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Unique natural exopolysaccharides for biomimetic protective effect against urban pollution

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Summary

Through natural selection, living organisms have evolved well-adapted survival strategies over time. The shallow salt waters of Moorea lagoon are the site of accumulation of microbial mats called "Kopara," in the native Polynesian language. This unique ecosystem is rich in film-forming exopolysaccharides (EPSs) secreted by microorganisms within the biofilm, as a mean to protect themselves from environmental stress (strong ultraviolet [UV], pH, salinity …). Using blue biotechnology, a manufacturing process was developed to obtain an EPS with skin benefits. The active ingredient (EPS-229) protects against urban pollution, including free radicals, heavy metals, hydrocarbons, and $PM_{2.5}$ (particulate matter with a size lower than 2.5 μm). Methods: The anti-lipid peroxidation action of EPS-229 was studied in an *in vitro* UVB-irradiated keratinocyte culture model, using lipophilic fluorescent probe. The chelating properties of EPS-229 were evaluated *in tubo* in the presence of cadmium and lead. The protective effect of EPS-229 on pollution-exposed skin explants was investigated through quantification of released malondialdehyde (MDA) and histological observation of skin morphology using optical microscopy. Clinical evaluation of the protective and cleansing efficacy of a water solution containing EPS-229 (0.02% and 0.01% w/v, respectively) was performed, against placebo, on a panel of 18 volunteers. For these studies, the forearms of volunteers were treated with EPS-229 before (anti-adhesion affect) or after (cleansing effect) application of $PM_{2.5}$ (iron particles of 1 μm). The presence of skin-adherent particles was observed and quantified by image analysis, using specific digital masks. Results: *In vitro*, EPS-229 significantly protected keratinocyte cell membranes from lipid peroxidation. A decrease of 28% was achieved when a concentration of 0.001% w/v EPS-229 was applied to the cell culture. *In tubo*, EPS-229 also presented strong chelating properties. Maximal adsorption was estimated at 154 mg/g (1.37 mmol/g) of EPS-299 for cadmium and at 250 mg/g (1.21 mmol/g) of EPS-229 for lead. In the skin explant model of pollution exposure, EPS-229 (0.03% w/v) reduced MDA production by 44%, preserved cell integrity, improved dermal–epidermal cohesion, and normalized the collagen network. *In vivo*, treatment of skin with EPS-229 before exposure to $PM_{2.5}$ created a protective film limiting particle adhesion. When used in a cleansing solution after exposure to $PM_{2.5}$, EPS-229 formed a mesh that entrapped particles and removed them from the skin surface. Conclusion: Inspired by the French Polynesia Kopara unique ecosystem, a bioactive exopolysaccharide (EPS-229) has been developed that offers protection from environmental aggression. As a biomimetic shield at the surface of the skin, EPS-229 provides an immediate multiprotective action that efficiently fights the harmful effects of urban pollution and smog.

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INTRODUCTION

In French Polynesia, the shallow salt waters of lagoons are the site of accumulation of organic matter of microbial origin that forms mats called "Kopara" in the native Polynesian language (1). Under stress conditions, such as fluctuations in temperature, water supply, pH, and salinity, microorganisms within the Kopara release exopolysaccharides (EPSs) as means of protection (2). EPS consist of high-molecular-weight carbohydrates that vary greatly in their sugar composition which impacts their chemical and physical properties (3). EPSs improve bacterial survival in many ways. They serve as a transport barrier to reactive chemicals. They can trap trace metals, thus reducing their toxicity (4). They strongly hold and redistribute water to prevent extreme cell dryness. They buffer against sudden osmotic changes. They contribute to oxidative defense (4). They also protect bacterial cells form ultraviolet (UV) induced damage by their capacity to absorb UV rays (5).

