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Odorant and pheromone binding by aphrodisin, a hamster aphrodisiac protein

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Abstract Aphrodisin is a soluble glycoprotein of hamster vaginal discharges, which stimulates male copulatory behavior. Natural aphrodisin was purified and its post-translational modifications characterized by MALDI-MS peptide mapping. To evaluate its ability to bind small volatile ligands, the aphrodisiac protein was expressed in the yeast *Pichia pastoris* as two major isoforms differing in their glycosylation degree, but close in conformation to the natural protein. Dimeric recombinant aphrodisins were equally able to efficiently bind odors (2-isobutyl-3-methoxypyrazine and methyl thiobutyrate) and a pheromone (dimethyl disulfide), suggesting that they could act as pheromone carriers instead of, or in addition to, direct vomeronasal neuron receptor activators. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Aphrodisin; Glycosylation; Hamster; Recombinant protein expression; Vaginal discharge protein; Vomeronasal organ

1. Introduction

Aphrodisin is a 17 kDa soluble glycosylated protein originally isolated from hamster vaginal discharges, in which it occurs in relatively high concentrations (5 µg/mg of discharge) [1,2]. The aphrodisin gene is expressed in vagina, uterus, Bartholin's glands and also in female hamster parotid glands [3,4]. It stimulates male copulatory behavior via the vomeronasal organ [5], but occurs in vaginal discharges before females reach fertility suggesting another unknown function for aphrodisin [4].

Aphrodisin consists of 151 amino acids with two disulfide bonds and a blocked N-terminus (pyroglutamic acid). Each N-glycosylation site (N41 and N69) is linked with only one N-acetylglucosamine (GlcNAc) residue [1]. Aphrodisin be-

longs to the lipocalin family, known as transporters of low molecular weight hydrophobic molecules. Lipocalins display a remarkable range of different molecular recognition properties and are variable in sequence, but share a typical 3D structure [6–8]. Among lipocalins, aphrodisin is highly homologous (40% sequence identity) to rat odorant binding proteins OBP-1 and 1F, which are thought to carry volatile odorants towards olfactory receptors through the nasal mucus [9,10]. Aphrodisin is also homologous to a recently described hamster submandibular gland protein [11].

It has not yet been definitely proved whether aphrodisin has a pheromonal activity by itself or needs the combination with low molecular weight ligands to be active [12,13]. Its homology with OBP suggests a role as a lipophilic compound carrier, but it could also intrinsically act as a pheromone by direct activation of neuron receptors of the vomeronasal organ. It has indeed been observed that another lipocalin, the rat major urinary protein, remains active [14] and able to induce receptor-mediated G protein activation of some vomeronasal receptor cells [15] when deprived of natural volatiles. Glycosylation of some olfactory active proteins suggests a potential role of oligosaccharide chains in the recognition process at the receptor site [16–22]. The lectin-assisted blockage of rat olfaction supports a potential role of carbohydrates in mediating ligand binding protein or binding protein–receptor interactions [23].

We report the purification of the natural aphrodisin and the characterization of its post-translational modifications and global folding. We compared these features with those of recombinant proteins expressed in the yeast *Pichia pastoris*. The recombinant aphrodisin was used to demonstrate its ability to bind small odorants and pheromones.

2. Materials and methods

2.1. Strains and chemicals

Escherichia coli strain DH5α was used for DNA subcloning and propagation of the recombinant plasmid. *P. pastoris* strain GS115 (*his4*) was used in the expression study. Sinapinic acid was provided by Aldrich and peptides for MALDI-MS calibration were purchased from PE Biosystems (France). All other chemicals were provided by Sigma.

2.2. Characterization of vaginal discharge proteins

Syrian golden hamster (*Mesocricetus auratus*) vaginal discharges were obtained by tactile genital stimulation of anesthetized females using a cotton swab impregnated with physiological NaCl solution. Cotton swabs were rinsed with MilliQ H₂O, the washing solution centrifuged at 10 000 × g for 20 min at 4°C and subjected to LC–MS

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Abbreviations: Aphro-Nat, natural aphrodisin; Aphro-RecG, recombinant glycosylated aphrodisin; Aphro-RecNG, recombinant unglycosylated aphrodisin; DMDS, dimethyl disulfide; GlcNAc, N-acetylglucosamine; IBMP, 2-isobutyl-3-methoxypyrazine; ES-MS, electrospray mass spectrometry; LC–MS, liquid chromatography coupled with mass spectrometry; MALDI-MS, time of flight matrix-assisted laser desorption ionization mass spectrometry; MTB, methyl thiobutyrate; OBP, odorant binding protein; RPLC, reversed-phase HPLC

and N-terminal sequencing. RPLC was performed at 40°C on a C18 RP 300 column (0.5 i.d. × 150 mm, Brownlee, Perkin-Elmer, France) equilibrated with eluent A (4 mM ammonium acetate, 0.1% v/v formic acid in H₂O). Elution was performed with a linear gradient of 5–95% eluent B (66% CH₃CN, 33% 2-propanol, 4 mM ammonium acetate, 0.075% formic acid) in 90 min. The flow rate was 5 µl/min and the absorbance recorded at 215 nm. ES-MS was performed using an API 100 Sciex apparatus (PE Biosystems). After being monitored for absorbance at 215 nm, the flow was split between the microion spray source (0.5 µl/min) and a fraction collector device (Perkin Elmer Biosystems Microblotter 173 A), which led to peptides blotted onto Problott PVDF membrane aimed at sequencing.

