

Odorant-binding protein as active ingredient for fragrance release regulated by sweat

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ABSTRACT

Mammalian odorant-binding proteins (OBPs) represent a fascinating class of small, soluble proteins that transport odors from the air to the olfactory receptors, through the nasal mucus. OBPs can accommodate diverse odorant molecules through their flexible binding pockets, undergoing conformational changes upon ligand binding. Acknowledge of release mechanism could be very useful to provide a controllable release trigger for cosmetic applications. However, OBPs face significant challenges when applied to keratin-based substrates (hair and wool) because OBPs demonstrate weak binding affinity to keratin. To overcome this challenge, pig OBP sequence was fused to keratin-binding peptide (KP) with a spacer (GQ₂₀), designated as OBP::GQ₂₀::KP. Protein structure revealed a high stability and high protein affinity to a ligand model (dissociation constants of $0.20 \pm 0.04 \mu\text{M}$ at 25 °C, $0.25 \pm 0.03 \mu\text{M}$ at 37 °C). However, when added a sweat solution, the dissociation constants decrease drastically ($1.69 \pm 0.01 \mu\text{M}$ at 25 °C, $2.04 \pm 0.02 \mu\text{M}$ at 37 °C) what didn't happen tested with individual components of sweat. To understand the OBP::GQ₂₀::KP functionality in cosmetic formulations, it was added to a body cream and a hair mask. Commercial products containing OBP::GQ₂₀::KP, when in the presence of sweat, promoted an increase of 90.7% linalool release existent in body cream, and an increase of 77.0% linalool release present in hair mask, versus absence of this OBP in the same cosmetics (GC–MS analysis). Olfactory assessment using volunteers supported OBP::GQ₂₀::KP capacity to management of fragrances in response to human sweat.

1. Introduction

Olfactory perception plays a role in detection of odor molecules, in warning mechanisms against potentially harmful substances and contributes to adaptive evolutionary responses. Mammalian odorant-binding proteins (OBPs) are fascinating components of the olfactory system by facilitating the transport and detection of odorant molecules in nasal mucus until to olfactory receptors. There are small (~20 kDa), globular, water-soluble carrier proteins belong to the lipocalins family, secreted by the epithelial cells in the nasal cavity at high concentration (~10 mM) and released for the nasal mucus [1–3]. In mammalian, it is known the existence of OBPs in a variety of mammalian species such as cows, pigs, mice, rats, rabbits, giant pandas, cats, chimpanzee, humans, and others deposited and available in UniProt and Ensembl [4,5]. Pig odorant-binding protein (pOBP) was used in this study because it has a

conserved structure composed by eight antiparallel β -sheets flanked by a short α -helix located close to the C-terminus, forming a β -barrel with a central apolar pocket, where hydrophobic compounds are transported [6–8]. pOBP was discovery in 1991 [9], 20 years before of human OBP discovery [10] counting with more information about pig OBP structure, stability and binding characterization than human OBP, making it useful to study the binding specificities and mechanisms within the OBP family. More, pOBP protein offers a recombinant expression more efficiently in *Escherichia coli*, producing higher yields of functional protein and consequently less costly associated and more attractive for commercial applications.

OBP action mechanism involves the capture of volatile odorants from the air and transport them through the aqueous mucus to the release of odor to olfactory receptors. The exact mechanisms involving odorant capture and release from OBP is not fully understood, but some

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proposals have been presented. Since nasal mucus is hydrophilic and the odorant molecules are hydrophobic, these molecules cannot dissolve directly in the mucus without help of OBP transport [1,11]. OBPs bind and transport hydrophobic odorant molecules by lowering the energy barrier through the aqueous environment to olfactory receptors [12]. The binding process involves a conformational change of OBP binding site. The capture (adsorption) of odorant molecule by OBP seems to be affected by the (i) molecular size of odorant, (ii) chemical structure and hydrophobicity of odorant, (iii) pH and temperature of mucus, (iv) OBP concentration and (v) competitive binding by other molecules. Molecular docking results showed that hydrophobic residues in the binding cavity are strongly involved in the stabilization of the odorant. In addition, a polar residue was identified as having a major role in the orientation of the odorant within the binding cavity [13]. The studied of Moitrier et al. also suggest that mOBP5 binding is primarily controlled by the size of the odorant, independent of its chemical function [14]. On the other hand, the composition of nasal mucus is also important to understand the ion changes that could occur in the adsorption mechanism. Nasal fluid is composed by sodium, potassium, chloride, bicarbonate, urea, calcium, magnesium and phosphate [15]. Upon odorant reaching the olfactory neurons, the desorption and interaction of odor to olfactory receptor includes another conformational change that enables odorant release. The desorption (dissociation) process seems to be influenced by the (i) presence and concentration of electrolytes, (ii) odor molecule properties, (iii) receptor surface properties, (iv) pH and temperature near of receptor, (v) electrostatic interactions, and (vi) specific protein-receptor recognition mechanisms. Once the odorant is released near the olfactory receptor, it binds to the receptor's binding site and occur a cascade of intracellular signaling events, resulting an electrical signal that can be interpreted by the brain as a specific smell [16]. This cascade involves the influx of sodium (Na^+) and calcium (Ca^{2+}) ions across the membrane followed by the efflux of chloride ions (Cl^-) [16].

The acknowledge action mechanisms of OBPs will allow to create new biotechnological applications, for example, to design target odor management strategies, to develop odor neutralization technologies, to create advanced olfactory sensors, to produce fragrance delivery technologies. Despite the potentially of this protein, OBPs face significant challenges when applied to keratin-based substrates such as hair and wool because OBPs demonstrate relatively weak binding affinity to keratin fibers. Addressing this challenge, we designed a new recombinant protein composed by the fusion of pig OBP sequence (PDB ID 1DZK) with to keratin-binding peptide (KP) containing a spacer (GQ_{20}) between them and designated as OBP::GQ₂₀::KP. GQ₂₀ spacer facilitates OBP movement [13] and KP exhibits binding affinity for keratin [17].

2. Material and methods

2.1. Reagents

GRS Protein Marker Blue and Terrific broth culture medium was purchased from GRISP, Portugal. 1-aminoanthracene (98%), kanamycin monosulfate (>94%), and D-(+)-Lactose monohydrate (>98%) were acquired from TCI chemicals, Belgium. MTS kit assay was provided by Promega (USA). Body cream (ATL) was purchased in a local store and hair mask (K18) was offered by the supplier. Tris-base, sodium phosphate, sodium chloride, dialysis membrane (14 kDa cutoff), SPME fiber (100 μm polydimethylsiloxane), HCl 37% and other reagents were obtained from Merck, Spain.

2.2. Design of OBP::GQ₂₀::KP fusion protein

The sequence of OBP-I (PDB ID 1DZK, from pig *Sus scrofa*) was fused with a spacer composed by 20 repetitions of glycine-glutamine residues (GQ₂₀) and with keratin-binding peptide (KP). KP has capacity to bind keratin [17]. The protein was designated as OBP::GQ₂₀::KP. The gene sequence was synthesized by GenScript, USA, and cloned in pET-28a

plasmid.

2.3. Fusion protein production and purification

The fusion protein was produced in *Escherichia coli* BL21(DE3), in a shake flask containing Terrific Broth (TB) culture medium and kanamycin (50 $\mu\text{g}/\text{mL}$), at 37 °C, 250 rpm. When the culture reaches the optical density between 4 and 6, the protein expression was induced with 2 g/L of lactose, for 16 h. Cells were harvest by centrifugation 7000g, 10 min and, lysed by sonication as described in Gonçalves et al. [13]. Insoluble and soluble fractions were separated by centrifugation at 9000g, for 45 min at 4 °C. The protein present in soluble fraction was purified using Nickel column with specificity to His-tag present in the protein's N-terminal. Pure fractions were dialyzed to remove salts and imidazole, as described by Gonçalves et al. [13].

