
ENZYME CATALYSIS AND REGULATION:

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# A Specific Bacterial Aminoacylase Cleaves Odorant Precursors Secreted in the Human Axilla*

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**Andreas Natsch‡, Hans Gfeller, Peter Gygax, Joachim Schmid, and Gonzalo Acuna§**

*From the Givaudan Dübendorf Ltd., Ueberlandstrasse 138, CH-8600 Duebendorf, Switzerland*

**Human axillary odor is known to be formed upon the action of *Corynebacteria* sp. on odorless axilla secretions. The known axilla odor determinant 3-methyl-2-hexenoic acid was identified in hydrolyzed axilla secretions along with a chemically related compound, 3-hydroxy-3-methylhexanoic acid. The natural precursors of both these acids were purified from non-hydrolyzed axilla secretions. From liquid chromatography/mass spectrometry analysis, it appeared that the acids are covalently linked to a glutamine residue in fresh axilla secretions, and the corresponding conjugates were synthesized for confirmation. Bacterial isolates obtained from the human axilla and belonging to the *Corynebacteria* were found to release the acids from these odorless precursors *in vitro*. A $Zn^{2+}$-dependent aminoacylase mediating this cleavage was purified from *Corynebacterium striatum* Ax20, and the corresponding gene *agaA* was cloned and heterologously expressed in *Escherichia coli*. The enzyme is highly specific for the glutamine residue but has a low specificity for the acyl part of the substrate. *agaA* is closely related to many genes coding for enzymes involved in the cleavage of N-terminal acyl and aryl substituents from amino acids. This is the first report of the structure elucidation of precursors for human body odorants and the isolation of the bacterial enzyme involved in their cleavage.**

The axilla region of humans contains a dense arrangement of apocrine, eccrine, and sebaceous glands, and it is an everyday experience, that volatile substances emanating from these areas make a key contribution to human body odor. Although this odor is perceived by today's society as mainly unpleasant, several studies indicate that it may contain chemical signals that affect the menstrual cycle (1) or that may be involved in a major histocompatibility complex allele-dependent mate selection (2). These studies point to an important role of body odors in the evolutionary history of man.

Sweat as it is secreted by axillary glands is odorless. Since the pioneering work of Shelley *et al.* (3), it is known that (*a*) the typical strong axilla odor can only be released from apocrine secretions, and (*b*) that the action of skin bacteria is needed to generate the odoriferous compounds from non-smelling molecules present in these secretions. Indeed, the axilla is a skin region supporting a dense bacterial population, which is dominated by the two genera *Staphylococcus* and *Corynebacteria* (4, 5). Most individuals carry a flora that is dominated by either one of these two genera, and a strong correlation was found between a high population of *Corynebacteria* and a strong axillary odor formation (4, 6). As a practical consequence of these findings, halogenated antibacterials and aluminum preparations for reducing the bacterial population have become the main active ingredient of commercial deodorants for the last 40 years. The scientific conclusion from this early work was that axilla secretions contain non-odoriferous precursors that must be transformed by bacterial enzymes only present in *Corynebacteria* and not in *Staphylococci*. Considerable progress has been made since then to identify the odoriferous compounds in human body odor, but the biochemistry of axillary odor formation has received relatively little attention. Indeed, no precursor structure has been isolated from axilla secretions and confirmed by re-synthesis, and no specific bacterial enzyme recognizing such a structure has been isolated and reported in the literature. Notwithstanding, several theories and indirect evidence have been published.

The focus of early studies was mainly on odoriferous steroids after the boar pheromone 5α-androst-16-en-3-one (7, 8) and a related odoriferous steroid 5α-androst-16-en-3α-ol (9) had been detected in human axilla secretions. Nevertheless, androstenone is only perceived by 50% of the human population (10) and thus cannot be the main odoriferous component. Release of androstenol from secreted sulfates and glucuronides in the axilla was proposed to be mediated by bacterial enzymes (11). Nevertheless, the steroid precursors were not isolated, and clear evidence for corynebacterial enzymes cleaving sulfate and glucuronide conjugates of steroids was not presented, although arylsulfatase and aryl glucuronidase activity was found in these bacteria. The involvement of thiols as key odor components and the putative implication of a pyridoxal phosphate-dependent β-lyase (probably related to cystathionine β-lyase) in the release of these thiols has been proposed (12), but without data on the structure of the secreted precursor or isolation of the bacterial enzyme. A very thorough analysis of the chemical composition of axillary odor was presented by Zeng *et al.* (13). They proposed that short, branched fatty acids make a major contribution with (*E*)-3-methyl-2-hexenoic acid (3M2H)[1] being the key odor component. This compound had initially been found in sweat of schizophrenic patients (14), and whether its presence is a specific marker of schizophrenia was the subject of a longer debate. Zeng *et al.* (13) could clearly

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF534871.*

‡ To whom correspondence should be addressed. Tel.: 41-1-824-21-05; Fax: 41-1-824-29-26; E-mail: andreas.natsch@givaudan.com.

§ Present address: Hoffmann-La Roche, Basel, Switzerland.

[1] The abbreviations used are: 3M2H, (*E*)-3-methyl-2-hexenoic acid; MTBE, methyl-*tert*-butyl-ether; HMHA, 3-methyl-3-hydroxyhexanoic acid; 3M2H-Gln, $N^{\alpha}$-(*E*)-3-methyl-2-hexenoyl-glutamine; HMHA-Gln, $N^{\alpha}$-3-methyl-3-hydroxy-hexanoyl-glutamine; bis-Tris, bis-(2-hydroxyethyl)-amino-tris-(hydroxymethyl)-methane; LC-MS, liquid chromatography/mass spectrometry; GC-MS, gas chromatography-mass spectrometry; ESI-MS, electrospray ionization-mass spectrometry; Z, carbobenzyloxy.

show its contribution to axillary odor of healthy donors. They could also show (15) that it is non-covalently associated with apolipoprotein D, the major protein present in axilla secretions. Furthermore, they showed that 3M2H is released upon incubation of the aqueous fraction of apocrine secretions with *Corynebacteria* or hydrolysis with NaOH, indicating a water-soluble, covalent precursor must be present (16). Yet its structure and the bacterial enzyme involved in its cleavage were not described. Here we report the isolation of a new branched fatty acid related to 3M2H and covalent linkage of both acids to L-glutamine in human axilla secretions. Furthermore, a specific zinc-dependent aminoacylase, which triggers the release of these odoriferous components from odorless axilla secretions, was purified from axilla isolates of *Corynebacteria,* characterized, and heterologously expressed in *Escherichia coli.*

## EXPERIMENTAL PROCEDURES

*Materials*—Unless otherwise noted, all chemicals were purchased from Fluka (Buchs, Switzerland). Z-protected amino acids and peptides as enzyme substrates were from Aldrich and from Senn Chemicals (Dieseldorf, Switzerland).

*Collection of Axilla Secretions*—Fresh axilla secretions were sampled from healthy human donors (27 male and 12 female) by placing a stainless steel cylinder (internal diameter 3.6 cm) onto the axilla, adding 4 ml of 10% ethanol and massaging the area with a metal spatula for 1 min. This procedure does not discriminate between apocrine and eccrine secretions. The samples were immediately frozen and concentrated by lyophilization. The samples were redissolved in 400 $\mu$l of water and then extracted twice with MTBE and twice with hexane to remove interfering lipids. At this stage the samples were split, and half was saved for analysis of the potential precursor compounds. For analysis of the odoriferous components, 50 $\mu$l of NaOH (5 M) was added to the remaining half, and the samples were heated for 20 min at 100 °C. They were then re-acidified by adding 50 $\mu$l of HCl (5 M) and applied to a solid phase extraction C-18 Bond Elute solid phase extraction cartridge (Varian). The column was washed with $H_2O$ and eluted with 50 $\mu$l of MTBE. For analysis, the samples of several donors were pooled.