2.3. Anion exchange chromatography purification of natural aphrodisin

Vaginal discharges were clarified by centrifugation at 10 000 × g for 30 min at 4°C and were equilibrated with 20 mM Tris-HCl, pH 8.0 by dialysis for 4 days at 4°C after filtration. Natural aphrodisin was purified by anion exchange chromatography according to Briand et al. [10] except that the gradient was 0–0.25 M NaCl in 25 min. The aphrodisin-containing fractions were identified by MALDI-MS as further described and pooled.

2.4. Expression and purification of recombinant aphrodisin

The *P. pastoris* expression vector pPIC9Aphro containing an aphrodisin cDNA was kindly donated by Prof. Dr. Forssmann. The construct contained the mature aphrodisin sequence with the preprosequence of the α-mating factor without spacer. Transformation of *P. pastoris*, screening for aphrodisin expression and large-scale production were achieved as recently described [10]. The culture supernatant was chilled and clarified by centrifugation at 10 000 × g for 30 min at 4°C. The recombinant protein was purified by anion exchange chromatography as described for natural aphrodisin. The fractions containing aphrodisin were identified by MALDI-MS, pooled and chromatographed by gel filtration to remove any possible ligand. Exclusion-diffusion chromatography was performed through a 24 ml bed volume (10 i.d. × 300 mm) Pharmacia Superose 12 column. The gel was equilibrated with 50 mM ammonium acetate, pH 7.0, and elution carried out at 0.5 ml/min. Finally, the aphrodisin fractions were extensively dialyzed against MilliQ H₂O and lyophilized.

2.5. Characterization of recombinant aphrodisin

SDS-PAGE (16% acrylamide) was performed using a Mini-Protein II system (Bio-Rad, France) according to the method of Schägger and von Jagow [24] with modifications [25]. LMW and PMW calibration kits (Pharmacia, France) were used as molecular weight standards and the proteins stained with Serva blue G. ES-MS coupled with sequencing was performed as described above. The colorimetric reaction using 5,5'-dithiobis(2-nitrobenzoic acid) developed by Ellman [26] was performed to quantify the amount of free thiols of the recombinant aphrodisin according to Briand et al. [27]. Oligomerization was studied by exclusion-diffusion chromatography on a 24 ml bed volume Superose 12 (10 i.d. × 300 mm) column (Pharmacia) as described [10].

2.6. Circular dichroism (CD)

CD spectra were recorded on a Jasco J-810 spectropolarimeter at 20°C in a 0.1 mm path length cell. Aphrodisin concentration (0.5–1 mg/ml in 50 mM phosphate buffer pH 7.0) was determined using UV spectroscopy employing an extinction coefficient of 15 910 M⁻¹ cm⁻¹ at 276 nm, calculated according to Pace et al. [28]. Three successive scans were step-collected with an integration time set at 2–8 s. The baseline, made with phosphate buffer, was subtracted from the averaged spectrum. Results were expressed in mean residue molar ellipticity and analyzed according to Deléage and Geourjon [29].

2.7. Peptide mapping

In order to locate the glycosylation sites, natural aphrodisin was reduced by 10 mM dithiothreitol in 8 M urea, 0.25 M NH₄HCO₃ during 2 h at 40°C and alkylated by 20 mM iodoacetamide for 1 h at room temperature in the dark. Trypsinolysis was conducted for 18 h at 37°C after dilution to 2 M urea with a 1/50 enzyme/substrate ratio. Recombinant aphrodisin was reduced with 10 mM dithiothreitol in 200 µl 67 mM Tris-HCl, pH 8.4, 0.7 mM EDTA, 6 M guanidine-HCl during 2 h at 40°C and alkylated by 20 mM iodoacetamide for 1 h at room temperature in the dark. Alkylation was halted by addition of dithiothreitol to 20 mM final concentration. Reduced and alkylated protein was desalted by size exclusion chromatography on a Superdex (3.2 i.d. × 300 mm) peptide column. Formic acid (0.1%) was used as eluent at a flow rate of 25 µl/min with UV detection at 280 nm. The collected protein was lyophilized and subjected to trypsinolysis. The digestion was performed in 50 µl of 200 mM ammonium hydrogenocarbonate at pH 8.0 using 0.5 µg of modified trypsin (Promega sequencing grade) at 37°C for 18 h. The reaction was stopped by formic acid addition. Natural aphrodisin was subjected to the same treatments in the absence of the desalting step. Tryptic peptides were separated and analyzed by LC-MS and N-terminal sequencing. RPLC was run as described in Section 2.2 except the gradient began at 5% of eluent B and linearly increased to 42% in 90 min.