2.4. Characterization of the OBP::GQ₂₀::KP fusion protein

2.4.1. SDS-PAGE analysis

Protein size and purity were analyzed by SDS-PAGE gel. The lyophilized protein was solubilized in solubilization buffer (6 M urea, 20 mM Tris-HCl pH 8.0, 0.5 M NaCl and 10 mM DTT), loaded on SDS-PAGE gel (12.5% acrylamide) and stained with Coomassie Blue. Protein purity was measured by intact mass analysis using X500B Q-TOF MS platform Mass Spectrometry Unit (UniMS), ITQB/IBET, Oeiras, Portugal.

2.4.2. Binding affinity of OBP::GQ₂₀::KP protein to 1-aminoanthracene, sweat mixture, sweat compounds and other odors

The binding capacity of OBP::GQ₂₀::KP was determined by direct titration with 1-aminoanthracene (1-AMA, ligand model used for lipocalin superfamily), as reported by Gonçalves et al. [13,18]. Resuming, 1 μM of protein dissolved in 50 mM Tris-HCl buffer, pH 7.5 was added to increasing concentrations of 1-AMA and incubated at 25 or 37 °C for 1 h. The fluorescence of the OBP-ligand complex was measured at 481 nm of emission (excitation at 295 nm). Measurements were recorded in triplicate, in three independent experiments, on a microplate spectrofluorometer (BioTek Synergy MX) equipped with a temperature controller. Dissociation constants (K_d) were calculated from a plot of fluorescence intensity versus concentration of 1-AMA, obtained with a standard non-linear regression method, described in Malpeli et al. [19]. To determine the effect of sweat solution in protein binding, a competitive assay was performed following previous work [13], when increased concentrations of artificial sweat were added to protein-1-AMA complex. The artificial sweat solution was prepared following the AATCC method 15-2009 "Colorfastness to Perspiration". Statistical analysis of protein binding without and with sweat was performed using one-way ANOVA and selecting Tukey's multiple comparisons test. Competition binding assays with sweat solution and the individual components of artificial sweat (sodium chloride, lactic acid, sodium phosphate and L-histidine) were performed to compare the action of individual components and the sweat mixture in the binding performance of OBP. For that, titration of individual sweat components was done, from 0 g/L to maximal concentration of artificial sweat described in the recipe. Once that mixture of artificial sweat has a pH 4.3, the 1-AMA binding was performed at pH 7.5 and pH 4.0 to compare the pH effect on OBP binding. Additionally, 1 malodor (isovaleric acid), 2 fragrances (linalool and β -citronellol) and a blend named Fructis Rev2 containing linalool in its composition were also tested in competition binding assays. Briefly, in competitive assay, 1 μM of OBP::GQ₂₀::KP in 50 mM of Tris-HCl, pH 7.5, was incubated with 2 μM of 1-AMA at 25 °C, for 1 h. Then, increased concentrations of competitor were added and incubated in the same conditions. For all assays, the fluorescence of the OBP-ligand complex was measured at 481 nm (excitation at 295 nm). All measurements were recorded in triplicate, in three independent experiments, on a microplate spectrofluorometer (BioTek Synergy MX) equipped with a temperature controller. Dissociation constants (K_d) for competitive assays were

calculated Cheng–Prusoff equation: K_d of ligand (μM) = $\text{EC}_{50}/(1 + [1\text{-AMA}/K_{d1\text{-AMA}}])$, where, EC_{50} (in μM) is defined as the ligand concentration that reduces the initial fluorescence by 50%, calculated by non-linear regression using a one-binding-site model in which fluorescence is plotted as a function of ligand concentration (in μM). $[1\text{-AMA}]$ denotes the concentration of the fluorescent probe used in the competitive assay (2 μM), and $K_{d1\text{-AMA}}$ is the dissociation constant of the 1-AMA probe.

2.4.3. Structural stability of the fusion protein

The structure of the fusion protein was evaluated by circular dichroism spectroscopy using a Jasco J-1500 spectropolarimeter equipped with a temperature controller, as reported by Gonçalves et al. [18]. The structure was evaluated at different temperatures (18, 25, 37, 45, 60, 70, 80 and 90 °C), range of pH (pH 4.0, 5.5, 7.5 and 9.0), and in the presence of artificial sweat. The analysis of the spectra in term of secondary structure was performed using the DichroWeb [20,21].

2.5. Cell viability in vitro in presence of OBP::GQ20::KP protein

2.5.1. Cell culture conditions

Human hair follicle dermal papilla (HFDP) cells were obtained from Applied Biological Materials (abm). The cell line was cultured in DMEM containing 4.5 g/L glucose, L-glutamine, 1.5 g/L sodium bicarbonate and sodium pyruvate supplemented with 10% FBS. The human skin keratinocytes (HaCaT) cell line was obtained from Cell Line Service (CLS). The cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 4.5 g/L glucose, L-glutamine, 1.5 g/L sodium bicarbonate and sodium pyruvate supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human hTERT-immortalized foreskin fibroblast (BJ-5ta) cells were purchased from American Type Culture Collection (ATCC) and maintained in a 4:1 mixture of DMEM with 4 mM L-glutamine, 4.5 g/L glucose and 1.5 g/L sodium bicarbonate supplemented with 10% heat-inactivated FBS and 0.01 mg/mL hygromycin B. Cell culture mediums were supplemented with 1% of an antibiotic-antimycotic solution (penicillin-streptomycin-amphotericin). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 .

2.5.2. Cell viability assay

Cell viability was assessed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyl oxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent in accordance with the manufacturer's instructions. Cells were seeded into 96-well cell culture plates and incubated for 24 h at 37 °C in 5% CO_2 to promote cell adhesion. After, the cells were treated with different concentration of OBP::GQ20::KP protein (0.05 mg/mL to 0.4 mg/mL) for 48 h. The MTS assay was performed in triplicate from two independent experiments. Then, 20 μL of MTS reagent was added to each well and incubated for 3 h at 37 °C. Absorbance was measured at 490 nm using a microplate reader (Synergy H1 multimode reader, Bio-Tek). Cell viability was expressed as a percentage relative to the negative control (untreated cells). Statistical analysis of the untreated cells and culture cells treated with different concentration of OBP::GQ20::KP were assessed using GraphPad Prism 8.0.1 (GraphPad Software, Inc., La Jolla, CA, USA) through a one-way ANOVA followed by Dunnett's multiple comparisons test against untreated cells and a confidence level of 95%. The obtained data are presented as mean $\pm$ standard deviation (mean $\pm$ SD).

2.6. Application of OBP::GQ20::KP protein onto hair

The fusion protein (OBP::GQ20::KP) was applied on bleached Asian hair, with virgin Asian hair used as control. The bleaching of Asian black hair was performed using KAY PRO Polvere Decolorante powder and 3.5 g of VIBA Professional cream according to brand specifications. The hair tresses were washed before and after each bleaching cycle (7 cycles) with a classic commercial shampoo (Pantene Basic). Tresses (300 $\pm$ 1 mg) of bleached hair were treated with 1 mL of wild-type OBP and OBP::

GQ20::KP proteins (1 mg/mL dissolved in dH_2O) and incubated for 10 and 60 min, followed by drying with a hairdryer. Control samples were obtained by adding 1 mL of water to the hair tresses. Wild-type OBP (without KP fusion) was used as the control protein to validate the role of KP.