*GC-MS Analysis of Hydrolyzed Axilla Secretions*—For qualitative GC-MS analysis of the solid phase extraction extracts, a combination of a Hewlett-Packard 5890 II gas chromatograph and a Finnigan MAT95 mass spectrometer (low resolution, 70-eV EI mode, ion source temperature 230 °C) was applied. A DB-5 column (J & W Scientific) with a length of 60 m, inner diameter of 0.25 mm, and film thickness of 0.25 $\mu$m was used. 1.5 $\mu$l of the MTBE solution were injected in the splitless injection mode (230 °C). The temperature of the column oven was initially set to 30 °C for 5 min and subsequently increased to 50 °C (10 °C/min) and then to 250 °C (2 °C/min).

*Fractionation and LC-MS Analysis of Aqueous Axilla Secretions*—The concentrated, pooled, non-hydrolyzed sample was separated on a Superdex Peptide HR10/30 gel filtration column (Amersham Biosciences) using $(NH_4)_2CO_3$ (100 mM) as elution buffer. Individual fractions of this separation step were tested for the content of an axillary odor precursor by hydrolysis of aliquots in 1 M NaOH. The fractions developing strong odors upon hydrolysis were subjected to LC-MS/MS analysis using a Finnigan LCQ mass spectrometer operated in the atmospheric pressure chemical ionization mode and equipped with a Flux Rheos 2000 high pressure liquid chromatography pump. High pressure liquid chromatography separation was performed on a $C_{18}$ RP column modified for proteins and peptides (Vydac, Hesperia, CA). The mobile phase consisted of $H_2O$ (A) and MeOH (B) each containing 1% HOAc (v/v). The solvent flow was 0.25 ml/min and the following gradient was used: 0–1 min, 100% A; 1–6 min from 100% A to 100% B, 6–11 min 100% B, 11–13 min from 100% B to 100% A. MS/MS spectra were recorded with 30% relative collision energy.

*Synthesis of Reference Compounds*—The precursor structures 3M2H-Gln and HMHA-Gln were prepared by the coupling of the activated sweat acids (*N*-hydroxysuccinimide ester) with L-glutamine. Whereas 3M2H is commercially available (Narchem Corp., Chicago), 3-methyl-3-hydroxyhexanoic acid (HMHA) was synthesized in a $Zn^{2+}$-mediated reaction of ethyl bromoacetate with 2-pentanone, followed by saponification in ethanolic sodium hydroxide solution. $Zn^{2+}$ powder in cyclohexane was heated to reflux and activated with a little ethyl bromoacetate. Then a mixture of ethyl bromoacetate and 2-pentanone was added dropwise. After 2 h of reflux the mixture was allowed to stand overnight and worked up in the usual manner. The resulting ethyl ester was dissolved in ethanol, treated with aqueous sodium hydroxide (2 N), and heated to reflux for several hours to yield the desired 3-methyl-3-hydroxyhexanoic acid, after acidification with aqueous hydrochloric acid and extraction with ether.

The acids and *N*-hydroxysuccinimide were dissolved in dioxane, and a solution of 1,3-dicyclohexylcarbodiimide in dioxane was added dropwise under ice-water cooling. The resulting suspension was allowed to warm to ambient temperature and stirred for 24 h. 1,3-Dicyclohexylurea was filtered, and the filtrate was concentrated to give the corresponding *N*-hydroxysuccinimide ester. This was dissolved in dioxane and added dropwise to a solution of L-glutamine and triethylamine in water. After stirring for 24 h, work up in the usual way furnished the compounds 3M2H-Gln and HMHA-Gln.

*Isolation of Bacterial Strains and Culture Conditions*—For isolation of axilla bacteria, the same method as described above for sampling axilla secretion was applied, with the exception that phosphate buffer, pH 7, containing 1% Tween 80 was used as the sampling liquid. The samples of axilla washings were spread plated on tryptic soy agar (Difco) amended with 5 g/liter Tween 80 and 1 g/liter lecithin. Single isolates obtained after 48 h of incubation were subcultured and characterized. Strains that had a coccoid cell morphology were identified with the ID Staph 32 kit (BioMerieux, France) and strains with a typical coryneform morphology (short, irregular rods) with the API coryne kit (BioMerieux, France). Gram reaction was determined with the KOH string technique (17), and lipophilicity was determined based on differential growth on media with or without Tween 80 as lipid source. For enzymatic assays and enzyme purification, the strains were grown overnight in Mueller-Hinton broth (Difco) amended with 0.05% Tween 80. As a reference strain *Corynebacterium xerosis* (DSMZ 20170; obtained from the German type strain collection) was used.

*Enzyme Assays*—To evaluate enzymatic activity in intact bacteria, an overnight culture was harvested by centrifugation and resuspended to a final $A_{600}$ of 1.0 in a semisynthetic medium (per liter: 3 g of $KH_2PO_4$, 1.9 g of $K_2HPO_4$, 0.2 g of yeast extract, 0.2 g of $MgSO_4 \times 7$ $H_2O$, 1.4 g of NaCl, 1 g of $NH_4Cl$, 10 mg of $MnCl_2$, 1 mg of $FeCl_3$, 1 mg of $CaCl_2$). Aliquots of this stationary culture were then amended with a final concentration of 2 mM HMHA-Gln, 3M2H-Gln, or alternative substrates (160 mM stock solution dissolved in $Me_2SO$). After 24 h of incubation (with shaking at 300 rpm; 36 °C), the samples were acidified with HCl and extracted with an equal volume of MTBE, and the amount of released acids was determined by GC as described above. No HCl was added if carbamates (*e.g.* Z-Gln) were used as substrates. To evaluate activity in cellular extracts, cells were washed, resuspended in as small volume of Buffer A (50 mM NaCl; 50 mM $NaH_2PO_4/K_2HPO_4$; pH 7), amended with a 10-fold volume of glass beads (425–600 $\mu$m, Sigma), and mechanically disrupted by vortexing them at maximal speed for 30 min. The lysates were centrifuged and the supernatants saved and diluted, and enzymatic assays were run with these crude extracts. Alternatively, enzyme assays were also done using the pure enzyme in Buffer A, either purified from the wild-type strain *Corynebacterium* Ax20 or from the recombinant strain (see below). As an alternative to the GC detection of the released acid, a fluorescent test was used. The enzyme reaction was stopped by adding 0.5 volumes of acetonitrile containing 2.5 mM fluorescamine (Fluka, Switzerland) thus derivatizing the free amino group of the released L-glutamine. Fluorescence was then measured with an LS-50 fluorescence spectrometer (PerkinElmer Life Sciences) with an excitation wavelength of 381 nm and an emission wavelength of 470 nm.

*Purification of the Aminoacylase—Corynebacterium striatum* Ax20 was selected to isolate and purify the enzyme responsible for the cleavage of HMHA-Gln. Cell lysates from 2-liter cultures were prepared as described above, and the proteins were fractionated by precipitation with an increasing concentration of $(NH_4)_2SO_4$. The precipitate obtained between 50 and 80% saturation of $(NH_4)_2SO_4$ contained the active enzyme. This enriched fraction was dissolved in Buffer A and desalted by repetitive dilution and concentration by ultrafiltration (Amicon membrane YM10, Millipore, Bedford, MA). The sample was then sequentially passed over four chromatography columns as follows: 1) DEAE-Sepharose CL-6B anion exchange resin (Amersham Biosciences), elution with a linear gradient from 0 to 800 mM KCl in Buffer A; 2) phenyl-Sepharose hydrophobic interaction resin (Amersham Biosciences), elution with a linear gradient from 1000 to 0 mM $(NH_4)_2SO_4$ in Buffer A; 3) Mono Q strong anion exchange column on the fast protein liquid chromatography system (Amersham Biosciences), elution with a gradient from 0 to 800 mM KCl in Buffer A; and finally Mono P weak anion exchange column on the fast protein liquid chromatography, elution with a gradient from 0 to 800 mM KCl in a 50 mM bis-Tris