2.8. MALDI-MS analysis and N-terminal sequencing

After acidification with formic acid, 1 µl of tryptic peptides were mixed with 24 µl of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.15% v/v trifluoroacetic acid). 1 µl of the mixture was applied to a MALDI-MS sample plate and allowed to air dry. Spectra were obtained using a PE Voyager-STR DE spectrometer. Automated Edman sequencing was performed using a PE Biosystems Procise 494A sequencer with reagents and methods of the manufacturer.

2.9. Ligand binding test

Volatile odorant binding assays were performed as recently described [10]. Briefly, purified recombinant aphrodisin, recombinant rat OBP-1F and α-lactalbumin were dissolved in 50 µl of 500 mM K phosphate buffer, pH 7.5 to a final concentration of 1.65 mM. Twelve 500-µl glass tubes containing either control buffer, OBP-1F or α-lactalbumin solutions were incubated overnight at 25°C in a 2-l sealed glass chamber containing a pure odorant (8 µl in the chamber) which evaporated freely. Odorants trapped in the control buffer and in the various protein solutions were then extracted at room temper-

Table 1
MALDI-MS analysis of reduced and carboxyamidomethylated natural aphrodisin digested with trypsin

Peptide sequence	Calculated mass	Measured mass	Difference
Pyroglu1-K9	1017.47	1017.47	
W10-K22	1534.80	1534.81	
W10-R30	2386.25	2386.23	
I23-R30	869.46	869.46	
F31-R34	631.31	631.31	
H35-K59	3153.37	3356.41	203.04
H35-K59	3153.37	3502.48	349.11
T60-K67	893.52	893.51	
G68-K91	2734.29	2937.35	203.06
I92-K97	768.42	768.40	
N98-R101	534.22	534.04	
A102-K114	1316.68	1316.70	
G115-K133	2138.06	2138.14	
K134-E151	2039.06	2039.06	

Indicated masses are monoisotopic.

ature with 50 μ l of chloroform and their amounts determined by gas chromatography [10].

3. Results

3.1. Characterization of hamster vaginal discharge proteins

Hamster vaginal discharges, collected with a cotton swab and diluted with water, were subjected to RPLC coupled with ES-MS and N-terminal sequencing. Fig. 1 shows the resulting chromatogram with molecular weights and N-terminal sequences obtained from the corresponding peaks. The first peak of 5396.1 ± 1.4 Da exhibited an N-terminal sequence homologous to that beginning at position 82 of the precursor of the human acid-stable proteinase inhibitor observed in mucous fluid (identification: ALK1_HUMAN; SwissProt accession number: P03973 and P07757; theoretical M_r beginning at position 82: 5607 Da). The second and third peaks were attributed to oxytocin-neurophysin and neurophysin, respectively. Their N-terminal sequences were homologous to the murine oxytocin-neurophysin 1 precursor sequence (identification: NEU1_MOUSE; SwissProt accession number: P35454; theoretical M_r : 10970 and 9638 Da, respectively). The last peak showed aphrodisin features. It was blocked to Edman degradation and its predominant M_r was 17621.3 ± 1.3 Da. Two other proteins observed around 80 kDa on SDS-PAGE (Fig. 2C, lane 1) were not identified by LC-MS.

3.2. Purification and characterization of natural aphrodisin from vaginal discharges

Vaginal discharge dilutions were subjected to ion exchange HPLC and the resulting fractions analyzed by MALDI-MS. Fig. 2A shows that the natural protein eluted at 95 mM NaCl in agreement with its acid isoelectric point calculated to be 4.86. SDS-PAGE analysis of the purified protein (Fig. 2C, lane 2) revealed two close bands migrating around 17 kDa. A LC-MS experiment was conducted on the ion exchange chromatography fraction containing aphrodisin. Fig. 3A shows a natural aphrodisin reconstructed mass spectrum, which exhibited a predominant protein peak at $17621.3 \pm$

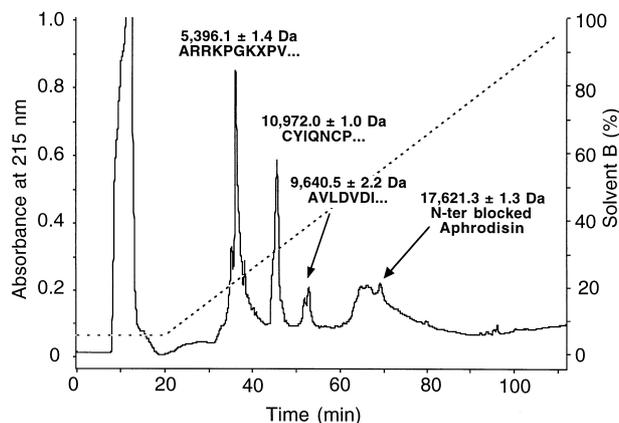


Fig. 1. Identification of hamster vaginal discharge proteins. LC-MS of the soluble fraction obtained from vaginal discharges was achieved by RPLC. Elution was performed with a linear gradient (dashed line) up to 95% solvent B (CH_3CN 66%, 2-propanol 33%, 4 mM ammonium acetate, 0.08% formic acid) mixed with solvent A (4 mM ammonium acetate, 0.1% formic acid in H_2O) in 90 min. Molecular weights and N-terminal sequences are indicated above the corresponding peak.