Several EPS found in the Kopara have been studied for skin care applications. Inspired by their natural shield function, research was oriented, early on, toward applications related to the protection of skin from environmental stress. One of these EPS (EPS-229) proved to be most promising *in vitro*, and was further characterized *ex vivo* and *in vivo*. EPS-229 is a highly ramified polysaccharide produced by *Alteromonas macleodii* living in the Kopara. However, the coral reef and lagoon ecosystems being extremely fragile, it was primordial to develop ways of exploiting the properties of this EPS without compromising its natural habitat. For industrial scale production, fermentation protocols were developed in collaboration with a local Polynesian biotech. Fermentation conditions, such as pH, temperature, oxygen concentration, agitation as well as the composition of the culture media, had to be carefully optimized to reproduce natural bacterial growth conditions. The current paper presents a brief summary of the research supporting the use of EPS-229 as an antipollution skin care ingredient, including new clinical data providing evidence of protection against $PM_{2.5}$ particles (particulate matter with a size lower than 2.5 μm).

EXPERIMENTAL

ALTEROMONAS FERMENT EXTRACT (EPS-229)

This extract (EPS-229) is a highly ramified EPS produced by *A. macleodii*, as part of a protective shield against environmental aggressions. It is composed of neutral sugars (57%), uronic acids (25%), and sulfates (8%), presents a slight white color, and has a molecular weight of 1000 kDa. The bioactive EPS is obtained through biotechnology, using a fermentation process reproducing natural synthesis conditions.

PROTECTION OF KERATINOCYTES FROM FREE RADICAL-INDUCED DAMAGES

Confluent human keratinocytes (NHEK) were incubated for 24 h with the test product EPS-229 (0.001% w/v) or butylated hydroxy anisole (BHA) (50 μM), a synthetic antioxidant used as a positive control. A C11-fluor probe was then introduced into the culture media and unbound probe was removed by washing, 45 min later. Following reintroduction of EPS-229 or BHA into the media, cells were challenged by exposure to UVB (201 mJ/cm^2). Cells were further cultured for 1 h, then rinsed and trypsinized. Fluorescence of

the C11-fluor probe was monitored on a fixed number of cells (10,000) by flow cytometric analysis, using a FACS array system.

CHELATION OF HEAVY METAL PARTICLES

EPS-229, at a concentration of 0.05% w/v (to reach saturation level), was incubated for 3 h under constant agitation (200 rpm) at 25°C, in the presence of 0.3 μg/ml of Cd or Pb, in a final volume of 30 ml, at pH 6. At the end of the incubation period, solutions were filtered by centrifugation at 3000 *g*, using Vivaspin 20 centrifugal filter units (Vivascience) with a 30 kDa molecular mass cutoff. The concentration of Cd and Pb in the supernatants was measured by flame atomic absorption spectrometry.

PROTECTION OF SKIN EXPLANTS FROM POLLUTANT-INDUCED LIPID PEROXIDATION

Skin explants were obtained from a woman (age 64) undergoing plastic surgery and maintained in culture. Every day from D0 to D4, a lotion containing the test product EPS-229 (0.03% w/v) or tocopherol (positive control) was applied at the surface of the skin explants. On D4, a small filter paper containing a mixture of various heavy metals plus hydrocarbons (benzene, toluene, xylene, anthracene, and naphthol) was additionally applied at the surface of the skin explants. On D5, filter papers were removed and malondialdehyde (MDA) levels, the end product of lipid peroxidation, were quantified in the culture media of each skin explant, using enzyme-linked immunosorbent assay.

PROTECTION OF SKIN EXPLANTS FROM POLLUTANT-INDUCED MORPHOLOGICAL CHANGES

On D5, at the end of the pollutant challenge experiment described earlier, skin explants were treated with Bouin's histological reagent for 48 h, dehydrated, and paraffinized. Morphological studies were done on cut paraffin sections, following Masson's trichrome staining.

CLINICAL EVALUATION

The efficacy of EPS-229 to protect the skin against air pollutants was evaluated on a panel of 18 healthy women, aged 42–72 years. Microparticles of black iron oxide with a size of 1 μm were used, as a mimic of the PM ($PM_{2.5}$) released in the atmosphere by industrial activity and motor vehicle emissions (6).

