion exchange surface (SAX2), binding buffer, PBS pH 7.2; (B) weak cation exchange surface (WCX2), binding buffer, sodium acetate pH 4.0; (C) reversed phase surface (H4), binding buffer, 50 mM sodium phosphate pH 6.5.

selected due to the opportunity to use a faster preparation protocol.

3.2. Identification of other DCD-related peptide species within sweat

Fig. 1 demonstrates DCD-1 to be the dominant peptide species in sweat below 10 kDa and is readily seen at a low laser intensity by SELDI on all chip surfaces tested and at a good signal/noise ratio. This peptide had previously been sequenced using Edman degradation by Schittek et al. (2001) in order to confirm its identity as DCD-1. However, in order to analyse other peptide species present, the same sample as in Fig. 1C was remeasured using a higher laser energy (Fig. 2A). Several peaks within the *m/z* 4000–6000 range were detected, at least five of which have been identified so far by automated Edman degradation to be of DCD origin (Fig. 2B and C). These were *m/z* 4302.5 (theoretical average mass 4302.6 Da); 4319.1 (theoretical average mass 4318.9 Da); 4418.6 (theoretical average mass 4418.0 Da); 4606.8 (theoretical average mass 4606.2 Da); and DCD-1 itself at 4705.9 (theoretical average mass 4705.3 Da). Three of the five peaks identified contained species relating to DCD-1 with variations occurring at the N- and/or C-termini (**b**, **c** and **d** in Fig. 2B and C), whereas peak **a** (*m/z* 4302.5) contained the sequence from positions 20–61 of the full DCD sequence and is therefore unrelated to DCD-1 (positions 63–109).