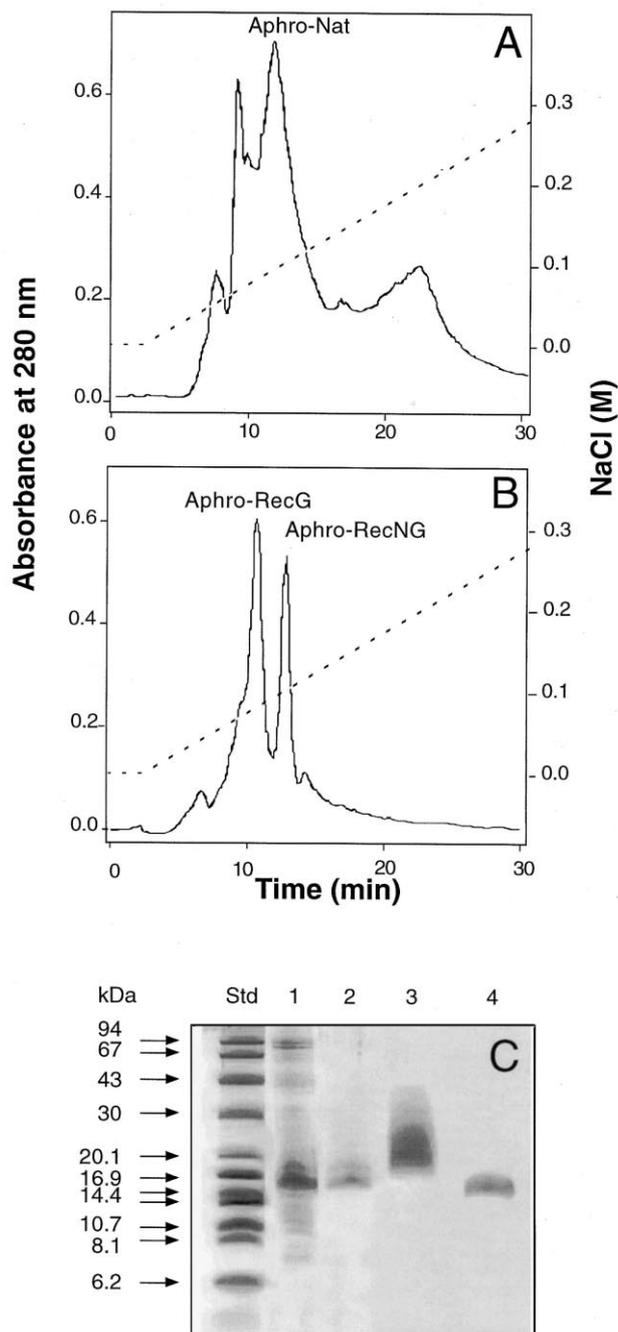


Fig. 2. Purification and characterization of natural and recombinant aphrodisin. A: Purification of natural aphrodisin (Aphro-Nat) by ion exchange chromatography; elution was performed with a 0–0.25 M NaCl gradient in 20 mM Tris-HCl at pH 8.0 (dashed line) in 25 min. B: Separation by ion exchange chromatography of recombinant glycosylated (Aphro-RecG) and unglycosylated (Aphro-RecNG) aphrodisin. C: Coomassie blue-stained SDS-PAGE of natural and recombinant aphrodisin. Std, molecular weight standards (Pharmacia PMW kit). Lane 1, vaginal discharge total proteins; lane 2, natural aphrodisin purified by ion exchange chromatography; lanes 3 and 4, purified recombinant aphrodisin (glycosylated and unglycosylated forms, respectively).

1.3 Da accompanied by two minor forms with M_r 17418.5 ± 2.0 Da and 17766.6 ± 2.6 Da. The major form corresponded to aphrodisin (theoretical M_r 17213.5 Da with formed disulfide bonds and a pyroglutamic acid at the N-terminus) with a mass excess of 406 Da suggesting linkage of two GlcNAc. The

first minor peak (17418.5 ± 2.0 Da) was 203 Da lighter than the major form indicating it contained only one GlcNAc instead of two. A larger molecule in low abundance showed a deviation of 145 Da from the major peak suggesting linkage of an additional deoxyhexose, probably fucose, on one of the two GlcNAc. In order to confirm the identification of this protein and locate the glycosylation sites, it was subjected to trypsinolysis and the resulting peptides analyzed by MALDI-MS (Table 1). The measured molecular weights of all the peptides were in perfect agreement with those calculated from the cDNA sequence, except in the case of peptides H35–K59 and G68–K91. Two peptides with monoisotopic molecular weights of 3356.41 Da and 3502.48 Da were assigned to H35–K59. They corresponded to the addition of one GlcNAc with and without one deoxyhexose added (theoretical M_r 3356.44 and 3502.50 Da, respectively). The G68–K91 peptide exhibited a monoisotopic molecular weight of 2937.35 Da in accordance with linkage of one GlcNAc (theoretical M_r 2937.36 Da). The natural glycosylation was observed to vary from one discharge to another. In some cases, both sites were observed to simultaneously bear an extra fucose (not shown).