For the anti-adhesion study (preexposure action), two zones (A and B) were defined on the forearms of volunteers. Zone A was treated with EPS-229 (0.02% w/v) in a water solution and zone B with a water placebo for 20 min. Next, a solution containing black iron oxide microparticles was applied on both zones using a make-up sponge. Three minutes later, both zones were rinsed with water (4 μl/cm^2) and then wiped to remove nonadherent particles. Pictures of both zones were taken before and after rinsing, with a Hirox® video microscope. The percentage of nonadherent $PM_{2.5}$ particles was calculated using the following formula: ((number of adherent $PM_{2.5}$ particles before rinsing - number of adherent $PM_{2.5}$ particles after rinsing)/number of adherent $PM_{2.5}$ particles before rinsing) × 100.

Statistical analysis was done using normality test, Shapiro–Wilk test, Student's *t* test and Wilcoxon signed-rank test.

For the cleansing study (postexposure action), the protocol was modified as follows: water (2 µl/cm²) was first applied on zone A and zone B, and skin left to dry. A solution containing black iron oxide microparticles was next dropped on both zones using a make-up sponge. Three minutes later, zone A was rinsed with EPS-229 (0.01% w/v) in water solution and zone B with water, massage, and wiped with a dry cotton tissue to remove nonadherent particles. Pictures of both zones were taken before and after rinsing, with a Hirox® video microscope. The percentage of removed $PM_{2.5}$ particles was calculated using the following formula: ((number of adherent $PM_{2.5}$ particles before cleansing – number of adherent $PM_{2.5}$ particles after cleansing)/number of adherent $PM_{2.5}$ particles before cleansing) × 100. Statistical analysis was done using normality test, Shapiro–Wilk test, Student's *t* test and Wilcoxon signed-rank test.

RESULTS AND DISCUSSION

In Figure 1, results obtained in fluorescence have been transformed to express protection of keratinocyte membranes from radical formation. Not surprisingly, UV exposure of cells resulted in a significant increase (expressed as 100% from baseline) in the level of lipid peroxidation at the membrane of cells. Addition of the synthetic antioxidant BHA (positive control) reduced the formation of UV-induced lipid peroxidation by 76%, validating the assay. In the presence of EPS-229, protection reached 28% for a concentration of 0.001% w/v. Thus EPS-229 is able to effectively protect skin cells from UV-induced lipid peroxidation. Protection from UV-induced lipid peroxidation is associated with prevention of skin photo-aging (7).

In Figure 2, data obtained *in tubo* from the heavy metal adsorption studies confirmed the chelation potential of EPS-229 toward cadmium and lead. In both cases, metallic retention reached a plateau allowing for the calculation of the maximal adsorption (Qmax) potential of EPS-229 for these cations, in accordance with the Langmuir adsorption model. For Cd, Qmax was estimated at 154 mg/g (1.37 mmol/g). For Pb, Qmax was estimated at 250 mg/g (1.21 mmol/g). The ability of EPS-229 to adsorb divalent cations may facilitate heavy

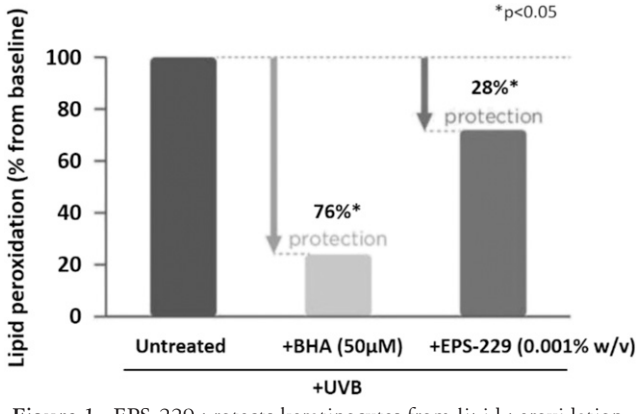

Figure 1. EPS-229 protects keratinocytes from lipid peroxidation.