2.6.1. Quantification of the OBP::GQ20::KP-functionalized hair

After the incubation of wild-type OBP, OBP::GQ20::KP and water in hair tresses, for 10 and 60 min, the excess of volume, corresponding to the non-functionalized protein, was recovered and quantified using DC Protein Assay (Bio-Rad). The amount of protein functionalized onto hair fiber was calculated by the difference between the initial concentration of protein (1 mg/mL) and the concentration of non-functionalized protein, measured by DC Protein Assay kit. Statistical analysis of the amount of wild-type OBP and the amount of OBP::GQ20::KP functionalized on hair were assessed using GraphPad Prism 8.0.1 through a two-way ANOVA comparing each cell mean with the other cell mean in that row and correction using Sidák test and a confidence level of 95%. The obtained data are presented as mean $\pm$ standard deviation (mean $\pm$ SD).

2.6.2. Penetration degree of OBP::GQ20::KP-functionalized hair

The penetration degree of OBP::GQ20::KP protein into hair was evaluated through the transversal cuts (20 μm) of individual bleached hair fibers treated with protein FITC (fluorescein-5-isothiocyanate)-labelled (1 mg/mL) and embedded in epoxy resin. Protein was labelled with FITC following the instruction of the manufacture (Merck, Spain). Fibers treated with water were used as control. Transversal cuts were prepared using a microtome (Microtome Leitz, Oberkochen, Germany) and analyzed by Confocal Scanning Laser Microscope (Olympus BX61, Model FluoView 1000), using the program FV10-Ver4.1.1.5 (Olympus) integrated with Line Series Analysis at a magnification of 40 $\times$ [22,23]. All fluorescence microscopy images were recorded using a laser excitation line at 488 nm and emissions filters BA 505–605 and using identical filter, exposure, brightness and gain settings. Quantitative fluorescence analysis of images was evaluated using ImageJ, an image processing program. In this program, the fluorescence intensity profile of the protein along the fibers' horizontal section, defining a line along of fiber's horizontal section and then measuring the plot profile. The same procedure was done to bleached hair without protein treatment. The data were saved and the graphics represented in GraphPad Prism 8.0.1.

2.6.3. Thermal analysis of the OBP::GQ20::KP-functionalized hair

The thermal resistance of the hair treated with OBP::GQ20::KP protein was accessed by dry Differential Scanning Calorimetry (DSC) following the procedure indicated in Tinoco et al. [23]. For these assays, middle region of the hair fibers, treated with and without protein, was cut into small portions and weighted 2–5 mg in aluminum pans (max. Pressure: 1 bar), and analyzed using DSC instruments (DSC 6000, Perkin Elmer), under a nitrogen atmosphere, at a temperature range from 50 to 250 °C (heating rate: 5 °C/min). The DSC instrument calibration was performed using high-purity indium and zinc and all samples were measured in duplicate, with the mean value and standard deviations calculated. Statistical analysis of the delta H obtained with hair and the hair treated with OBP::GQ20::KP were assessed using GraphPad Prism 8.0.1 through a two-way ANOVA comparing each cell mean with the other cell mean in that row and correction using Sidák test and a confidence level of 95%. The obtained data are presented as mean $\pm$ standard deviation (mean $\pm$ SD).

2.6.4. Mechanical behavior of damaged (bleached) hair functionalized with OBP::GQ20::KP protein

Individual hair fibers (30 monofilaments) were selected from hair tresses to perform the mechanical tests using the TA.XTplusC Texture Analyzer as reported in Tinoco et al. [24]. Young's modulus was calculated based on the average of all hair single fibers using a load cell of 5 kg and a clamping distance of 55 mm, according to guidelines outlined in

ASTM C1557-14 for fiber tensile testing. Each hair was individually mounted in the tensile jig by means of a paper template with a fixed gauge length of 55 mm. Measurements were performed under controlled conditions ($25 \pm 1^\circ\text{C}$; $55 \pm 5\%$ relative humidity) at a rate of 3.0 mm/min. Results were presented in Young's modulus value that define the relationship between stress and strain, which is related to material strength and elasticity. Statistical analysis between the Young's modulus values for virgin hair, bleached hair and bleached hair treated with OBP::GQ₂₀::KP were assessed using GraphPad Prism 8.0.1 through a one-way ANOVA comparing the mean of each column with the mean of every other column and correction using Tukey test and a confidence level of 95%. The obtained data are presented as mean $\pm$ standard deviation (mean $\pm$ SD).

2.7. Fragrance release from OBP::GQ₂₀::KP protein formulated in commercial cosmetics in presence of sweat

Following the observed release of 1-AMA from OBP::GQ₂₀::KP upon artificial sweat addition, we sought to evaluate the performance and efficacy of this protein in commercial cosmetic formulations.

2.7.1. Incorporation of the OBP::GQ₂₀::KP protein in cosmetics

OBP::GQ₂₀::KP protein (0.01%, w/v) was incorporated in two commercial products containing fragrances in their composition - ATL body cream (0.1 g) and K18 hair mask (0.1 g) - followed by the addition of artificial sweat (5 puffs in spray). Artificial sweat solution (pH 4.3 ± 0.2) was prepared according to AATCC Test Method 15-2009. The samples tested were: (i) the commercial product (only), (ii) the commercial product + the protein + artificial sweat, and (iii) the commercial product + artificial sweat. After exposition of the GC SPME fiber to these samples for 1 h at 25°C , the peak areas of the fragrances present in the commercial products (ATL body cream or K18 hair mask) were analyzed by GC-MS. The analysis was performed in duplicate. ATL body cream contains on its composition Aqua, Petrolatum, Decyl Oleate, Dimethicone, Glycerin, Polyglyceryl3 Methylglucose Distearate, Glyceryl Stearate, Stearyl Alcohol, Phenoxyethanol, Caprylyl Glycol, Lanolin, Panthenol, Lactic Acid, Parfum, Alpha-Isomethyl Ionone, Citronellol, Coumarin, Geraniol, Hexyl Cinnamal, Hydroxycitronellal, Linalool. K18 hair mask contains Water (Aqua) (Eau), Alcohol Denat., Propylene Glycol, Cetearyl Alcohol, Dicaprylyl Ether, Cetyl Esters, Behen-trimonium Chloride, Polysorbate 20, sh-Oligopeptide-78 (K18 PEP-TIDE), Hydrolyzed Wheat Protein, Hydrolyzed Wheat Starch, Isopropyl Alcohol, Tocopherol, Phenoxyethanol, Potassium Sorbate, Citric Acid, Fragrance (Parfum), Geraniol, Linalool, Hexyl Cinnamal, Benzyl Alcohol. These compositions were retrieved from the websites of the brands.

2.7.2. GC-MS analysis

To identify the fragrances existent in each cosmetic, 0.1 g of ATL body cream and 0.1 g of K18 hair mask were inserted in GC vial and incubated at 50°C for 1 h. The identification of fragrance peaks was done using the NIST library (version 17). No internal standard was added in the samples since most of the standards belong to the odorant class having the capacity to bind to the OBP pocket, thus acting as competitors of odorants [25]. The calibration curves were prepared using increasing concentrations of β -citronellol, linalool and geraniol (10–100 μM , 50 μL) in the same conditions of the samples. The amount of each fragrance was determined by integration of the peaks from chromatograms and quantified against the calibration curves.