3.3. Expression and purification of recombinant aphrodisin

Aphrodisin was expressed using the methylotrophic yeast *P. pastoris* under the control of the methanol-inducible alcohol oxidase (*AOX1*) promoter. The protein was secreted using the yeast prepropeptide signal of the *Saccharomyces cerevisiae* α -mating factor peptide without spacer. Approximately 250 His⁺ transformants of the GS115 strain were obtained by electroporation and 24 transformants corresponding to the methanol utilization slow phenotypes selected. Clones were screened by SDS-PAGE for their capability to secrete large amounts of aphrodisin in the extracellular medium. Only two bands of recombinant protein, migrating in the range 17–19 kDa, were detectable by Serva blue G staining. The electrophoretic profile (data not shown) revealed that the proteins regularly accumulated, reaching approximately 230 mg/l over an expression period of 4 days as determined after purification, while other proteins were only present in trace amounts. No proteolytic degradation of the recombinant protein was detectable during an expression period of 5 days, as confirmed by MALDI-MS (not shown).

After centrifugation, the yeast culture filtrate was subjected to ion exchange HPLC. Fig. 2B shows that two proteins eluted at 90 mM and 110 mM NaCl, respectively. A last purification step was conducted on each fraction through gel filtration in order to remove small ligands. SDS-PAGE analysis of the purified fractions is shown in Fig. 2C. The fastest migrating band (lane 4) showed an apparent molecular weight of approximately 17 kDa while the slowest one (lane 3) migrated as a scattered 19 kDa band. We named these isoforms Aphro-RecNG for recombinant unglycosylated aphrodisin and Aphro-RecG for recombinant glycosylated aphrodisin, respectively. The latter was three times more abundant than Aphro-RecNG in the culture medium.

3.4. Recombinant protein characterization

Sulfhydryl titration using the method of Ellman showed that disulfide bridges were indeed formed since a content of approximately 0.1 thiol/protein was measured, which was confirmed by MS. N-terminal sequencing and LC-MS were per-

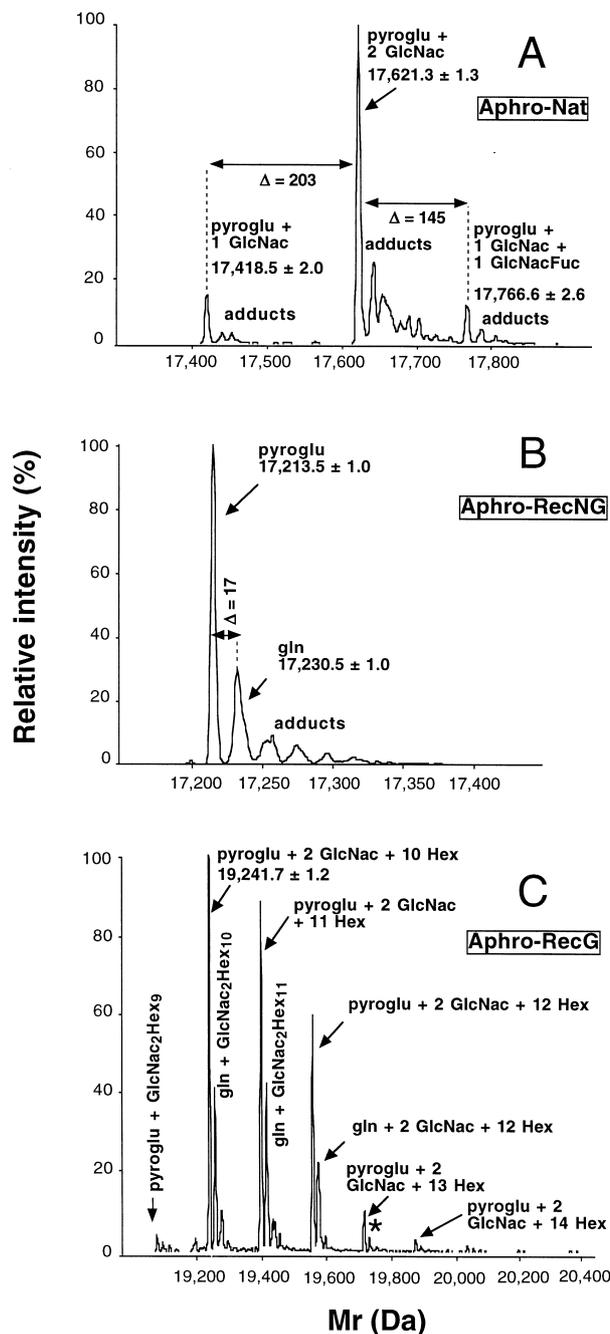


Fig. 3. Reconstructed mass spectra of natural and recombinant aphrodisin. A: Natural aphrodisin (Aphro-Nat) isolated from hamster vaginal discharge. B: Unglycosylated recombinant aphrodisin (Aphro-RecNG). C: Glycosylated recombinant aphrodisin (Aphro-RecG). Pyroglu and gln indicate the occurrence of either pyroglutamic acid or glutamine as N-terminal residue, respectively; The asterisk indicates a protein with 13 hexose residues and N-terminal Gln; Δ , mass difference between peaks shown by arrows. Na and K adducts are shown.