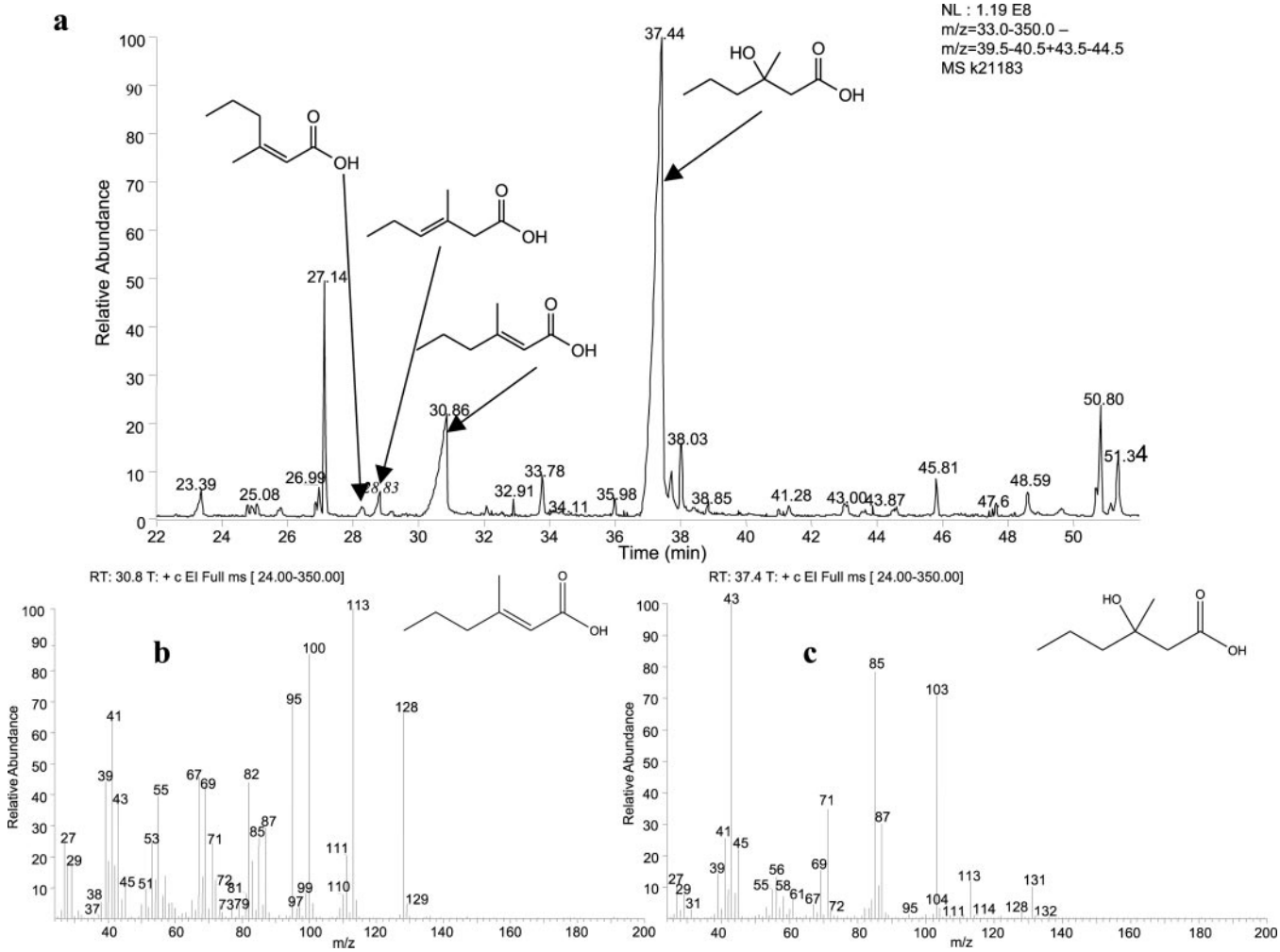


FIG. 1. **GC-MS analysis of hydrolyzed axilla secretions.** A pooled sample from nine individual donors is shown. *a*, the total ion count; *b*, the mass spectrum recorded at a retention time of 30.8 min corresponding to 3M2H; *c*, the mass spectrum recorded at a retention time of 37.4 min corresponding to HMHA.

buffer instead of Buffer A. After each column separation the active fractions (determined by the fluorescent assay) were pooled and desalted by dilution/ultrafiltration. The resulting apparently pure enzyme was subjected to tryptic digestion, and the sequence of two internal peptides was determined with LC-ESI-MS/MS analysis, and the N terminus was determined by automated Edman degradation. The molecular weight of the isolated enzyme was determined with nano-ESI-MS.

*Protein Determination and SDS-PAGE*—Protein concentrations were determined with the Bradford reagent (Bio-Rad) using bovine serum albumin as standard. SDS-PAGE was performed according to Laemmli (18) with 4% stacking gels and 9% separation gels. Protein bands were detected by Coomassie Blue staining.

*Molecular Biology Methods*—Chromosomal DNA of Ax20 was obtained from cell lysates by proteinase K digestion and subsequent extraction with hexadecyl-trimethylammonium bromide/NaCl and chloroform/isoamyl acetate (19). Based on the partial amino acid sequence analysis of the aminoacylase, degenerated primers were designed to amplify fragments between the N terminus and the internal peptide sequences. Standard PCR conditions were used according to the manufacturer (*Taq* polymerase, Sigma). The annealing temperatures were optimized on a gradient cycler (T-Gradient, Biometra, Göttingen, Germany). The amplified DNA was cloned into the vector pGEM-T Easy (Promega, Madison, WI), and the nucleotide sequence was determined on the ABI-Prism model 310 (PE Biosystems, Rotkreuz, Switzerland) using standard methods. Based on the obtained partial sequence, specific nested oligonucleotides were designed to clone the upstream and downstream regions. Chromosomal DNA of Ax20 was digested with *Sma*I and *Pvu*II and ligated to the GenomeWalker Adaptor (Clontech Laboratories, Palo Alto, CA). The upstream and downstream regions were then amplified as described in the instructions to the Universal GenomeWalker™ kit (Clontech Laboratories, Palo Alto, CA) and cloned into the vector pGEM T-easy, and the nucleotide sequence was determined. The full-length sequence of the open reading frame coding for the enzyme was then amplified with PCR from chromosomal DNA of Ax20 using the specific primers 5′-CAT GCC ATG GCA CAG GAA AAT TTG CAA and 5′-CCC AAG CTT TCA CTT CAT CAA CCA GGG CG. The amplified DNA fragment was digested with *Nco*I and *Hin*dIII and ligated into the vector pBAD/gIIIA (Invitrogen) pre-digested with the same enzymes. The resulting plasmid pBAD/gIIIA-AMRE was transformed into the host strain *E. coli* TOP10 (Invitrogen). This strain was grown in LB broth until it reached an $A_{600}$ of 0.5. The culture was induced with arabinose (0.2% final concentration), incubated for 4 h, harvested by centrifugation, and disrupted by ultrasonication. The extracts were used to purify the recombinant enzyme as described above but only with two chromatographic steps, namely phenyl-Sepharose and Mono Q. Data base comparisons of the deduced open reading frame were made to public protein sequence data bases (Swiss-Prot and GenBank™ bacterial sequences and to data bases containing the genomes of different bacteria) using the gapped blast algorithm (20) on the Biology WorkBench (biowb.sdsc.edu) platform.

## RESULTS

*Analysis of Hydrolyzed Axilla Secretions*—The organic extracts obtained from axilla secretions before hydrolysis were almost odorless, and only upon hydrolysis and re-acidification was a strong axillary odor developed, indicating that the volatiles were mainly present in the collected secretions as covalently linked, water-soluble precursors. The organic phase obtained from extraction of the hydrolyzed and re-acidified