The fragrance release from cosmetic samples were quantified by headspace gas chromatography–mass spectrometry (GC-MS) using manual injection of the SPME fiber (100 μm polydimethylsiloxane) as performed in Gonçalves et al. [25]. The cosmetics, calibration solutions and the samples were prepared, in triplicate, and the SPME fiber was inserted in the middle (~ 40 mm from top) of the GC vial (22.5×75.5 mm, septa of silicone blue transparent/PTFE white, Enzymatic,

Portugal) for exposure during 1 h. Gas chromatographic analyses were carried out using a Bruker 450/320 GC with a split/splitless injector coupled to a mass spectrometer. Injections were operated at 250°C in the split mode 1:10 using a Rxi-5Sil MS (Restek) column (30 m $\times$ 0.25 mm, and 0.25 μm film thickness), with a column-head pressure of 7.3 psi using helium as carrier gas. The oven temperature was held at 45°C and for 5 min, programmed at $7^\circ\text{C}/\text{min}$ to 250°C . A full scan mode (50–750 m/z) was applied for the identification of the target compound. The mass spectrometer (MS) was operated in electron ionization (EI) mode at 70 eV with total ion chromatogram (TIC) detection mode for quantitative determination and S/N ratio of 5. The amount of released fragrance from the commercial cosmetics (ATL body cream and K18 hair mask) was determined by integration of the peaks from chromatograms and the area value was compared with the control without protein.

2.7.3. Olfactory assessment of protein-coated fibers using human sensory panels

A sense test with volunteers was performed to assess the perception of fragrance release and reduction of sweat odor. Wool towels were used as hair/keratin model due to similar composition between these two matrices [26] and due to a bigger surface facilitating the tests with several conditions.

In a double-blind olfactory assessment, 14 volunteers with age between 21 and 45 years were instructed to smell 4 towels' pieces 3 times each one and then to describe the sensorial aspect in 4 possible options, evaluated in a single session of approximately 10 min for each volunteer. The four towels' pieces were: (A) towel (without odor or natural human sweat), used as blind control; (B) towel with human sweat; (C) towel with human sweat and Frutis Rev2 fragrance; and (D) towel with human sweat and OBP::GQ₂₀::KP/Frutis conjugate (0.01%, w/v protein versus 0.02% fragrance, 1:2 ratio). This conjugate was prepared by incubation of 0.01% of protein with 0.02% of Frutis Rev2 fragrance, 1 h at 25°C . Condition A (towel without any treatment) was used as blind control to validate the volunteers against absence of odor. Due to the difficulty to have a high number of volunteer producing sweat in similar amounts and with same smell, we selected one person to jogging/running and which natural sweat presented odor, and the armpit sweat was cleaned with the towels. The 4 options to describe the olfactory sense were: 1) no smell any odor; 2) smell at sweat odor; 3) smell at sweat and at fragrance; or 4) smell at fragrance and no smell at sweat. Data were collected, anonymously, on paper and the results compiled in GraphPad in a table 4×4 (4 conditions versus 4 odor perception). Statistical analysis between each condition and the answer of volunteers were assessed through Chi-square test and a confidence level of 95%.

3. Results

3.1. Purity of the OBP::GQ₂₀::KP fusion protein

The purified protein was loaded in SDS-PAGE gel electrophoresis, and it was observed an intense band correspondent to the studied protein (Supplementary material Fig. 1). The purity analysis of lyophilized protein using an intact mass analysis by X500B Q-TOF MS platform determined at 96.1% of purity (data not shown).

3.2. Binding affinity of the OBP::GQ₂₀::KP protein to 1-AMA, sweat mixture, sweat compounds and other odors

The dissociation constants (K_d) of the OBP::GQ₂₀::KP to 1-AMA, a ligand model used for mammalian OBPs characterization, was of $0.20 \pm 0.04 \mu\text{M}$ at 25°C and of $0.25 \pm 0.03 \mu\text{M}$ at 37°C . These values correspond to the high affinity values at both temperatures. In the presence of artificial sweat, the dissociation constants, at both temperatures, increase significantly (Table 1), indicating that artificial sweat composition promote 1-AMA release. The analysis of K_d values comparing without and with sweat are statistically significant (P values < 0.0001 ,

Supplementary material Fig. 2).

Individual components of artificial sweat exhibited relatively high dissociation constants (Kds): $15.23 \pm 0.01 \mu\text{M}$ for NaCl, $227.94 \pm 0.06 \mu\text{M}$ for lactic acid, $143.23 \pm 0.04 \mu\text{M}$ for sodium phosphate, and $575.70 \pm 0.03 \mu\text{M}$ for L-histidine, indicative of weak binding (Fig. 1A). The fluorescence response by NaCl, lactic acid, sodium phosphate, and L-histidine didn't reach 50% at the highest concentration tested and for this reason the Kd were estimated, and these compounds would be more appropriate to classify as "very weak binders". In contrast, artificial sweat, as a whole, exhibited significantly lower dissociation constants towards OBP::GQ₂₀::KP (Kd = 1.69–2.04 μM). These results indicate that the combined ionic strength of the sweat solution, rather than any individual component, is responsible for the observed decrease in 1-AMA affinity (i.e. probe displacement). On the other hand, the effect of pH on binding activity of OBP::GQ₂₀::KP was performed comparing the 1-AMA binding at pH 7.5, work pH used to OBP, and at pH 4.0, pH of artificial sweat solution (Fig. 1B). pH doesn't interfere in binding capacity of 1-AMA to OBP::GQ₂₀::KP, presenting a dissociation constants very similar at both pHs (Kd = $0.14 \pm 0.03 \mu\text{M}$ at pH 7.5 and Kd = $0.16 \pm 0.05 \mu\text{M}$ at pH 4.0). The dynamics of OBP and the modification in binding and in dissociation processes have been little studied. The results, here presented, suggest that the mechanism of odor release by OBP::GQ₂₀::KP isn't affect by pH but occurs in the presence of sweat solution, most probably mediated by ions combination (ions like Na⁺, K⁺, Ca²⁺, Cl⁻, Mg²⁺) present in perspiration solution and due to the charge combination that might affect hydrogen bonds, electrostatic and hydrophobic interactions and van der Waals interactions inside of binding pocket and promote alteration in protein hydration, creating favorable conditions for odorant release [19]. Previous work, using molecular dynamics assay for OBPs observed that the occurrence of a high energy barrier to unbind the 1-AMA from OBP, probably have as causes the hydrogen bond loss at the complexed state [18].

The evaluation of competitive activity of a malodor present in native human sweat - isovaleric acid - was performed. Isovaleric acid is a component identified in body malodor, resulted by microbial degradation (by *Staphylococcus epidermis* e.g.) of the leucine from sweat to isovaleric acid, a cheesy-smelling compound [27]. The dissociation constant for isovaleric acid was $0.57 \pm 0.03 \mu\text{M}$, indicating significant binding between OBP::GQ₂₀::KP and 1-AMA. At this concentration, half of the binding sites would be occupied by the isovaleric acid. The analysis of pleasure odors, like β -citronellol, linalool, and Fructis Rev2 blend (blend rich in linalool fragrance), exhibited dissociation constants of 1.52 ± 0.01 , 0.51 ± 0.01 and $0.56 \pm 0.03 \mu\text{M}$, respectively. These dissociation values indicate that the 2 fragrances, and the blend attenuated the fluorescence in a dose-dependent-manner (Fig. 1C). Kd of β -citronellol indicates moderate to good binding affinity between this fragrance and the protein representing a good competitor [28]. Notably, linalool and the Fructis Rev2 blend presented similar affinity, suggesting that linalool is a primary contributor to binding. It is not clear the factors that determine whether one molecule binds to a OBP better than another. It is proposal that molecules with low size and better geometric complementarity (spatial arrangement) means stronger binders [14]. Crystallography studies of the pig OBP with several molecules show that different ligands fit into the protein pocket through different orientations, interacting with different residues of OBP [6,29]. This binding is stronger when more hydrogen bonds, electrostatic and hydrophobic

interactions occurred. Molecules more hydrophobic, such as β -citronellol tend to maximize the interactions resulting in the strongest affinity [6,29,30].