formed to analyze these recombinant aphrodisin isoforms. Fig. 3B shows the reconstructed mass spectrum of the Aphro-RecNG fraction. Two different molecular weights were observed together with derivatives corresponding to Na and K adducts. The most abundant (approximately 80%, based on ion count) named Aphro-RecNG.pyroglu exhibited a M_r of 17213.5 ± 1.0 Da, in perfect agreement with that deduced

from the cDNA sequence (17213.5 Da) with pyroglutamic acid at the N-terminus and two disulfide bridges formed. An additional peak (approximately 20%) named Aphro-RecNG.gln of 17230.5 ± 1.0 Da corresponding to a mass excess of 17 Da was assigned to the same molecule with a glutamine residue instead of pyroglutamic acid at the N-terminus, as confirmed by sequencing. Approximately 20% of the amount of the analyzed recombinant aphrodisin was observed not to be blocked to Edman sequencing. Neither of these two isoforms was found to be glycosylated.

Fig. 3C shows the reconstructed mass spectrum of the Aphro-RecG fraction. Six molecular weight doublets were observed. They all comprised a major peak with a minor one having a mass excess of 17 Da likely due to the presence of glutamine instead of pyroglutamic acid at the N-terminus. The major peak exhibited a M_r of 19241.7 ± 1.2 Da in perfect agreement with that deduced from the cDNA sequence with pyroglutamic acid at the N-terminus, two disulfide bridges formed and two GlcNAc linked to 10 hexoses (theoretical M_r 17241.3 Da). The other doublets were separated from each others by 162 Da due to variable hexose addition (9–14 hexoses). Compared to the natural molecule, the recombinant aphrodisin produced by *P. pastoris* was hyperglycosylated. Protein with glycosylation at the other site was also observed by ES-MS, but in a very low amount (not shown).

Reduced and alkylated Aphro-RecG was trypsinolysed and the resulting peptides identified by LC-MS (Table 2). The size of all the peptides was in perfect agreement with the molecular weight calculated from the amino acid sequence, except that of peptide G68–K91, which was found to be 4764.9 ± 0.7 Da, corresponding to two GlcNAc linked to 10 hexoses (theoretical M_r 4765.1 Da). In addition, as for the undigested protein (Fig. 3C), we observed additional peaks separated by 162 Da due to variable hexose addition in the range 9–14 (data not shown). Peptide G68–K91 was subjected to Edman sequencing. All expected residues were observed except N69, indicating that it was the glycosylation site. The N41–K59 peptide sequence revealed that N41, the other potential *N*-glycosylation site, was not glycosylated.

The correct folding of recombinant aphrodisins was attested by their CD spectra. Fig. 4A shows the overlaid CD spectra of the natural and the two forms of recombinant aphrodisin. The shape of the spectra, i.e. a positive band at 196 nm and a negative one centered at 217 nm, clearly showed

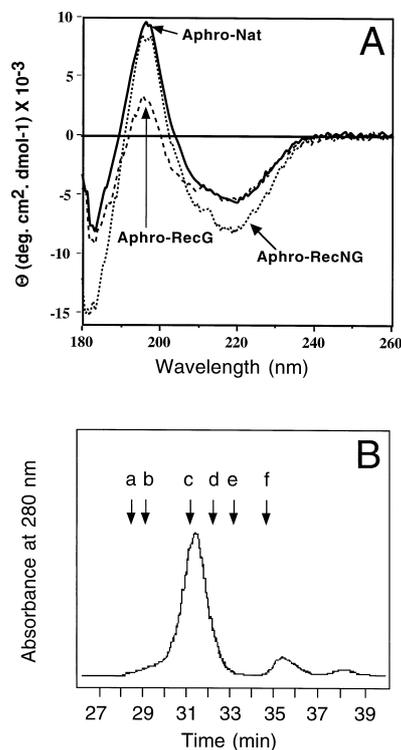


Fig. 4. Secondary and quaternary structures of natural and recombinant aphrodisin. A: Far-UV CD spectra of natural aphrodisin (Aphro-Nat, solid line), recombinant glycosylated aphrodisin (Aphro-RecG, dashed line) and recombinant unglycosylated aphrodisin (Aphro-RecNG, dotted line). Protein concentration was 0.5–1 mg/ml and path length 0.1 mm. B: Exclusion–diffusion chromatography on Superose 12 of Aphro-RecG. Elution positions of molecular weight standards are indicated by arrows: a, bovine serum albumin (67 kDa); b, chicken egg ovalbumin (43 kDa); c, dimeric bovine β -lactoglobulin (36 kDa); d, carbonic anhydrase (30 kDa); e, soybean trypsin inhibitor (21.5 kDa); f, bovine ribonuclease A (13.7 kDa).