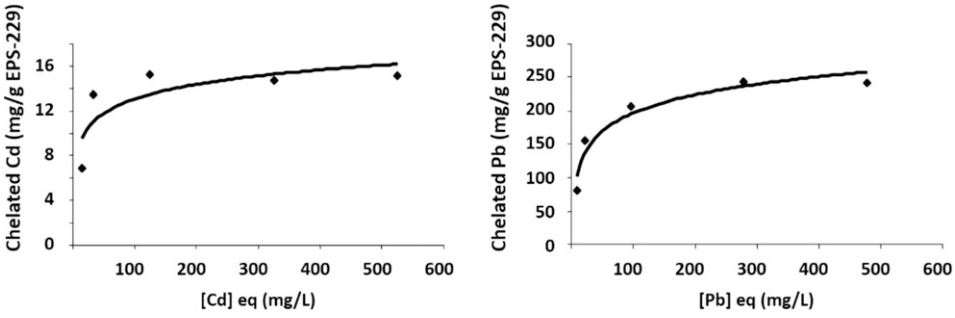

Figure 2. EPS-229 chelates heavy metal particles.

metal removal from the surface of the skin, thus offering a protection from common urban pollutants such as Cd and Pb. Heavy metal exposure has been linked to increase oxidative stress and lipid peroxidation in skin (8).

In Figure 3, when exposed to a mixture of urban pollutants, skin explants produced a high quantity of MDA (expressed in picomole/ml) reflecting the occurrence of oxidation and lipid peroxidation. In the presence of α-tocopherol (positive antioxidant control), MDA production was reduced by 76%, validating the assay. In the presence of EPS-229 (0.03% w/v), MDA production was lowered by 44%. Thus, EPS-229 demonstrated a strong antioxidant activity and protected from lipid peroxidation, under urban pollutant challenge. Preserving skin lipids from oxidation is known to be important for proper maintenance of the barrier function at the stratum corneum (9).

In Figure 4, using histochemical techniques, apparition of pycnotic nuclei (deep magenta color) was observed in the epidermis of skin explants following exposure to a mixture of heavy metals and hydrocarbons. As a marker of severe cellular damage, pyknosis has been associated with environmental insults such as urban pollution or heavy metal exposure (10,11). Pollutant exposure also resulted in clear dermal–epidermal separation at the dermal–epidermal junction and reduced collagen network (blue color) in the dermis. Pretreatment of skin explants with EPS-229 (0.03% w/v) for 4 days prior to pollutant challenge prevented the formation of pyknotic nuclei and improved dermal–epidermal cohesion, as well as collagen fiber density. Such results attest of the potential of EPS-229 to protect and restore normal skin structure and physiology under urban challenging conditions.

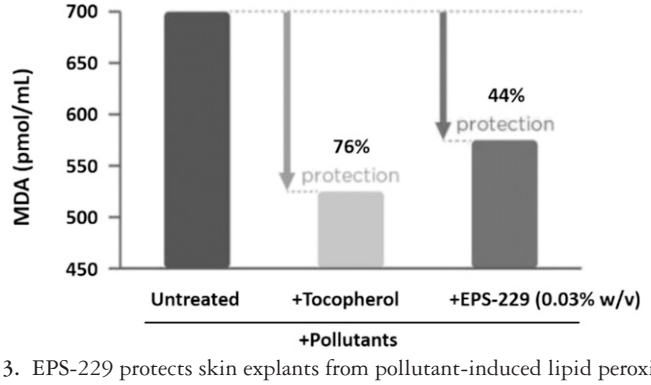

Figure 3. EPS-229 protects skin explants from pollutant-induced lipid peroxidation.