3.3. Secondary structure of the fusion protein

The secondary structure analysis of OBP::GQ₂₀::KP was assessed by circular dichroism (CD) spectroscopy. CD spectra show typical peaks for proteins rich in β -sheets (Fig. 2), characteristic of mammalian OBPs [31]. The structure is stable until 60 °C. At temperatures higher than 70 °C the protein loose the structure what it is accordance to Capo et al., (2018) that studied the secondary structure of pig wild-type OBP by CD spectroscopy and proved that this protein is stable up to 65 °C [32]. The analysis of secondary structure of OBP::GQ₂₀::KP using the DichroWeb software reveals that the increase of temperature from 60 °C to 90 °C, change the native structure of this protein, decreasing the number of helix (from 16.4% to 0%) and strands (from 30.0% to 19.8%) content and increasing the turns, from 22.5% to 46.0%, and unordered structures, from 31.0% to 33.9% (Supplementary material Fig. 3).

In terms of pH stability, OBP::GQ₂₀::KP demonstrated remarkable stability across the tested pH range (4.0–9.0), covers physiologically or industrially relevant conditions (Fig. 2). Additionally, the spectra showed that tested pH didn't affect OBP::GQ₂₀::KP conformation what support the dissociation constant values very similar at pH 4.0 and pH 7.5.

In the presence of artificial sweat, only minor spectral changes were observed at 215 nm at the highest tested concentration. It might indicate that in the dissociation process of an odorant the sweat solution doesn't alter the protein structure, but it can weaken the interaction of odorants and protein pocket. In the previous studies, X-ray structure of pig OBP with 2-isobutyl-3-methoxypyrazine (IBMP) molecule and dynamic simulation studies revealed that the interaction of different molecules established interactions that didn't change significantly the conformation of protein [6,29]. These results confirm the high stability of OBP, supporting its suitability for technological applications.

3.4. Cytotoxicity of OBP::GQ₂₀::KP protein

The cytotoxicity of the fusion protein (OBP::GQ₂₀::KP) was evaluated by measuring the cell viability of HFDP, HaCaT and BJ-5ta cell lines (Fig. 3A, B and C). While the mechanistic action of DMSO towards cells could differ from the possible mechanisms towards protein cytotoxicity, it was applied, together with the positive control, to prove the scientific methodology applied, both regarding viability (positive control) and toxic effects (negative control). Since a possible toxic effect of this fusion protein was never detailed, finding a control that mimics the same possible effects is not possible. The MTS assay was performed in triplicate, in two independent experiments, after incubating the cells with increasing concentrations of OBP::GQ₂₀::KP (0.05, 0.1, 0.2 and 0.4 mg/mL) for 48 h. The number of replicates is consistent with similar studies in the field and provided reproducible results with acceptable variability as corroborated by the statistical method utilized. For all tested protein concentrations and in the three cell lines tested, cell viability remained above 90%, indicating that OBP::GQ₂₀::KP is non-cytotoxic (Fig. 3A, B and C).

3.5. Quantification of fusion protein functionalized onto hair fiber

For future application of OBP in hair cosmetics, the KP peptide was fused to the protein sequence (OBP::GQ₂₀::KP) and compared with wild-type OBP. For that, 1 mg/mL of each protein was functionalized onto hair for 10 and 60 min. The amount of protein functionalized onto hair was calculate by subtracting the unbound amount of proteins to the initial amount. After 10 min incubation with OBP::GQ₂₀::KP it was measured that around 40% of protein is functionalized onto hair reaching 49% of OBP::GQ₂₀::KP functionalized after 60 min of

Table 1

Dissociation constants (Kd) of 1-AMA for OBP::GQ₂₀::KP at 25 °C and 37 °C, in the absence or presence of artificial sweat. The values represent the mean $\pm$ standard deviation ($n = 3$).

	25 °C		37 °C	
	Without sweat	With sweat	Without sweat	With sweat
Kd (μM)	0.20 ± 0.04	1.69 ± 0.01	0.25 ± 0.03	2.04 ± 0.02

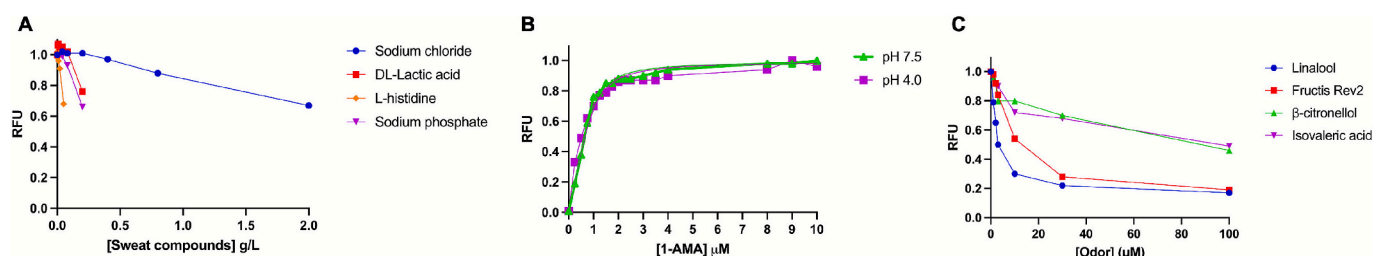


Fig. 1. Binding activity of OBP::GQ₂₀::KP. (A) Competitive assay of OBP::GQ₂₀::KP to individual components of artificial sweat, tested from 0 g/L to maximal concentration of artificial sweat recipe. (B) Binding assay of OBP::GQ₂₀::KP to 1-AMA at pH 7.5 versus 4.0. (C) Competitive assays of OBP::GQ₂₀::KP to odor compounds (fragrances and malodor). RFU: relative fluorescence unit. The values represent the mean of triplicate from 3 independent experiments.

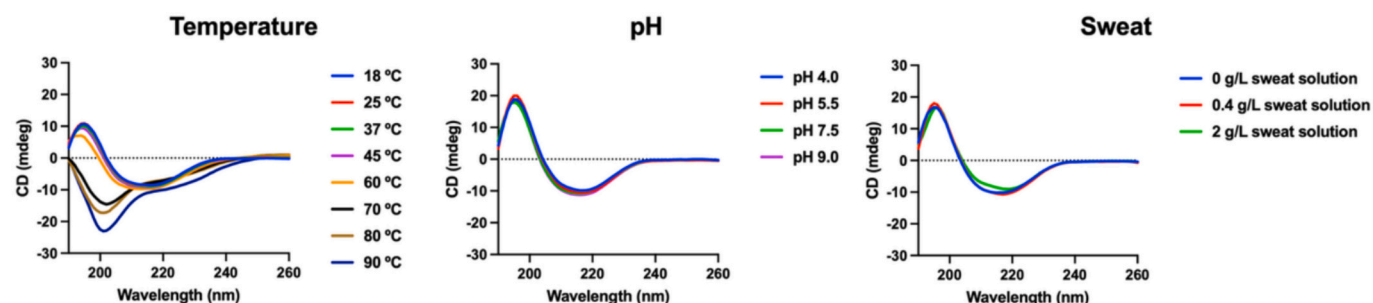


Fig. 2. Secondary structure of OBP::GQ₂₀::KP assessed by circular dichroism (CD) spectroscopy using a Jasco J-1500 spectropolarimeter equipped with a temperature controller. Spectra were recorded between 190 and 260 nm to investigate the structural effects of temperature variation, pH changes, and sweat addition on OBP::GQ₂₀::KP. Artificial sweat solution legend indicates the concentration of sodium chloride, reagent used in most concentration to prepare the solution (AATCC method 15–2009 recipe). The spectra represent the mean of 3 accumulations.