the presence of a high abundance of β -sheet. The spectra were not strictly superposable in shape and intensity. The positive band of Aphro-Nat and Aphro-RecNG, on the one hand, and the negative band of Aphro-Nat and Aphro-RecG, on the other hand, were superimposable. The spectrum deconvolution revealed very close proportions of secondary structure in Aphro-Nat and Aphro-RecG (40% β -sheet and 10% α -helix) whereas Aphro-RecNG was shown to be composed of approximately 50% β -sheet and less than 10% α -helix. Calibrated exclusion–diffusion chromatography of purified Aphro-RecG at 1.0 mg/ml (Fig. 4B) gave an apparent molecular mass of 32.8 kDa, which is approximately twice the value obtained from MS, demonstrating dimerization of the recombinant glycosylated protein at neutral pH.

3.5. Volatile odorant binding assay

The large quantity of aphrodisin secreted by *P. pastoris* made it possible to use a recently described assay [10] to show the uptake of airborne molecules by aphrodisin. We tested two sulfur-containing compounds that are released in abundance in vaginal secretions at estrus, methyl thiobutyrates (MTB) and dimethyl disulfide (DMDS) [30], and also an odorant, 2-isobutyl-3-methoxypyrazine (IBMP). For this purpose, purified Aphro-RecG, recombinant rat OBP-1F and a control protein (bovine α -lactalbumin, a protein that does not

Table 2

ES-MS analysis of reduced and carboxyamidomethylated recombinant glycosylated aphrodisin (Aphro-RecG) digested with trypsin

Peptide sequence	Calculated mass	Measured mass
Q1–K9	1035.1	1034.9 \pm 0.4
Pyroglu1–K9	1017.0 ^a	1017.6 ^a
W10–K22	1535.8	1535.1 \pm 0.1
I23–R30	869.5 ^a	869.4 ^a
F31–R34	631.3 ^a	631.4 ^a
H35–K40	834.4 ^a	834.4 ^a
N41–K59	2338.6	2338.5 \pm 0.4
T60–K67	893.5 ^a	893.6 ^a
G68–K91	2735.9	4764.9 \pm 0.7
I92–K97	768.4 ^a	768.6 ^a
N98–R101	534.2 ^a	534.4 ^a
A102–K114	1317.5	1317.0 \pm 0.3
G115–K133	2139.4	2139.4 \pm 0.3
I135–E151	1912.2	1911.5 \pm 0.2

^aMonoisotopic mass.

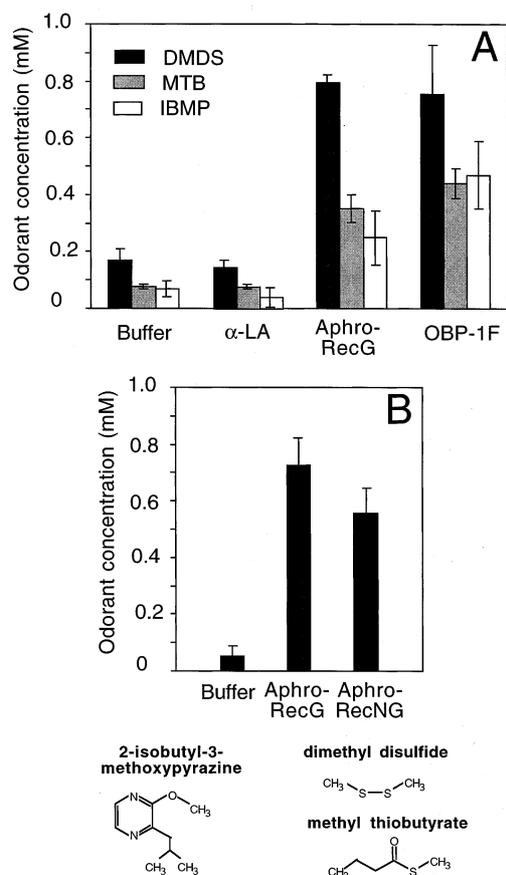


Fig. 5. Binding of airborne odorants onto aphrodisin using volatile odorant binding assay. A: Comparison of binding onto α -lactalbumin (α -LA), Aphro-RecG, recombinant rat OBP-1F and buffer (500 mM phosphate, pH 7.5) of DMDS, MTB and IBMP. B: Comparison of DMDS binding onto Aphro-RecG and Aphro-RecNG. Volatile ligand concentrations were determined by GC. Bars indicate standard deviations. The chemical structures of tested odorants are shown.

bind hydrophobic molecules such as odorants) were tested at a high concentration similar to that of OBP in the nasal mucus (1.65 mM). Fig. 5A exhibits the comparison of the ability of Aphro-RecG, OBP-1F and α -lactalbumin to bind the three tested molecules (IBMP, DMDS and MTB). Odorant amounts trapped by the bovine α -lactalbumin solution were not different from those solubilized by the control buffer alone. In contrast, all tested odorants bound very strongly to recombinant aphrodisin and OBP-1F, although their amounts in the aqueous buffer remained very low, except DMDS, known to be slightly soluble in water. The affinity of the odorant molecules for aphrodisin and OBP-1F appeared very close, except in the case of IBMP. In addition, we observed that Aphro-RecNG was also able to bind DMDS with the same apparent affinity as Aphro-RecG (Fig. 5B).