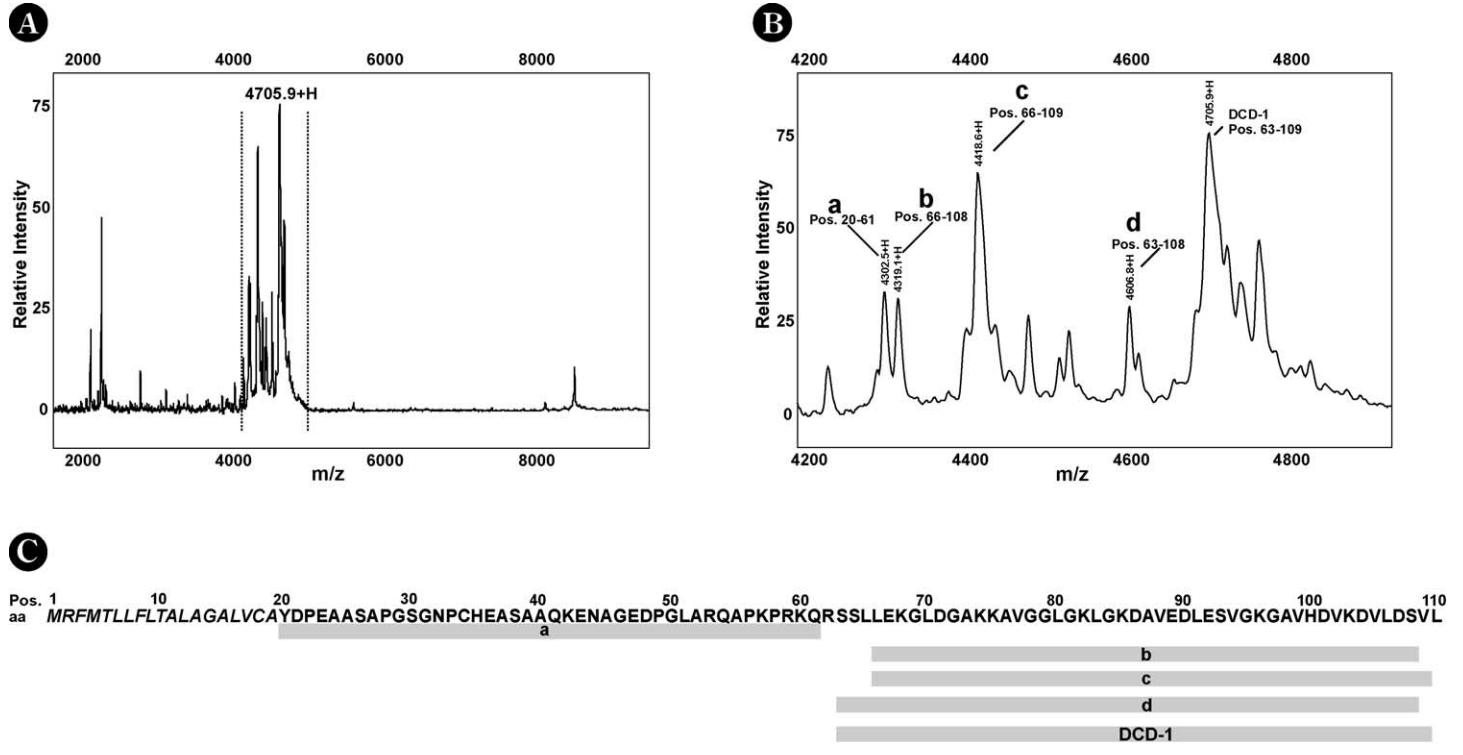


Fig. 2. Analysis of dermcidin-related peptides in sweat on an H4 ProteinChip Array: (A) The SELDI spectrum of the sweat sample shows the DCD-1 peptide at *m/z* 4705.9 as the dominant species. (B) Higher resolution of the region between *m/z* 4200 and 5000 (dotted lines in row A) shows four additional peptides (**a**–**d**) that derived from the DCD protein. (C) Entire amino acid sequence of DCD (110 aa), the signal peptide is highlighted by italics. Grey boxes represent portions of the DCD protein that have been identified within the sweat sample.

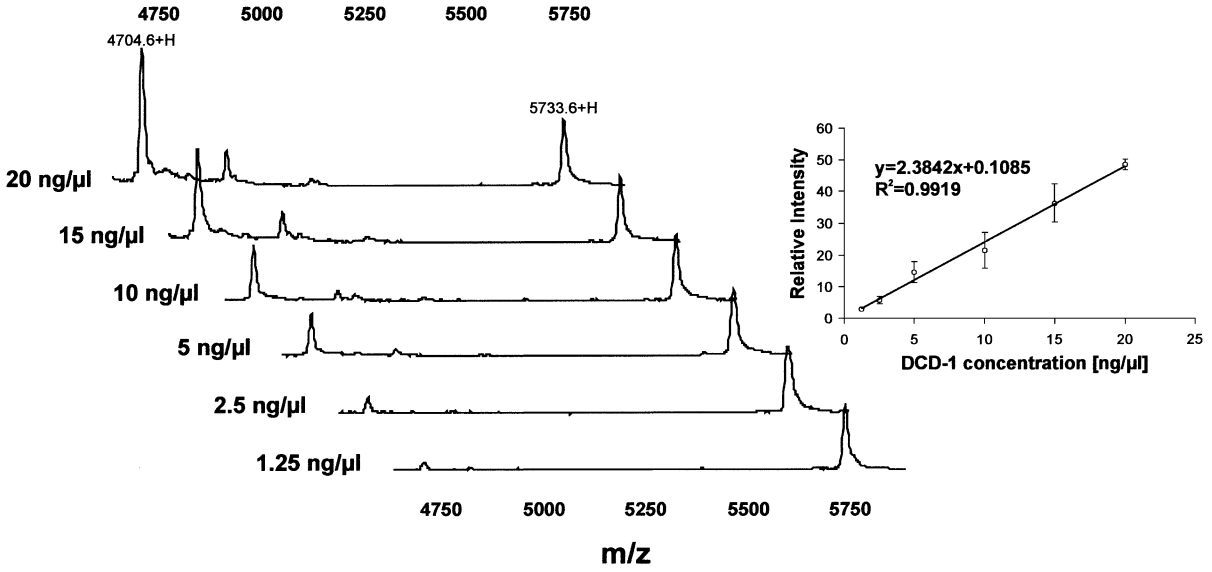


Fig. 3. Semiquantitation of the DCD-1 peptide by SELDI. SELDI analysis was performed in triplicate. The spectra show the median value for each concentration of the synthesized DCD-1 peptide (concentrations as indicated; the small peak at *m/z* 4912.4 is due to a minor impurity from the peptide synthesis). For the calibration curve (insert on the right), the average value of the triplicates was used. Standard deviations for each concentration are shown as error bars. The average DCD-1 value for the sweat sample analyzed was 10.4 ng/µl (9.89, 11.53 and 9.77 ng/µl) with a standard deviation of 0.98 ng/µl.