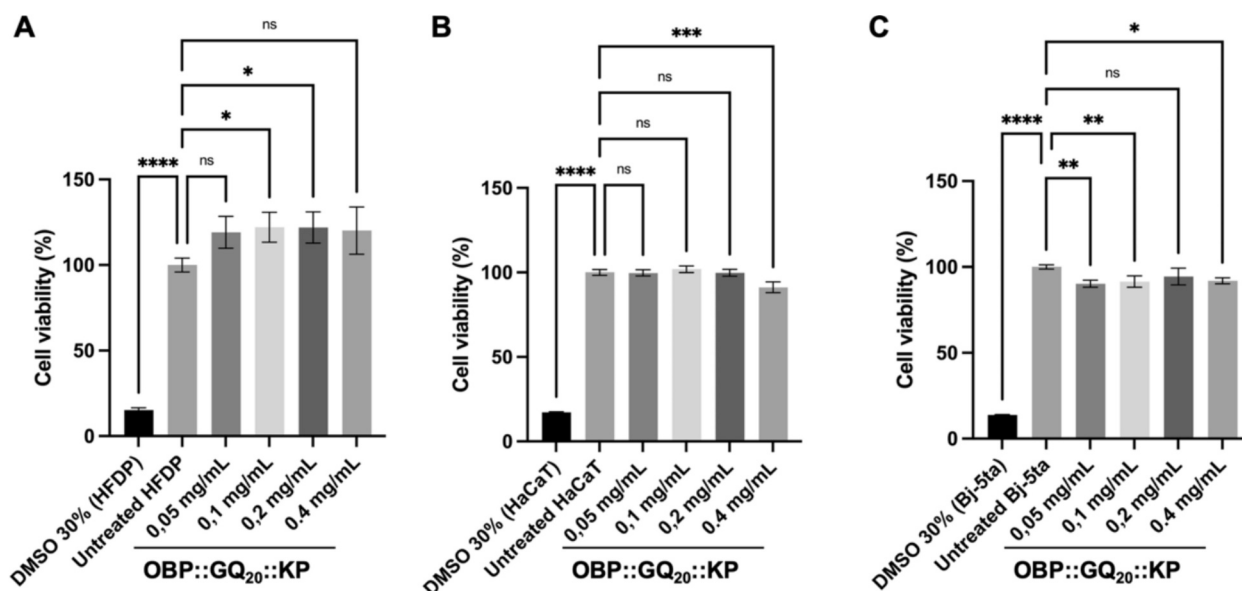


Fig. 3. Cell viability of (A) HFDP (human hair follicle dermal papilla), (B) HaCaT (human keratinocytes) and (C) BJ-5ta (human immortalized fibroblasts) cell lines after 48 h of incubation with OBP::GQ₂₀::KP at different concentrations (0.05 to 0.4 mg/mL), determined by the MTS assay. Values are the triplicate mean of 2 independent experiments. The data were analyzed by ANOVA statistical test with *P*-value in A of <0.0001 (****), 0.0350–0.0359 (*), 0.0565–0.0727 (ns), *P*-value in B of <0.0001 (****), 0.0007 (***) and 0.6779–0.9998 (ns) and *P*-value in C of <0.0001 (****), 0.0034–0.0091 (**), 0.0131 (*) and 0.0948 (ns), compared with 3 untreated cell lines.

incubation (Fig. 4A). The functionalization level with wild-type OBP (protein without KP) was statistically very low due to the absence of KP peptide responsible for the binding of the protein to hair (Fig. 4A). The interaction between OBPs and keratin involves primarily weak van der Waals forces and hydrogen bonding, which are easily disrupted by washing, mechanical agitation, or environmental conditions. This leads

to rapid protein desorption from the fiber surface as it is the example of wild-type OBP. On the other hand, the results highlight the role of KP as a peptide binding mediator of proteins to keratinaceous materials. This peptide contains 2 cysteine residues in its sequences what promote strong disulfide bonds with hair and consequently improve the interaction of protein to hair what was already demonstrated in the

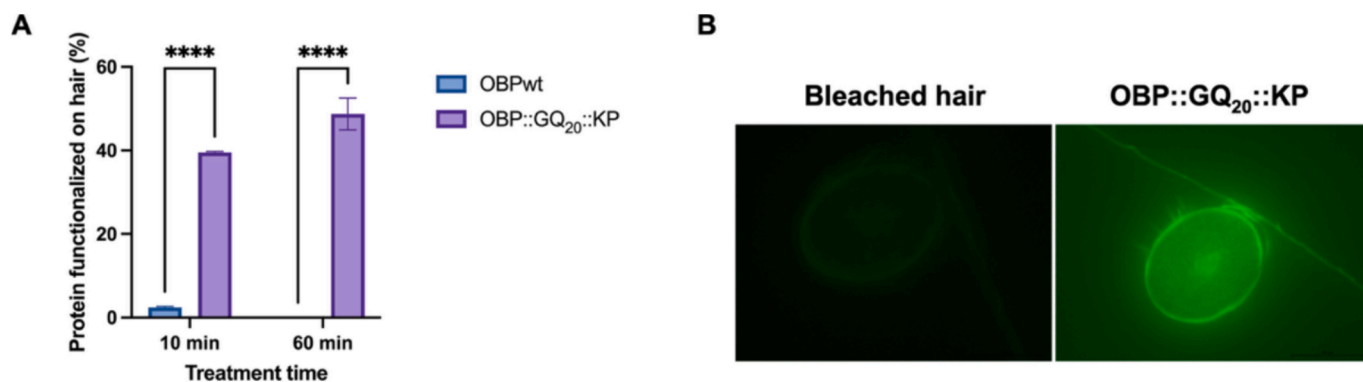


Fig. 4. OBP::GQ₂₀::KP protein functionalized onto hair. (A) Percentage of wild-type OBP (OBPwt) and OBP::GQ₂₀::KP proteins functionalized onto hair, after 10 and 60 min of incubation. The chart represents the mean $\pm$ standard deviation ($n = 3$). The data were statistic analyzed by two-way ANOVA test with P -value < 0.0001 . (B) Fluorescence images of human hair cross sections of untreated bleached Asian hair (left), and bleached hair treated with OBP::GQ₂₀::KP (1 mg/mL) (right side). Bar scale: 50 μ m.

previously research using laboratory techniques and molecular-level evidences [33,34].

The functionalization of the OBP::GQ₂₀::KP protein onto hair was also observed by fluorescence microscopy in transversal cuts of treated bleached hair. The images show higher fluorescence intensity in the cuticle and cortex of hair treated with OBP::GQ₂₀::KP, compared with the control (hair without protein) (Fig. 4B). The fluorescence intensity of the protein along the fibers' horizontal section was analyzed using ImageJ analysis plot profile and revealed higher green intensity in fiber treated with protein than fiber without protein (Supplementary material Fig. 4).

3.6. Effect of OBP::GQ₂₀::KP functionalization on hair physicochemical properties

Differential Scanning Calorimetry (DSC) analysis of hair samples revealed an increase in the enthalpy (ΔH) required to break the alpha-helix structure of keratin after treatment with OBP::GQ₂₀::KP. Specifically, ΔH increased by 54.4% and 61.7% following 10 and 60 min incubation periods with the protein, respectively (Fig. 5A). This elevated energy requirement indicates that the OBP::GQ₂₀::KP protein confers a thermal protective effect on the hair, enhancing its thermal stability. The DSC thermograms are presented in Supplementary material Fig. 5.