4. Discussion

In addition to aphrodisin, three major proteins were identified among abundant soluble proteins of hamster vaginal discharges: a proteinase inhibitor homologous to the human acid-stable proteinase inhibitor observed in mucous fluid, oxytocin-neurophysin and neurophysin. However, we did not ob-

serve the recently described human serine proteinase inhibitor expressed in vaginal gland and Bartholin's glands [31]. Three natural aphrodisin isoforms, which vary in glycosylation degree, were purified and characterized. In agreement with the results obtained by Singer et al. [1], the major natural isoform exhibited two sites (N41 and N69), each glycosylated by a single GlcNAc. Unlike these authors, we found two minor isoforms, the former with one GlcNAc at N69 and one GlcNAc linked to a deoxyhexose at N41 and the latter with only one GlcNAc whose linkage site could not be determined.

The origin of the aphrodisiac properties of aphrodisin may be found either in its intrinsic properties, in its ability to behave as a small pheromone carrier or in both as described for the mouse major urinary protein [15]. Large amounts of recombinant protein, which has never been in contact with any putative active small organic molecule, are necessary to carry electrophysiological or biochemical investigations to demonstrate the intrinsic aphrodisiac property. For such a goal, proper post-translational modifications akin to those of the natural aphrodisin must be performed by the heterologous expression system. The *P. pastoris* system was therefore chosen because it allows producing large quantities of disulfide-bonded *N*-glycosylated proteins [27,32–35].

Recombinant aphrodisin was produced and purified in large amounts (230 mg/l) consisting of two major isoforms that could be separated. One form was shown to be glycosylated at only one of the two glycosylation sites (N69) with two GlcNAc linked to 9–14 hexoses. Hexose residues should likely be mannose since Montesino et al. [36] have shown that *N*-glycosylated proteins secreted by *P. pastoris* are essentially glycosylated with such residues. The other major isoform was found to be unglycosylated. Only 80% of both fractions was blocked by a pyroglutamic acid residue, contrary to the natural aphrodisin, which was found to be totally blocked. These results confirm that *P. pastoris* is able to accomplish glycosylation of heterologous polypeptide chains by addition of *N*-asparagine-linked oligosaccharides composed of mannose-type isomers [33,37], but with dissimilarity with the natural vertebrate glycosylation process.

Recombinant aphrodisins appeared to be properly folded with their disulfide bonds formed, but CD showed that their conformation was slightly dependent on their glycosylation degree with a lipocalin secondary structure [6–8,38]. The presence of carbohydrate moieties on Aphro-Nat and Aphro-RecG could explain differences in CD due to some local conformational changes of the three-dimensional structure, as previously observed for other proteins [39]. The aphrodisin glycosylation degree seems nevertheless not to interfere with the odorant binding properties.

The large amounts of produced aphrodisin permitted us to use a volatile odorant binding assay [10] to test unlabeled ligands since the fluorescent probe 1-AMA proved not to interact with aphrodisin. Aphrodisin was observed to be as efficient as a rodent OBP to bind volatile ligands of different chemical structures and odors. Not only IBMP but also odorants naturally present in vaginal secretions were observed to bind aphrodisin with an affinity close to that of rat OBP-1F. DMDS, a highly volatile sulfur-containing constituent, is known to function as a sex attractant (pheromone role) but does not elicit copulatory behavior in hamster males [40–42]. DMDS binding strengthens the idea that aphrodisin might also be a pheromone carrier, like α_{2u} -globulin, a rat major

urinary protein. Krieger et al. [15] have indeed recently demonstrated that this lipocalin functions as a volatile pheromone carrier protein, in addition to its own pheromonal role. Recombinant glycosylated aphrodisin was found to exist as a dimer at neutral pH like recombinant rat OBP-1 [43], OBP-1F [10] and bovine OBP [44,45]. The question of whether the odorant binding site is located at the monomer interface or in the β -barrel has now to be addressed.

Our results provide the first evidence that aphrodisin is also able to bind small volatile compounds which could bear the pheromonal activity attributed to the natural protein. The identification of these putative active compounds bound to the natural aphrodisin is under way. In addition, heterologous expression of aphrodisin opens the possibility for site-directed mutagenesis of specific residues in order to investigate and clearly define the relationships between the structure and the function of this aphrodisiac protein.

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