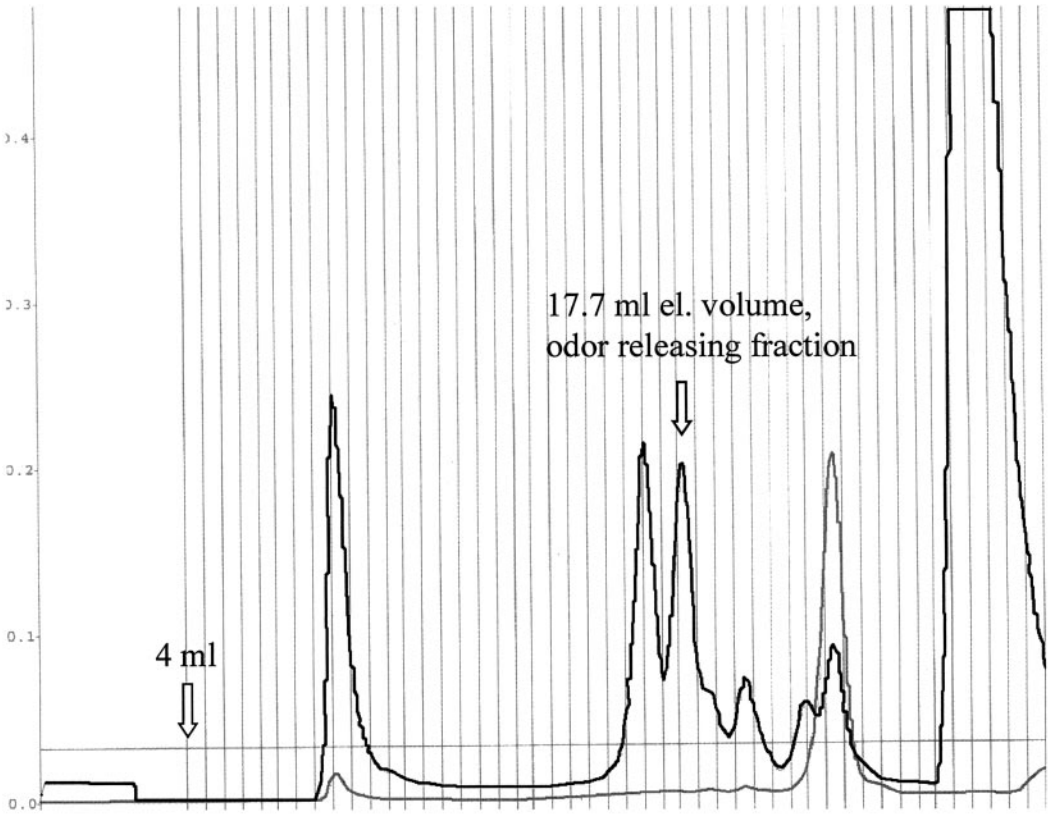


FIG. 2. **Gel filtration chromatogram of non-hydrolyzed aqueous fraction of axilla secretions.** Amersham Biosciences Peptide HR 10/30 column was used with 24-ml total bed volume, $(NH_4)_2CO_3$ (100 mM) elution buffer. The absorbance of the eluate at 214 nm is shown in *black*, and the absorbance at 280 nm is shown in *gray*.

samples of axilla secretions was therefore first olfactorily evaluated with a gas chromatograph equipped with a sniff port, and peaks of effluents which exhibited typical axillary odor were marked on the chromatograms. These peaks were then further analyzed by GC-MS. A typical chromatogram is shown in Fig. 1*a*. The presence of 3M2H could be confirmed to be an important odoriferous component (retention time 30.8 min) based on mass spectrum and retention time compared with a synthetic sample. At a retention time of 37.4 min a second and larger peak with a hypothetical molecular weight of 146 was found. The mass spectra of this unknown peak and that of 3M2H are shown in Fig. 1, *b* and *c*. Based on the fractionation pattern and comparisons to MS data bases, this new compound was proposed to be a hydrated derivative of 3M2H, namely 3-hydroxy-3-methylhexanoic acid (HMHA). This latter compound was synthesized, and its retention time and mass spectrum were found to be identical with the one of the unknown peak (data not shown). In addition, the hydrolyzed axilla secretion samples contained small amounts of (*Z*)-3 methyl-2 hexenoic acid and (*E)*-3-methyl-3-hexenoic acid based on MS analytics of the corresponding peaks (data not shown).

*Analysis of Non-hydrolyzed Axilla Secretions*—Non-hydrolyzed samples of axilla secretions were subjected to gel filtration on a Superdex Peptide HR10/30 column. The resulting chromatogram of the pooled samples of eight donors is shown in Fig. 2. The fraction at an elution volume of 17.7 ml (of 24 ml total gel bed size) was found to release a strong odor upon hydrolysis. This elution volume is between the peaks for $Gly_6$ and $Gly_3$ according to the manufacturer. The corresponding peak does not absorb at 280 nm. The typical axilla secretion proteins did elute early (7 ml of elution volume) as determined by SDS-PAGE (data not shown), and this fraction released no perceivable odor upon hydrolysis. Thus, these data indicate that the precursor is not a protein but a smaller molecule in the range of 200–400 Da. The presence of 3M2H and HMHA was verified in a hydrolyzed aliquot of the fraction of interest by GC-MS analysis (data not shown). This fraction was then subjected to LC-MS analysis. It contained one peak with a pseudo-molecular ion at $m/z$ 257 and a peak with a pseudo-molecular ion at $m/z$ 275. Assuming an ester or amide linkage of the two acids, the molecular masses of 256 and 274 suggested that 3M2H and HMHA are bound to a molecule with molecular mass of 146. Several common biological molecules with this mass were considered, and glutamine was found to fit best the MS fractionation pattern observed. This led to the hypothesis that 3M2H is linked to the $N^{\alpha}$ atom of L-glutamine (*i.e.* $N^{\alpha}$-3-methyl-2-hexenoyl-L-glutamine-; 3M2H-Gln), whereas the second peak with a mass of 274, based on its mass and fractionation pattern, corresponds to the hydrated analogue $N^{\alpha}$-3-hydroxy-3-methylhexanoyl-L-glutamine (HMHA-Gln). These two compounds were then synthesized, and their MS spectra and retention times in the LC-MS/MS analysis were compared and found to be identical to the compounds present in axilla secretions. The presence of these compounds in the hydrophilic phase of non-fractionated axilla secretions of individual donors was then also verified. In Fig. 3*b*, the LC-MS/MS analysis of such a sample is shown, and the analytical data for the two synthetic references are given in Fig. 3*a*. The synthesized compounds partly released the acids upon hydrolysis with NaOH and re-acidification which perfectly fits the observations made previously by us and others (16) on the nature of the precursor. Thus, from all this analytical evidence one could predict the enzymatic activity needed for the release of the odoriferous component, namely a bacterial $N^{\alpha}$-acylglutamine aminoacylase, and the corresponding scheme and structures are shown in Fig. 4.