The mechanical resistance of bleached Asian hair before and after treatment with the OBP::GQ₂₀::KP was evaluated to determine the effect of the protein in hair's mechanical properties. Virgin hair was used as a control to evaluate the effect of bleaching. Young's modulus of bleached

hair decreased by 14.3% compared to virgin hair, indicating structural damage to the cortex caused by the bleaching process (Fig. 5B). This reduction is attributed to the alkaline conditions used during bleaching, which disrupt the keratin structure and lead to a loss of the hair's mechanical properties [35]. Hair treated with the recombinant protein showed an increase of the Young's modulus comparatively to the bleached hair and virgin hair (Fig. 5B). The data indicate that OBP::GQ₂₀::KP is capable of restoring and enhancing the mechanical properties of damaged hair, with a 43.3% improvement relative to untreated bleached hair. This effect could be associated with the formation of covalent bonds between the cysteine of KP and the cysteine of hair improving the interaction of protein to hair what was already demonstrated in the previously research using laboratory techniques and molecular-level evidences [33,34]. Also here it was observed high intensity of fluorescence of bleached hair treated with OBP::GQ₂₀::KP (Fig. 4B). On the other hand, the high content in β -strand structures from OBP increases hair resistance and stiffness of hair because these pleated sheet structures are more crystalline and are stabilized by a network of hydrogen bonds, requiring more force to unfold [36,37].

3.7. Fragrance release from OBP::GQ₂₀::KP protein promoted by sweat in cosmetic formulations

Skin and hair cosmetics are in our daily routine and are considered indispensable feature of modern lifestyle of individuals. The use of fragrances creates an elegant and welcoming environment, particularly in dairy social interplay. The increase of fragrance sense in air creates a

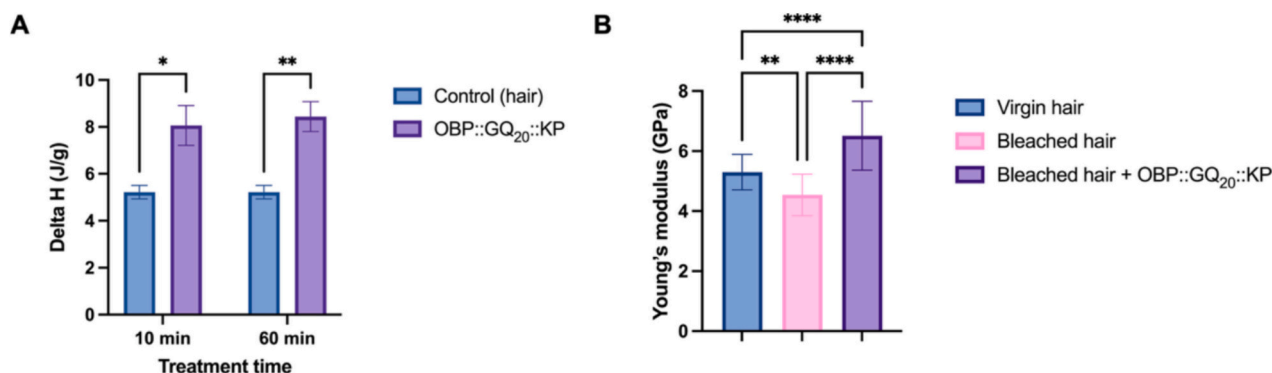


Fig. 5. Physicochemical characterization of hair treated with OBP::GQ₂₀::KP. (A) Delta H (J/g) obtained from thermal study of hair treated with OBP::GQ₂₀::KP using a power compensated differential scanning calorimetry (DSC) instrument, at a temperature range from 50 to 250 °C (heating rate: 5 °C/min). The chart represents the mean $\pm$ standard deviation ($n = 3$). The data were statistic analyzed by two-way ANOVA test with P -values of 0.0148 (*) and 0.0095 (**). (B) Mechanical property (Young's modulus) of virgin Asian hair and bleached Asian hair before and after treatment with 0.1% (w/v) of OBP::GQ₂₀::KP recombinant protein. Values are the mean $\pm$ SD of 30 independent measurements. The data were statistic analyzed by one-way ANOVA test with P -values of 0.0022 (**) and < 0.0001 (***).

pleasure environment for the utilizer, interesting for manufactured items, such as fine perfumery, body care, detergents, household products. Thus, after the tests of OBP::GQ₂₀::KP performance in solution and to do a proof-of-concept of the release of 1-AMA molecules in presence of artificial sweat, we measured the performance of this recombinant protein in commercial cosmetic products (Fig. 6). The cosmetic products (ATL and K18 mask) were analyzed by GC–MS to identify the fragrances contained in and detected by our method. In ATL body cream were identified β -citronellol, linalool and geraniol. In K18 mask were identified linalool. These fragrances are just identified in ingredient list of the cosmetic products. The calibration curves for β -citronellol, linalool and geraniol using increasing concentrations of each odorant (10–100 μ M, 50 μ L) was injected in GC–MS, in the same conditions of the samples (Supplementary material Fig. 6). From the GC data and using NIST database (version 17), a unique peak was obtained corresponding to a mass spectra characteristic of each fragrance, with retention time of 13.9 min for linalool, 16.4 min for β -citronellol and 17.2 min for geraniol (also known as 2,6-octadien-1-ol, 3,7-dimethyl).

OBP::GQ₂₀::KP was added to commercial skin and hair cosmetic products followed by the addition of artificial sweat (Fig. 6) and the amount of released fragrance from OBP::GQ₂₀::KP was measured by headspace-GC–MS. The GC–MS revealed high increase of released fragrance when the cosmetics containing OBP::GQ₂₀::KP recombinant protein versus absence of this polypeptide in the body cream or in the hair mask. The analysis of released fragrances in presence of artificial sweat showed that the body cream with the protein promoted an increase of the fragrances' release presents in body cream. It resulted in an increase of 90.7% of linalool, as well as the more detection of citronellol (+13.4%) and geraniol (+83.9%), compared with body cream, without protein. The incorporation of OBP::GQ₂₀::KP in K18 hair mask resulted in higher release of linalool present in this mask, in approximately 77.0%, compared with mask without protein. The differences in the release of different fragrances are associated with the volatility of fragrances [30] and the force of binding between fragrance-OBP [38]. Linalool is the most volatile of the three compounds, followed by

geraniol, and then citronellol being the least volatile. Linalool has a vapor pressure of approximately 0.159 mmHg at 23.5 °C, indicating high volatility than geraniol (vapor pressure of ~0.03 mmHg at 25 °C) and citronellol (0.02 mmHg at 25 °C) [39]. These differences in volatility affect how readily these compounds evaporate which is important for their roles as fragrance compounds and their interactions with odorant-binding proteins. Linalool's higher vapor pressure is consistent with its widespread use in perfumery (perfumes, lotions, soaps, and shampoos) and its strong olfactory impact even at low concentrations.

3.8. Human olfactory perception of wool towels functionalized with OBP::GQ₂₀::KP

GC–MS instrumentation allows us the quantification of analytes. However, it does not give information about human perception. Thus, a double-blind olfactory assessment involving 14 volunteers was performed to determine their odor perceptions relative to the towels treated according to 4 tested conditions. To avoid inter-individual variations in sweat odor between sweat donors, for example one sweat donor has smell and another has no smell, only one donor was selected which natural sweat present malodor. Four towel's conditions were presented to volunteers, individually. For each condition, the volunteers smelt the pieces of wool and answer their perception of odor between 4 possible odor responses (Fig. 7). Towels without any added treatment (Condition A) were used as blind controls. None of the volunteers reported "any odor" in Condition A, and therefore all volunteers were approved to go further with the study. In Condition B, for wool pieces exposed to human sweat, 80% of the volunteers detected a human "sweat odor". In Condition C, where the wool was used to clean human sweat and then treated with a commercial fragrance (Frutis Rev. 2), presents a mixed odor profile with a majority of volunteers (60%) detected "sweat odor" and a smaller group detected both human "sweat and fragrance". These results indicate that the commercial fragrance did not fully mask the human sweat odor, highlighting the need for more effective solutions to neutralize malodors. In Condition D, the wool was used to clean