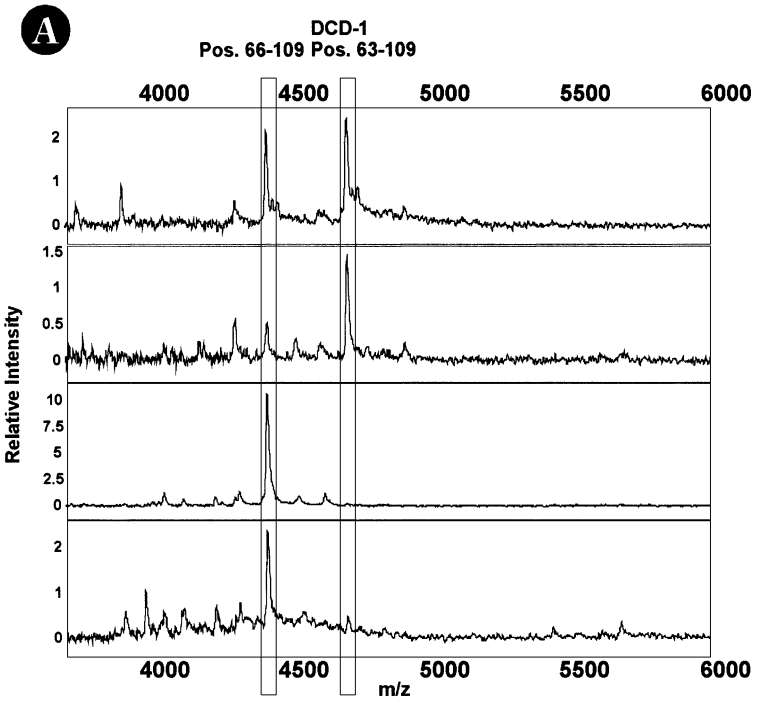
3.3. Semiquantitation of DCD-1 in sweat

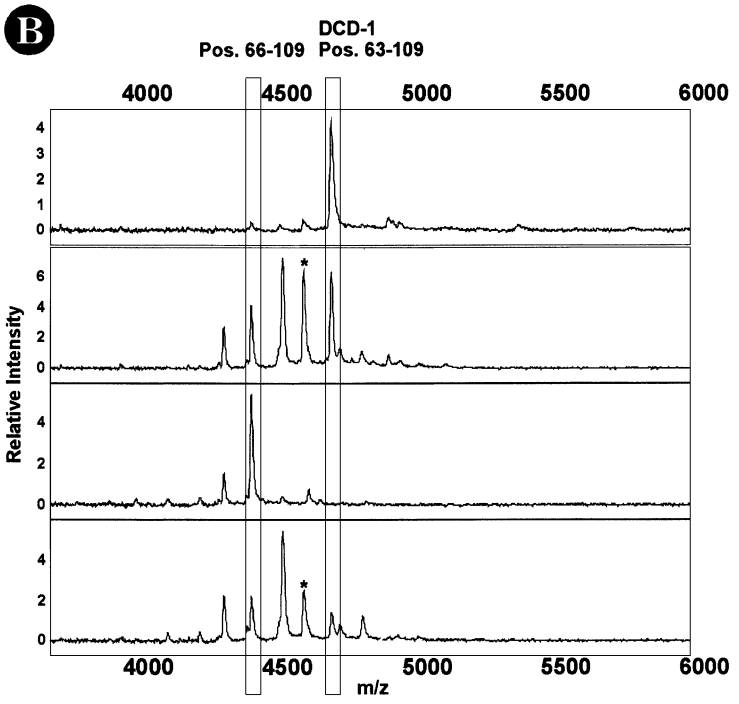
Previously, the concentration of DCD-1 in sweat was estimated to be in the range of 1–10 ng/µl based on the ultraviolet signal of the RP-HPLC fractionation of the sweat sample (Schittek et al., 2001). We assessed the same sample for its DCD-1 content using SELDI technology. Therefore, a calibration curve measuring the concentration of a synthetic DCD-1 peptide in proportion to signal intensity, after normalisation to the signal intensity of 100 fmol bovine insulin added to each sample, was measured using various DCD-1 concentrations (1.25–20 ng/µl) on an H4 chip. Fig. 3 demonstrates a good correlation between signal intensity and DCD-1 concentration. Experiments were performed in triplicate, giving rise to an average signal intensity in the calibration curve (Fig. 3, insert). Under the conditions employed, a linear range from 1.25 ng/µl to the highest concentration of 20 ng/µl was obtained. Spiking of synthetic DCD-1 into sweat samples devoid of DCD-1 gave similar peak intensities in SELDI analyses (data not shown) as an indicator for similar ionisation of DCD-1 under the conditions for the calibration curve and in complex sweat samples, where "quenching" effects could not be excluded. From the calibration curve, we determined that the concentration of DCD-1 in the sweat sample was about 10.4 ng/µl (standard deviation 0.98 ng/µl). This is at the upper limit of the range estimated in the previous study.