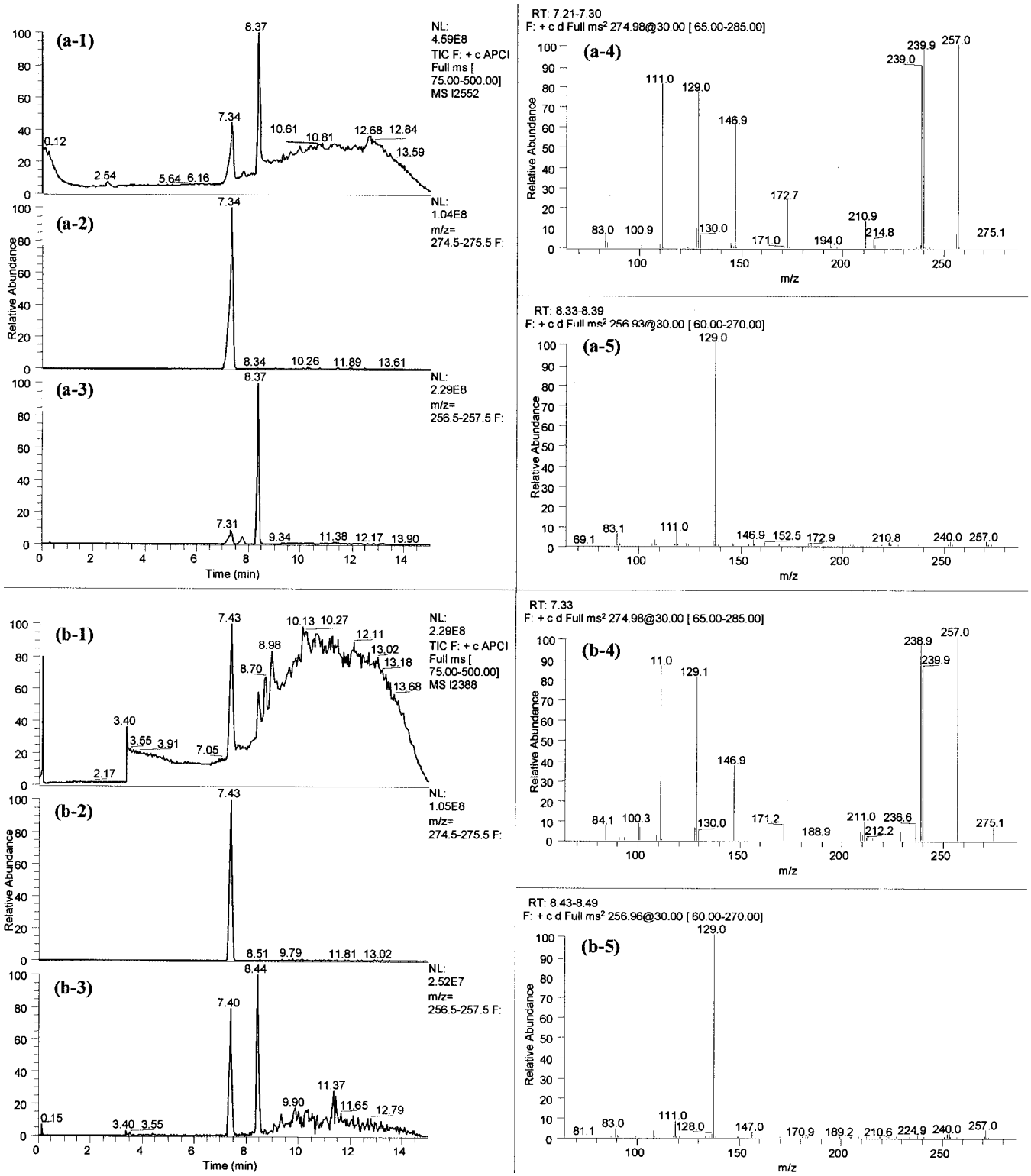


FIG. 3. **LC-MS/MS analysis of axilla secretions.** *a*, a mixture of the synthetic HMHA-Gln and 3M2H-Gln (each 50 ppm); *b*, the aqueous phase of an axilla secretion sample. *a-1* and *b-1*, the total ion chromatogram; *a-2* and *b-2*, the extracted mass chromatogram at *m/z* = 275; *a-3* and *b-3*, the extracted mass chromatogram at *m/z* = 257; *a-4* and *b-4*, the $MS^2$ spectrum of the peak at 7.3–7.5 min retention time corresponding to HMHA-Gln; and *a-5* and *b-5*, the $MS^2$ spectrum of the peak at 8.3–8.5 min retention time corresponding to 3M2H-Gln.

*Isolation of Axilla Bacteria Able to Cleave HMHA-Gln and 3M2H-Gln*—The axilla flora of 21 donors was isolated on tryptic soy agar, and single isolates obtained after 48 h of incubation were subcultured and identified. A total of 19 individual strains of *Corynebacterium* and 25 strains of *Staphylococcus* were obtained. Stationary cultures of the strains were amended with HMHA-Gln or 3M2H-Gln and analyzed after a 24-h incubation for released acids. Isolates from both the non-lipophilic *C. striatum* and the lipophilic *Corynebacterium jeikeium* and *Corynebacterium bovis,* but not all *Corynebacte-*

*rium,* released a significant quantity of 3M2H and HMHA, whereas none of the strains of *Staphylococcus* sp. or *Micrococcus* sp. exhibited this enzymatic activity. Table I gives the results for a subset of the strains tested. These data are in good agreement with the well known fact that only subjects with an axilla flora dominated by *Corynebacteria* produce the most typical axillary odor. Thus, $N^{\alpha}$-acylglutamine aminoacylase activity is indeed present in bacteria isolated from the human axilla, as predicted based on the identification of the precursor structure. If $N^{\alpha}$-lauroyl-L-glutamine was used as substrate in the same experiment, it was found that also other *Corynebacteria* and some *Staphylococci* can release lauric acid from this substrate, indicating that even more skin bacteria may have a related aminoacylase activity more specific for substrates substituted with a straight chain acid, and thus not directly involved in the formation of the typical odor of the branched chain acids.

*Characterization of the $N^{\alpha}$-Acylglutamine Aminoacylase Activity—C. striatum* Ax20 was selected to characterize the aminoacylase activity in more detail. No activity was observed in the supernatants of stationary phase cultures. On the other hand, the enzymatic activity could be obtained in the soluble fraction after mechanical disruption of the cells and subsequent removal of cell debris by centrifugation. This indicates that the cleavage is mediated by an intracellular, soluble enzyme that is not, or only loosely, associated with the cell membrane. Further experiments were thus done with cell extracts. The enzymatic activity was lost if these extracts were preincubated with 0.5 mM of EDTA or *o*-phenanthroline, but no inhibition was observed if an excess of $Zn^{2+}$ was simultaneously added. Only partial loss of activity was observed with the combination $Mn^{2+}$ and chelating agents. Activity was also lost in presence of 0.5 mM dithiothreitol. The extracts retained their activity if treated with the serine protease inhibitors phenylmethylsulfonyl fluoride and Pefablock (4-(2-aminoethyl)-benzenesulfonyl fluoride) or with pepstatin. Taken together, these observations indicate that the aminoacylase is related to the $Zn^{2+}$-dependent metallopeptidases. In the next step, an analysis of the substrate specificity in the cellular extracts was made. As mentioned above, in addition to the physiological substrates, $N^{\alpha}$-lauroyl-Gln was efficiently cleaved also. Furthermore, carbamates of glutamine were alternative substrates, in particular carbobenzyloxy-L-glutamine (Z-Gln) as well as some dipeptides with C-terminal Gln. Among other Z-substituted or lauroyl-substituted natural amino acids, only a very weak activity for Z-Ala was observed. No activity for Z-L-asparagine, Z-L-aspartate, *N*-lauroyl-L-aspartate, Z-L-glutamate, and other substituted essential amino acids was present in the cell extracts nor was there any cleavage of Z-D-glutamine, the methyl ester of Z-L-glutamine or Z-Gln-Gly (Table II). Thus the enzymatic activity is very specific for the glutamine residue; it is stereospecific and needs the free carboxy group of Gln as is typical for carboxypeptidases, and it has a low specificity for the acyl part of the substrates. Based on the

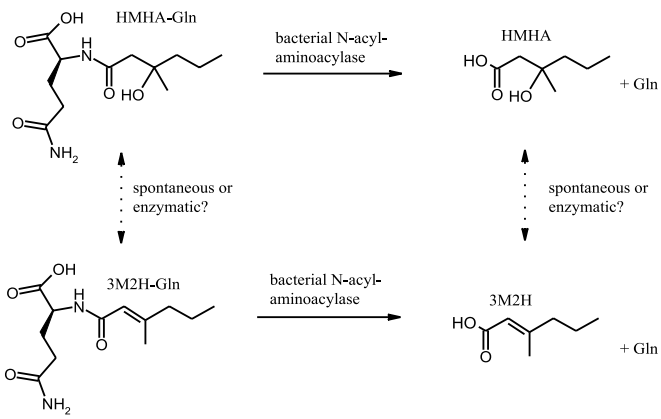


FIG. 4. **Proposed scheme for the release of odoriferous acids by skin bacteria.**

TABLE II
*Substrate specificity of the enzymatic activity in cellular extracts of C. striatum Ax20*

| Substrate | Cleavage by raw extract[a] |
|---|---|
| 3M2H-Gln | ++ |
| HMHA-Gln | ++ |
| $N^{\alpha}$-Lauroyl-L-glutamine | +++ |
| $N^{\alpha}$-Decanoyl-L-glutamine | +++ |
| Carbobenzyloxy-L-glutamine (Z-Gln) | ++ |
| Z-Ala-Gln | ++ |
| Tyr-Gln | + |
| $N^{\alpha}$-3,7-Dimethyl-6-octenyloxycarbonyl-L-glutamine | +++ |
| *N*-Lauroyl-L-aspartate | – |
| $N^{\alpha}$-Lauroyl-L-lysine | – |
| $N^{\alpha}$-Lauroyl-L-arginine | – |
| *N*-Lauroyl-L-alanine | +/– |
| Carbobenzyloxy-L-alanine | +/– |
| Carbobenzyloxy-L-glutamate | – |
| Carbobenzyloxy-L-asparagine | – |
| Carbobenzyloxy-L-aspartate | – |
| Carbobenzyloxy-L-serine | – |
| Carbobenzyloxy-L-tyrosine | – |
| Carbobenzyloxy-L-glycine | – |
| Carbobenzyloxy-L-histidine | – |
| Carbobenzyloxy-L-leucine | – |
| Carbobenzyloxy-D-glutamine | – |
| Carbobenzyloxy-L-glutamine-O-Me | – |
| Carbobenzyloxy-L-glutamine-glycine-OH | – |

[a] – indicates no cleavage; +/–, traces of product detected; +, indicates cleavage <10%; ++, cleavage 10–50%; and +++, cleavage over 50% with 2 mM substrate, 24 h incubation in 50-fold diluted cell extracts.