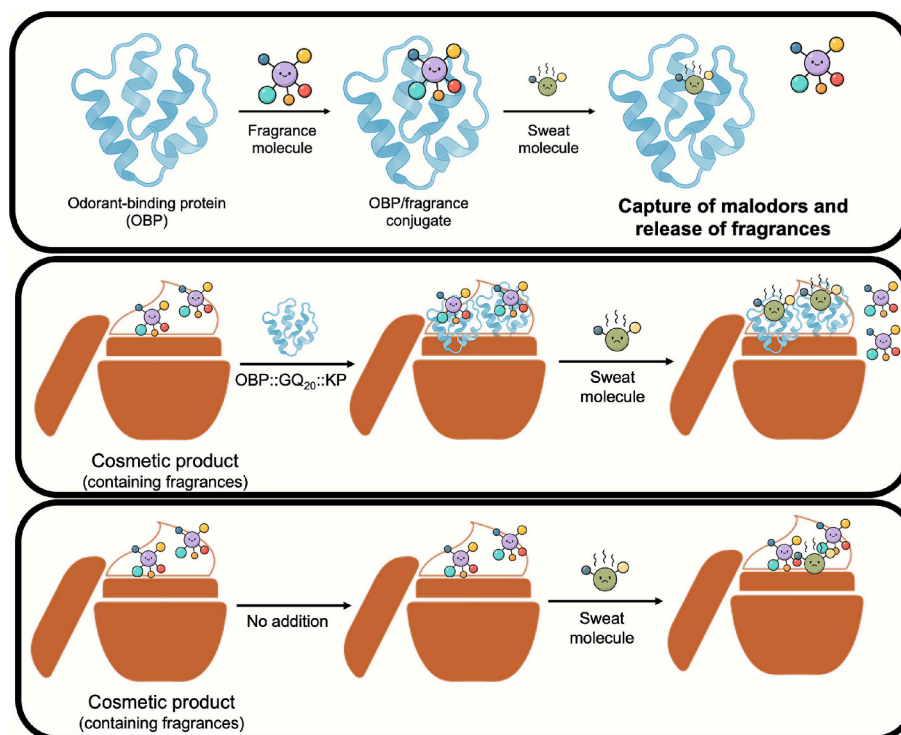


Fig. 6. Illustration of tests performed to measure the OBP::GQ₂₀::KP activity. The performance assays were done using the protein in solution (1st rectangle) and incorporated in commercial cosmetic products trigger by artificial sweat (2nd rectangle) and in the absence of OBP::GQ₂₀::KP (3rd rectangle).

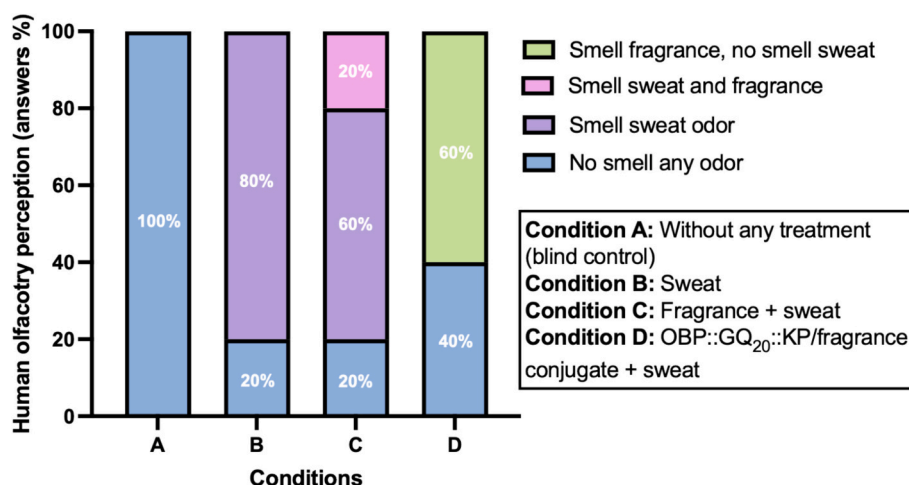


Fig. 7. Human olfactory perceptions to wool towels in different conditions (A-D). Condition A: Without any treatment (blind control); Condition B: human sweat; Condition C: Frutis Rev2 fragrance + human sweat; Condition D: OBP::GQ₂₀::KP/fragrance conjugate + human sweat. The perception description by 14 volunteers. Wool was used as hair model and the sweat is human natural sweat generated by a volunteer.

perspiration and then treated with the OBP::GQ₂₀::KP/Frutis conjugate. Conditions D is strongly associated with perception of “smell fragrance” with 60% of the volunteers reported not smelling human sweat and perceiving a pleasant fragrance, supporting the conclusion that OBP::GQ₂₀::KP can effectively capture malodors and release fragrance in the presence of human sweat. On the other hand, the remain 40% of the volunteers reported “no smell any odor”, suggesting that the recombinant protein was able to neutralize malodor compounds.

Statistical analysis of 4 conditions versus 4 odor perceptions confirms that observed differences are not due to random chance, indicating that the four conditions have distinct and perceivable effects on the smell of towels. Statistical analysis resulted in a Chi-square of 66.64, with a *P*-value <0.0001 indicating that it is statistically significant and that the data collected from 14 volunteers, shows a clear association between the experimental conditions and the type of odor perceived.

4. Conclusions

Recombinant OBP::GQ₂₀::KP protein was produced in *E. coli* with high purity and showed to be stability at range of pH and temperature, characteristics useful in industry. OBP::GQ₂₀::KP showed a mechanism of fragrance release in response to sweat, even in solution as in commercial cosmetics. This proof-of-concept was established using analytical methods and a human panel. Although our results clearly demonstrate sweat-triggered fragrance release, the precise molecular mechanism - whether driven by subtle conformational changes, competitive ligand displacement, ionic screening, or another process - remains to be established; resolving this will require higher-resolution structural and biophysical studies. Olfactory assessment using volunteers support that OBP::GQ₂₀::KP has capacity to mask the malodor of human sweat and promote the smell of fragrance creating a good environment and sense for individual's dairy line and social interactions. OBP demonstrated exceptional efficacy as a technology for reducing and masking body odors, including malodorous compounds generated by bacterial metabolism during perspiration. This technology promotes a highly competitive market and the creation of cosmetics products with high quality and novelty, being it is the first time that the mechanism of fragrance release by OBP trigger by sweat is described.

CRediT authorship contribution statement

Filipa Gonçalves: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Ana Tinoco:** Methodology, Investigation. **Diana Guimarães:** Methodology, Investigation.

Carla Silva: Writing – review & editing, Validation, Supervision. **Artur Ribeiro:** Writing – review & editing, Validation, Supervision. **Artur Cavaco-Paulo:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Artur Cavaco-Paulo, Carla Silva, Artur Ribeiro reports administrative support was provided by Foundation for Science and Technology. Artur Cavaco-Paulo, Carla Silva, Artur Ribeiro reports administrative support was provided by European Regional Development Fund. Artur Cavaco-Paulo, Carla Silva, Artur Ribeiro reports administrative support was provided by University of Minho Associate Laboratory in Biotechnology and Bioengineering and Microelectromechanical Systems. Carla Silva reports financial support was provided by Foundation for Science and Technology. Filipa Gonçalves reports financial support was provided by Foundation for Science and Technology. Artur Cavaco-Paulo has patent pending to WO/2022/003655 and WO/2024/147108. Filipa Gonçalves has patent pending to WO/2022/003655 and WO/2024/147108. Carla Silva has patent pending to WO/2022/003655. Artur Ribeiro has patent pending to WO/2022/003655. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2026.150469>.

Data availability

Data will be made available on request.

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