3.4. Profiling DCD-related peptides in human sweat from different donors

To evaluate whether the protein profile, including DCD-1 as the dominant species, obtained from the initial sweat sample was representative, a further 34 samples from normal donors were analysed. Fig. 4 shows eight representative donor profiles, four females (Fig. 4A) and four males (Fig. 4B). Clearly,

Fig. 4. Representative spectra of eight different sweat samples from four female (A) and four male (B) donors. The two most dominant peptide peaks encompass positions 66–109 (theoretical average mass 4418.0 Da) and 63–109 (theoretical average mass 4705.3 Da, DCD-1) of the DCD sequence and are indicated by boxes. Peaks labeled with an asterisk correspond to positions 63–109 of the DCD sequence (theoretical average mass 4606.2 Da).


A
DCD-1
Pos. 66-109 Pos. 63-109
4000
4500
5000
5500
6000
Relative Intensity
m/z


B
DCD-1
Pos. 66-109 Pos. 63-109
4000
4500
5000
5500
6000
Relative Intensity
m/z

DCD-1 (average mass 4705.33 Da) is not the only dominant peptide species in all the sweat samples. Of the eight samples shown in Fig. 4, it is the dominant species in only three, while being present in a further three and undetectable in two. A second peptide species, *m*/*z* 4418, corresponding to peptide **c** in Fig. 2 (average mass 4418.02 Da), was identified by Edman degradation as being positions 66–109 of the DCD protein. This peptide was detectable in all samples displayed in Fig. 4 and predominated in three samples. SELDI analyses with equimolar concentrations of synthetic DCD-1 and the synthetic peptide **c** showed almost equal peak intensities (data not shown) as an indicator for similar ionisation capabilities of both peptides. So far, we have observed no apparent linkage of the distribution of the DCD peptides with sex. A third peak, identified in Fig. 2B as positions 63–108 of DCD with an average mass of 4606.2 Da and labelled with an asterisk, was also seen with differing intensities between the samples, though not predominating in any. Further peaks were detected between 4 and 6 kDa, but without identification, it is not clear whether they are DCD related or not.

4. Discussion

We have applied SELDI mass spectrometric technology for the analysis of sweat samples for the presence of the novel antibiotic peptide DCD-1, derived from the recently described *Dermcidin* gene (Schittek et al., 2001). The technology enabled us to rapidly detect the peptide as well as profile the sample for other peptide species derived from DCD and required a sample volume of only 1 μl of sweat.