TABLE I
*Cleavage of $N^{\alpha}$-acylglutamine derivatives by axilla bacteria*

| Strain | Species assignment | Lipophilicity[a] | Release of 3M2H from 3M2H-Gln[b] | Release of HMHA from HMHA-Gln | Release of lauric acid from $N^{\alpha}$-lauroyl-Gln |
|---|---|---|---|---|---|
| Ax3 | *C. bovis* | + | 0.020 | <0.005 | 1.295 |
| Ax7 | *Corynebacterium group G* | + | <0.005 | <0.005 | 0.028 |
| Ax15 | *C. jeikeium* | + | 0.496 | 0.065 | 0.006 |
| Ax19 | *C. jeikeium* | + | 1.132 | 0.735 | 0.028 |
| Ax20 | *C. striatum* | – | 0.217 | 0.200 | 0.796 |
| Ax21 | *C. bovis* | + | 0.449 | 0.037 | 2.096 |
| Ax1 | *Staphylococcus capitis* | – | <0.005 | <0.005 | 0.021 |
| Ax6 | *Staphylococcus epidermidis* | – | <0.005 | <0.005 | 0.154 |
| Ax9 | *Micrococcus luteus* | – | <0.005 | <0.005 | 0.016 |

[a] + indicates growth only in media amended with Tween 80 as lipid source.
[b] Stationary phase cultures with $A_{600}$ = 1 were amended with 2 mM of the substrate and analyzed after 24 h of incubation. Given is the amount of acid released in mM.

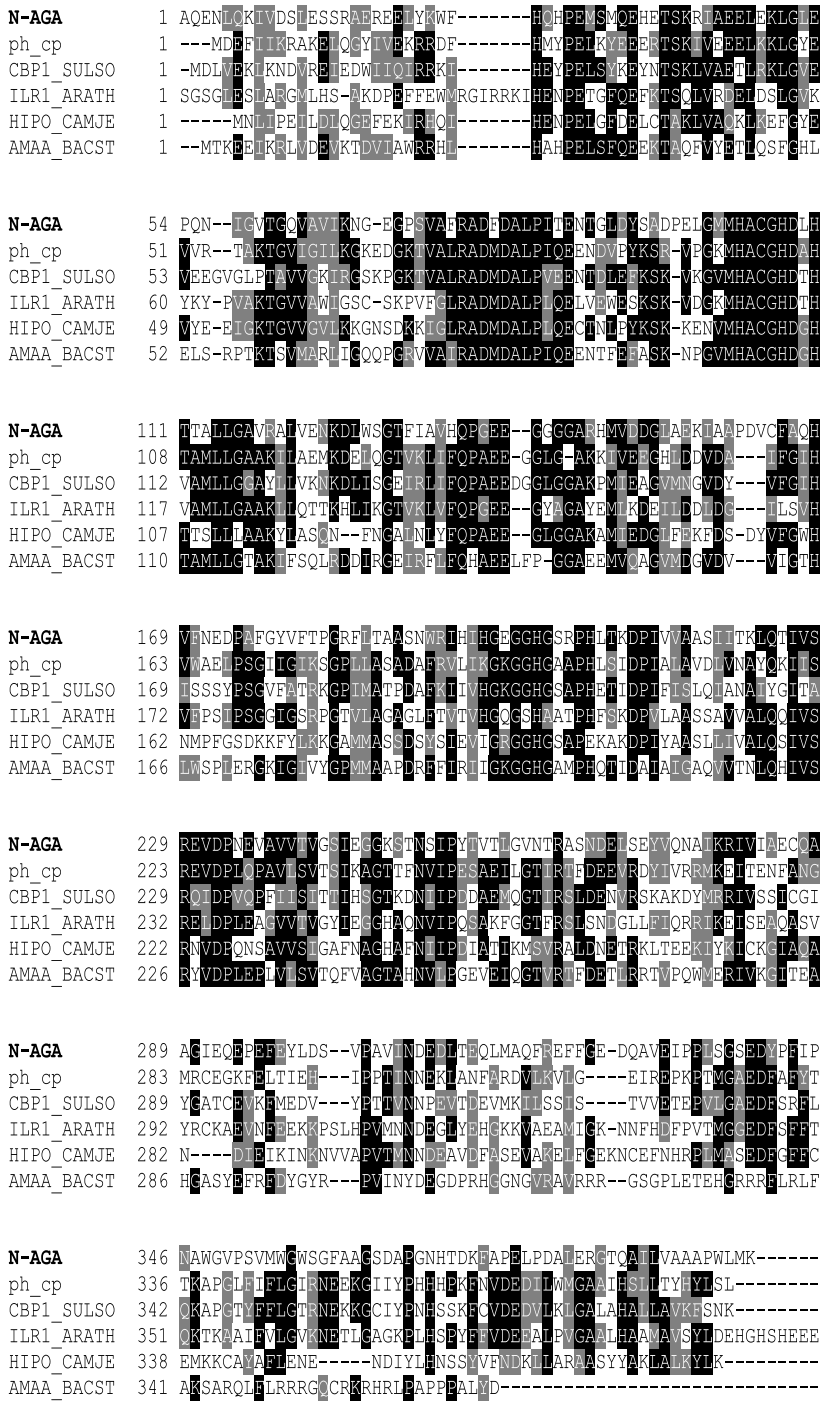


FIG. 5. **Sequence homology of the new aminoacylase to enzymes with known function.** Matching sequences were obtained from GenBank™ bacterial sequences and Swiss-Prot using the Biology Workbench platform and the BLAST function. Sequences were aligned to each other using the ClustalW algorithm. *N-AGA*, *N*-acyl-Gln aminoacylase, this work; *ph_cp*, carboxypeptidase from *Pyrococcus horikoshii* (25), GenBank™ accession number AB009503; *CBP1_SULSO*, carboxypeptidase from *S. solfataricus* (22), EMBL accession number Z48497; *ILR1_ARATH*, indole-3-acetic acid-conjugate hydrolase from *A. thaliana* (26), Swiss-Prot accession number P54968; *HIPO_CAMJE*, hippuricase from *C. jejuni* (24), Swiss-Prot accession number P45493; and *AMAA_BACST,* aminoacylase from *B. stearothermophilus* (23), GenBank™ accession number X74289.

substrate specificity, it belongs to the class of enzymes summarized in the official nomenclature under the number EC 3.5.1.14.