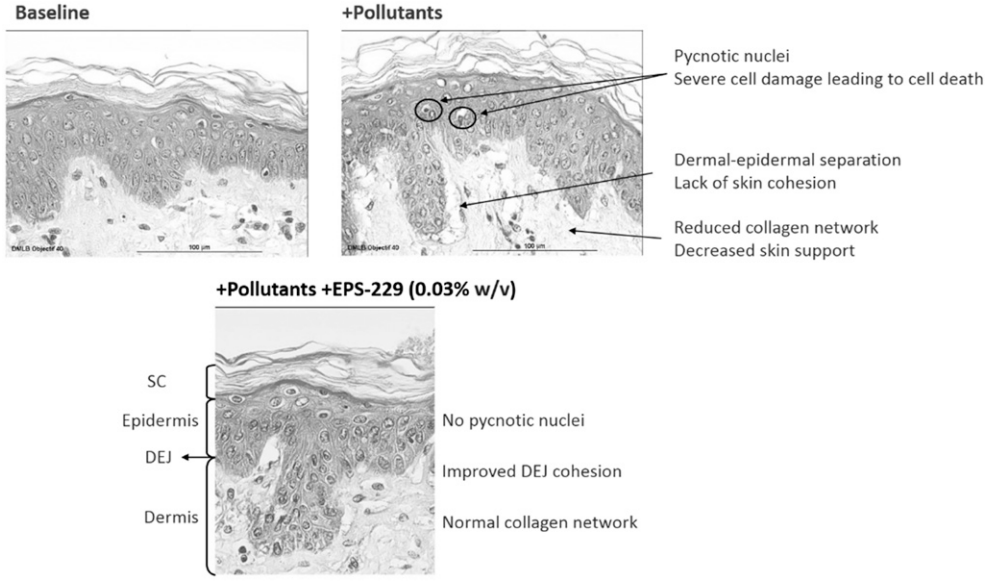

Figure 4. EPS-229 protects skin explants from pollutant-induced structural changes.

In Figure 5, application of EPS-229 (0.02% w/v) on the skin of human volunteers, before exposure to fine particles ($PM_{2.5}$) mimicking air pollution, reduced the number of adherent particles by 45% compared to placebo treatment. By forming an invisible film at the surface of the skin, EPS-229 creates a physical shield able to protect from pollution-induced damage. In Figure 6, cleansing the skin of human volunteers with a solution containing EPS-229 (0.01% w/v) following exposure to $PM_{2.5}$ could washed away 27% more particles than placebo treatment. Thus, when incorporated in a cleansing solution, EPS-229 forms a mesh able to entrap $PM_{2.5}$ particles and remove them from the skin surface to reduce pollution-induced damage. By limiting $PM_{2.5}$ adsorption to the skin, EPS-229 may help prevent melasma development and barrier disruption, since both have been associated with PM exposure (12,13).

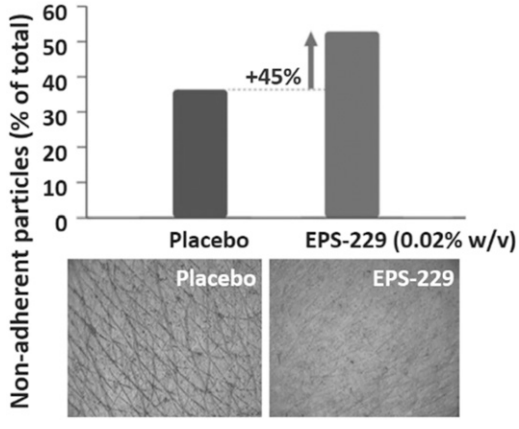

Figure 5. EPS-229 prevents the adhesion of $PM_{2.5}$ particles to the skin surface.

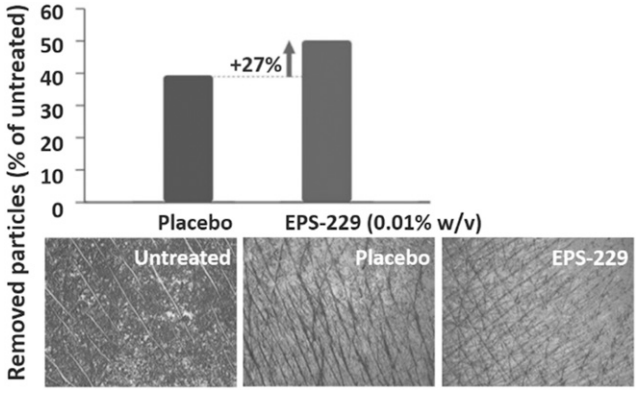

Figure 6. EPS-229 helps remove $PM_{2.5}$ particles from the skin surface.

CONCLUSION

In conclusion, EPS-229 protects from UV-induced oxidative stress, chelates heavy metals, preserves from lipid peroxidation and structural changes caused by exposure to pollutants, and shields the skin from fine particles. Such properties make it an ideal antipollution ingredient for a skin that looks luminous and healthy. The successful development of EPS-229, as a cosmetic active capable of shielding the skin from urban pollution, is a vibrant example of how biomimicry can lead to sustainable innovation.

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