In addition, the use of the ProteinChip Arrays also permitted us to semiquantify DCD-1. In SELDI, the arrays act as a surface to which the sample binds uniformly, and the matrix for mass analysis is placed on the chip after the proteins are bound. This gives more uniform and reproducible mass spectra and enables relative protein quantitation (Fung et al., 2001). Moreover, most mass spectrometric techniques encounter ionisation problems when confronted with high concentrations of salt. This would render the direct analysis of sweat by MALDI MS insensitive due to the naturally high salt concentration of sweat. The SELDI process, however, where proteins are retained on the array surface while washing steps in binding buffer are used to desalt the sample, improves and facilitates the direct analysis of the sweat sample. This "on-chip" step also means that protein loss is minimised, a point that can be crucial when attempting to quantify a single peptide species in a volume as small as 1 μl. The use of other commercially available techniques to desalt would involve an extra preparatory step that could lead to sample loss. However, we cannot exclude the possibility that the DCD-1 semiquantitation in some sweat samples might be influenced by substances that could cause a suppression of ionisation of the analyte, as the measuring of peptides/proteins in complex mixtures can lead to a "quenching" of signals, though we have not observed such effects in initial analyses that spike DCD-1 into sweat samples devoid of DCD-1.

A major outcome of this study is that, in addition to DCD-1, other DCD-derived peptides were detected simultaneously in differing quantities in the various sweat samples. This poses an interesting question as to whether DCD-1 is indeed the dominant peptide species in sweat. Fig. 2B shows the presence of other DCD-derived peptides, three of which are variations of DCD-1 itself and a fourth is derived from the N-terminus of the DCD protein after the signal peptide. Various proteases in sweat have been reported, including kallikrein and kininase II (Hibino et al., 1994), gelatinolytic proteases (Horie et al., 1986) and cysteine proteases (Yokozeki et al., 1987), which could lend support to a theory of DCD being processed to its active DCD-1 form soon after secretion into the sweat. The identification of a fourth peptide in the sample not related to DCD-1, but to the rest of the processed DCD, would also suggest a proteolytic cleavage of the protein to its active form after secretion.

From sweat sample analyses of different donors, we see that DCD-1 is not always present as the dominant peptide as it is also found either in conjunction with the other peptide species identified, or not at all. Indeed, the species corresponding to *m*/*z* 4418.0 in Fig. 2B is detected in all samples analysed in Fig. 4 and differs from DCD-1 by the absence of only three amino acids at the N-terminus. All of the DCD-1-related peptides detected in the individual samples exhibited cleavage at the ends, possibly due to the action of trimming proteases. Moreover, DCD-1 itself differs at the C-terminus from full DCD by the

absence of a single leucine (L), which could also be achieved by the activity of such an exopeptidase. Further exopeptidase activity would sequentially cleave amino acids from each peptide end to produce the species **b**, **c** and **d** in Fig. 2B and C. With variations in individual sweat secretion rates and induction methods, the activity of such enzymes could quite easily create the differences in the peptide profiles seen. However, this topic will require further analysis in order to deduce fully the method by which DCD is processed to forms with antimicrobial activity. The pooling of sweat from different regions of the body could also lead to variation. As with the site-dependent variation seen with interleukin 1 species (Didierjean et al., 1990), sweat collection from different sites could lead to a difference in the distribution of DCD-1 between samples. It is still unclear whether the same site-dependent variation is seen with DCD-1 species.

We have been unable to detect either a mass higher than that of DCD-1 corresponding to full-length DCD (positions 1–110), or DCD without signal peptide (positions 20–110), suggesting that DCD-1 or related peptides may still be secreted into sweat in a processed form. It is yet to be shown whether the other dominant peptide forms retain the full antimicrobial activity shown by DCD-1. Further studies with larger sample sets will be needed to investigate the expression pattern and processing of DCD-1. However, the methodology presented here should permit the expression, secretion and processing of DCD-1 and other dermcidin-derived peptides to be studied in more detail in order to unravel the detailed biological function of these novel antimicrobial peptides. Of special interest for the future is the application of this method to studies involving sweat from patients suffering with dermatoid diseases such as acne vulgaris, psoriasis and atopic dermatitis. This could lead to the discovery of novel diagnostic and prognostic factors, as well as providing an insight into novel treatments for these diseases.

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