*Purification of the Aminoacylase and Cloning of the Corresponding Gene*—The glutamine-specific aminoacylase was purified from the cellular extracts of *C. striatum* Ax20 according to the scheme described under "Experimental Procedures." The aminoacylase activity eluted from all the columns as a single peak indicating that only one enzyme is involved in cleavage of these substrates. The isolated enzyme was stable at 4 °C, and it was over 90% pure when analyzed by SDS-PAGE with one major band with an apparent molecular mass of 48 kDa. Its effective molecular mass was determined by nano-ESI-MS analysis and found to be 43,365 ± 5 Da. The N-terminal sequence was found to be AQENLQKIVDSLESSRAEREELYKWFHQHPEMSMQE, and LC-ESI-MS/MS analysis after tryptic digestion revealed the sequences DLWSGTFIAVHQPGEEIGGTK and WGWSGFAAGSDAPGN of two internal peptides. Based on each of these peptides, four degenerated oligonucleotides were designed, and a total of 32 primer combinations between the internal peptides and the N-terminal sequence were used for PCR amplification with chromosomal DNA of Ax20 as template. Six primer combinations gave a major band of 350 and 650 bp, respectively, whereas the others gave no or unspecific amplification. These amplicons were cloned, and the sequence was determined. Specific nested oligonucleotides were then designed to clone the upstream and downstream region using the Universal GenomeWalker™ kit (Clontech Laboratories, Palo Alto, CA) with libraries generated from *Pvu*II and *Sma*I digests of chromosomal DNA. An upstream

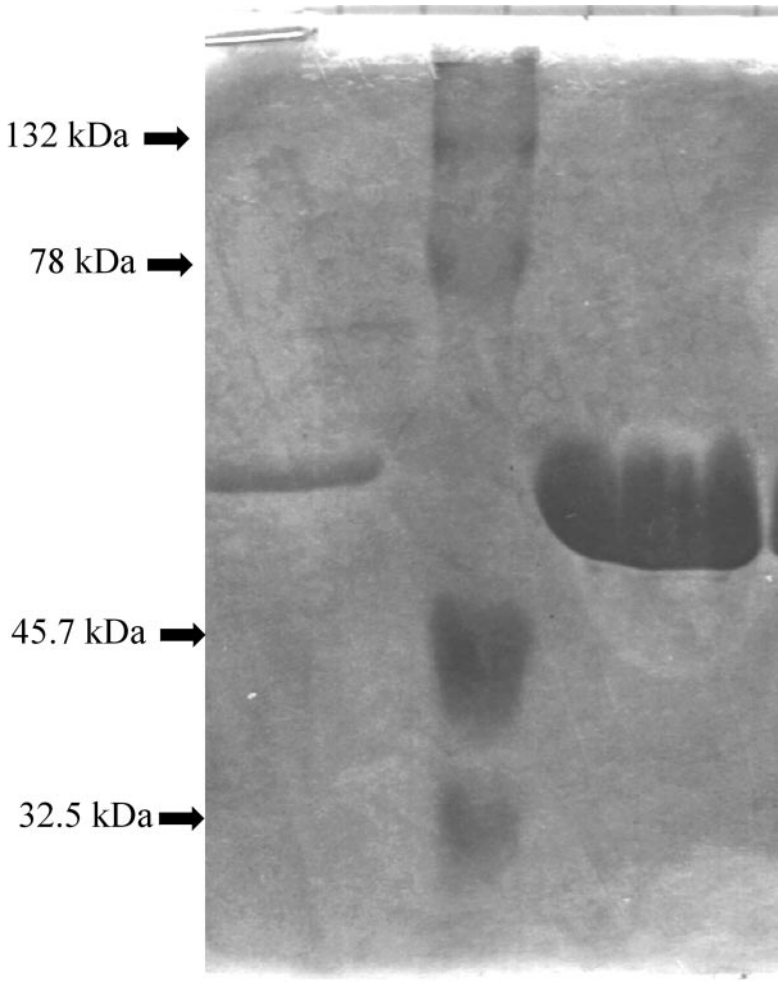


FIG. 6. **Purification of the recombinant aminoacylase from cell extracts of *E. coli* TOP10 transformed with (pBAD/gIIIA-AMRE).** *1st lane,* 0.5 μl of active fraction from the Mono Q separation step; *2nd lane,* molecular size marker; *3rd lane,* 20 μl of the same active fraction.

fragment of 1200 bp and a downstream fragments with 3000 bp were obtained. Within this sequenced region (4600 bp), an open reading frame coding for a polypeptide with 399 amino acid residues and with a high homology to known aminoacylases, some carboxypeptidases, and various putative peptidases was identified. The calculated molecular mass of the deduced polypeptide starting from the experimentally determined N terminus is 43,365 Da, corresponding well to the value measured with nano-ESI-MS analysis. The gene is named *agaA* (for acylglutamine-aminoacylase) and the complete sequence was deposited in GenBank™.

*Sequence Comparison to Related Proteins*—The protein deduced from the open reading frame has a very high homology to a large number of bacterial sequences, although the function of most of these genes has not been determined experimentally. Indeed, when comparing the sequence to 15 different bacterial genomes (*Streptococcus pneumoniae R6, Staphylococcus aureus Mu50, Pseudomonas aeruginosa, Neisseria meningitidis, Mycobacterium tuberculosis, Listeria monocytogenes, Helicobacter pylori, Haemophilus influenzae Rd, E. coli K-12, Corynebacterium glutamicum, Clostridium perfringens, Campylobacter jejuni, Borrelia burgdorferi, Bacillus subtilis,* and *Agrobacterium tumefaciens*), 11 of them, both Gram positives and Gram negatives, contain at least one gene with a significant homology (*E* value $<1^{-20}$ in the gapped blast comparison). All these genes have the highly conserved sequence MHACGHD*X*H and a second conserved motif with a consensus of RAD*X*DXLP*XX*E. In addition, they contain two more highly conserved histidines, four glutamates, and one aspartate. The open reading frame was also compared with genes with a known function only, and the alignment with the closest relatives with known function is shown in Fig. 5. The same conserved motifs as summarized above are also found in this alignment. The gene *cpsA* from *Sulfolobus solfataricus* codes for a $Zn^{2+}$-dependent carboxypeptidase able to cleave benzyloxycarbonylated amino acids similar to the aminoacylase described in this work but without a high specificity for the amino acid residue (21, 22). The gene *amaA* codes for an aminoacylase in *Bacillus stearothermophilus* able to cleave *N*-acyl derivatives especially of aromatic amino acids (23). *hipO* codes for hippuricase in *C. jejuni* (24) an enzyme present in many bacterial species and hydrolyzing hippurate (benzoyl-glycine). The related hyperthermostable carboxypeptidase/aminoacylase from *Pyrococccus horikoshii* OT3 has both carboxypeptidase activity especially for terminal Phe and aminoacylase activity for acetylated amino acids without strict specificity (25). Finally, ILL2 is one of six ILR1-like

TABLE III
*Kinetic data for the pure recombinant glutamine-aminoacylase*

| Substrate | $V_{max}$ | $K_m$ |
|---|---|---|
| | *mmol/min/mg protein*[a] | *mM* |
| 3M2H-Gln | 0.121 | 0.20 |
| HMHA-Gln | 0.461 | 0.74 |
| $N^{\alpha}$-Decanoyl-Gln | 0.752 | 0.08 |
| $N^{\alpha}$-Lauroyl-Gln | 0.325 | 0.06 |
| Z-Gln | 0.065 | 0.05 |

[a] The recombinant enzyme (diluted 1:50,000 to 250,000 depending on the substrate) was incubated with a dilution series of the substrates; the reaction was stopped after 15 min, and the released glutamine was determined by derivatization with fluorescamine.

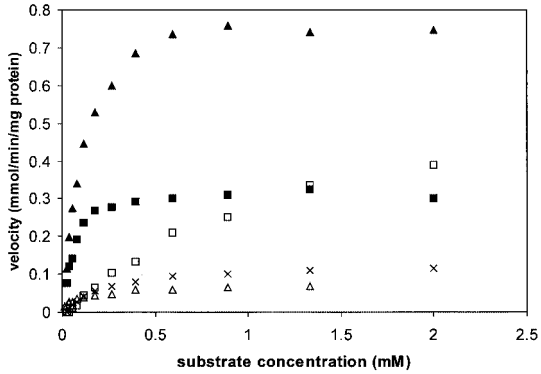


FIG. 7. **Kinetic analysis of the recombinant aminoacylase.** The following substrates were tested at varying concentrations: HMHA-Gln (*open squares*); lauroyl-Gln (*filled squares*); decanoyl-Gln (*filled triangles*); 3M2H-Gln (*crosses*), and Z-Gln (*open triangles*).

genes from *Arabidopsis thaliana* (26, 27). These genes code for enzymes cleaving the phytohormone indole-3-acetic acid from amino acid precursors. All the six related plant genes share all the conserved motifs with the bacterial sequences as described above. Based on the sequence comparison, the aminoacylase belongs to the peptidase family m40, also known as the ama/hipo/hyuc family of hydrolases.

*Characterization of the Pure Recombinant Enzyme*—A DNA fragment containing the complete open reading frame was then cloned into the expression vector pBAD/gIIIA and transformed in *E. coli* TOP10. The transformants produced a high yield of the protein, and it was purified to >95% purity with two chromatographic steps (Fig. 6). The recombinant enzyme was similarly inhibited by $Zn^{2+}$-chelating agents, and it exhibited the equal substrate specificity toward acylated amino acids as initially observed in the crude cell extracts (data not shown). A subset of the substrates was then used to determine enzyme kinetics. The kinetic parameters are listed in Table III, and the Michaelis-Menten plot is shown in Fig. 7. Among the substrates tested, $N^{\alpha}$-decanoyl-Gln has the highest $V_{max}$, and it has a high affinity to the enzyme ($K_m$ of 0.08 mM). Interestingly, the main natural substrate isolated from axilla secretions, HMHA-Gln, has a much lower affinity ($K_m$ = 0.74 mM) but also a high $V_{max}$, whereas the natural substrate 3M2H-Gln as well as the synthetic carbamate Z-Gln have a significantly lower $V_{max}$.

## DISCUSSION

Several short, 3-methyl-branched fatty acids were found to be released from odorless sweat upon hydrolysis. This confirms the finding of Zeng *et al.* (13) showing the important contribution of this class of molecules to the olfactive impression of axilla secretions. The nature of the precursor structures found in this work is in very good agreement with earlier observations (16) suggesting that a bond sensitive to alkaline hydrolysis must be involved and that the putative precursor is water-soluble. In addition, the isolation of an odorless precursor confirms the observation made 50 years ago that fresh axilla secretions are odorless (3). The fact that bacteria, but only *Corynebacteria*, cleave the precursor further confirms the large number of reports (*e.g.* Refs. 4 and 6) showing that a dense population of coryneforms is a prerequisite for strong odor formation, and this is the first report clearly proving a biochemical mechanism explaining these observations. However, the nature of the precursor identified in this work at first sight is in contradiction to the work of Zeng *et al.* (15) and Spielman *et al.* (28) showing association of 3M2H with apolipoprotein D in axilla secretions, and suggesting that this protein-fatty acid association indeed is the precursor. Nevertheless, once cleaved from the glutamine residue as shown in this work, the acids might subsequently associate non-covalently with apolipoprotein D, and this non-covalent association could be just one more mechanism of slow release of these volatiles from the axilla. A similar release mechanism is postulated for hamster aphrodisin and a putative hydrophobic pheromonal compound (29). Finally, apoD has an N-terminal glutamine residue (15), and we cannot rule out the possibility, that a covalent linkage to this residue exists and that the proteins are first cleaved by an endopeptidase thus releasing the terminal acid-Gln conjugate. In this case, the accumulation of a significant amount of 3M2H-Gln or HMHA-Gln in axilla secretions as shown in this work and the lack of odor released from the protein fraction by hydrolysis would indicate that cleavage of the glutamine conjugates is the rate-limiting step. However, such a mechanism would require that for the release of each volatile molecule of $M_r$ 128 or 146, a complete protein molecule with $M_r$ 23,000 needs to be synthesized and secreted.

It has been speculated that body odor formation originates from common catabolic pathways of the skin bacteria and therefore is just a by-product of bacterial metabolism of unspecific skin secretions, which themselves are by-products of the metabolism of the body. Examples of such catabolic processes put forward are the formation of isovaleric acid from L-leucine or the formation of short acids by incomplete degradation of skin lipids (30). The nature of the identified 3M2H-Gln and HMHA-Gln points in another direction. Indeed it is rather unlikely that these compounds are by-products of the human metabolism, and it appears more likely that they are synthesized specifically to exert their action once secreted in the axilla region. The actual benefit of the secretion in a precursor form instead of direct secretion of the acids could be manifold. On the one hand, a precursor leads to a controlled release making the chemical signal more long lasting. On the other hand specific secretion into the ducts of the apocrine glands could pose physiological problems for the low molecular weight acids that are overcome by a water-soluble precursor structure.

The fact that enzymes of human skin bacteria specifically recognize the precursor structures indicates that the bacteria have adapted their enzymes to the specific axilla secretions and points to a very close interaction and co-evolution of humans and their axilla flora. The aminoacylase described in this work is unique in its substrate specificity, being very selective for the glutamine residue but having a very broad substrate specificity regarding the acyl part. This specificity would open up the possibility that other potential Gln-containing precursor structures in sweat could be cleaved by the same enzyme. Other branched acids have been reported in axilla secretions (13), and we could confirm this finding (data not shown). Whether some of these acids are also linked to Gln in axilla secretions remains to be investigated. Indeed, the $K_m$ values for the substrates $N^{\alpha}$-decanoyl-Gln and $N^{\alpha}$-lauroyl-Gln not found in axilla secretions are clearly lower then for the natural substrates detected in the axilla secretions. The high $K_m$ values for the natural substrates could point to a suboptimal cleavage of HMHA-Gln and 3M2H-Gln in the axilla, because the maximal level detected on the skin of HMHA-Gln was 0.95 nmol/cm$^2$ (data not shown). Depending on the water film present on the skin, the actual concentration in sweat is then estimated to be 0.019–0.095 mM and thus clearly below the $K_m$. However, at a concentration of 0.031 mM HMHA-Gln, a diluted stationary phase culture of *C. striatum* ($A_{600}$ = 0.25) cleaves 10.5% of the substrate within 15 min (data not shown), which indicates that despite the high $K_m$ value, the enzyme may release significant amounts of HMHA under the physiological conditions in the axilla.

Closely related enzymes are present in the majority of the bacterial species (Gram-negatives, Gram-positives, and Archaea) and in plants. Most of them have not been investigated for their substrate specificity, but in all instances where the substrate specificity is known, these enzymes catalyze the release of an aliphatic or aromatic acid from the amino group of an amino acid and/or they cleave carbamates (21, 23–27). Besides this common denominator, the substrate specificity of these enzymes varies widely, and the different enzymes depend on different metal co-factors (manganese, cobalt, or zinc). The very high specificity for one particular amino acid residue has not been found for most of the related enzymes, which cleave a broad range of synthetic substrates. However, the physiological substrate is not known for most of the enzymes, especially those isolated form bacteria. The conserved sequences in this family of enzymes do not contain either of the common denominators H*EXX*H, H*XXE*H, H*XXE*, or H*X*H found in many zinc-dependent metallopeptidases (31), and thus the active site configuration and the residues involved in $Zn^{2+}$ chelation remain to be determined. The strikingly high conservation of the two motifs RAD*X*DALP and MHACGHD*X*H among species belonging to the Archaea, Eubacteria, and plants is especially interesting in this respect and deserves further attention.

Finally, the elucidation of a specific biochemical mechanism of human body odor formation brings back the question about its biological function. Although the role of chemical communication in animals has been studied in detail, the information on humans is scarce. Today, body odors are mainly perceived as offensive and negative. This might be the result of social learning but could also reflect the actual function that these compounds had in our evolutionary past. They may have always been involved in marking dominance and repelling rivals, although both the popular and the scientific discussion on the potential semi-chemical activity of human body odor has mainly focused on its potential attractiveness and importance in mate selection (2). In humans, a functional vomeronasal organ for perceiving pheromones cannot be found in adults. Whereas the family of genes coding for vomeronasal receptors contains up to 300 genes in rodents, only one is expressed in humans, and the remainder are lost or are pseudogenes (32). Thus it is likely that chemical communication in man solely depends on olfaction, and a more detailed understanding of chemical composition, biochemical formation, and function of body odor still deserves further attention.

From a practical point of view, the findings of this work also point to a novel way to combat body odors. Today prevention is based on the daily topical application of (mainly halogenated) antibacterial agents and aluminum salts. These agents unspecifically and transiently reduce the bacterial skin flora. The fact that an enzyme related to the class of metallopeptidases with a unique substrate specificity plays an important role in body odor formation points to the possibility of synthesizing specific inhibitors, because for metallopeptidases a rational inhibitor design has been successfully applied in many instances (33). Such compounds could be used as additional or alternative active ingredients in deodorants.

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