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Reduction of bacterial biofilm formation using marine natural antimicrobial peptides

K. Doiron[a], L. Beaulieu[b], R. St-Louis[c], K. Lemarchand[a,*]

[a] Institut des Sciences de la Mer de Rimouski, Université du Québec à Rimouski, 310 Allée des Ursulines, C.P. 3300, Rimouski, QC, G5L 3A1, Canada
[b] Institut sur la Nutrition et les Aliments Fonctionnels, Food Sciences, Pavillon Paul-Comtois, 2425 rue de l'Agriculture, Université Laval, Québec, QC, G1 V 0A6, Canada
[c] Département de biologie, chimie et géographie, Université du Québec à Rimouski, 300 Allée des Ursulines, C.P. 3300, Rimouski, QC, G5L 3A1, Canada

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ABSTRACT

There is an important need for the development of new "environmentally-friendly" antifouling molecules to replace toxic chemicals actually used to fight against marine biofouling. Marine biomass is a promising source of non-toxic antifouling products such as natural antimicrobial peptides produced by marine organisms. The aim of this study was to demonstrate the efficiency of antimicrobial peptides extracted from snow crab (SCAMPs) to reduce the formation of marine biofilms on immerged mild steel surfaces. Five antimicrobial peptides were found in the snow crab hydrolysate fraction used in this study. SCAMPs were demonstrated to interact with natural organic matter (NOM) during the formation of the conditioning film and to limit the marine biofilm development in terms of viability and bacterial structure. Natural SCAMPs could be considered as a potential alternative and non-toxic product to reduce biofouling, and as a consequence microbial induced corrosion on immerged surfaces.

1. Introduction

Biofouling occurs worldwide in various industries, from fishing equipment, offshore oil and gas industries, cooling systems to canalizations using water. Unprotected submerged marine metallic surfaces are inevitably subjected to biofouling and corrosion, leading to considerable economic and environmental consequences for marine industries. The cost of biofouling for marine industries is evaluated at several billions US dollar per year [1] and the development of environmentally friendly antifouling strategies is a great challenge today [2]. Up to recently, synthetic chemicals agents, such as tributyltin (TBT) and 2-méthylthio-4-tert-butylamino cyclopylamino-6-(1,3,5-triazine) (Irgarol 1051) were used in paint formulation to prevent and protect metallic structures from biofouling. However, due to their toxicity for non-target marine organisms, their use was restricted or prohibited (as for TBT in 2008) following the recommendations of the Marine Environment Protection Committee (MPEC) and the International Maritime Organization [3–5]. Although the use of synthetic biocide are still on-going (e.g. 4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (Sea-Nine 211)), "green" alternatives are now the focus of many researchers worldwide.

The first step of the biofouling is initiated by different bacterial species and the compound of seawater as natural organic matter organized into a microenvironment called a biofilm. When the biofilm settles on submerged or periodically submerged metallic surfaces, such as mild steel, it not only modifies physically the surface (and then changes the hydrodynamicity of the installation, e.g. boat hull or pipe) but it may in addition accelerate the corrosion through the microbiologically influenced corrosion process [6]. Christensen et al. [7] and Nielsen et al. [8] have demonstrated that the biofilm is a dynamic microenvironment, where intra and interspecific interactions directly influence its morphology and bacterial species survival. As a consequence, reducing bacterial adhesion and limiting the expansion of the biofilm are essential to limit the impacts of biofouling on submerged steel structures. Steinberg et al. [9], Holmström and Kjelleberg [10] and Callow and Callow [11] were pioneers in the development of alternative antifouling products from marine biomass, essentially from seaweeds. Since their discovery, over 100 marine natural products were identified as antifouling and several other products are studied for these properties [12,13].

One of the main advantages of using marine biomass as "green" antifouling strategies is the valorisation of marine by-products, which are considered as under-exploited wastes for marine industries. Many substances have been extracted from these products and several applications in antifouling paints have been developed [14,15]. Lactones, alkaloids, polysaccharides and fatty acids

* Corresponding author.
E-mail address: karine_lemarchand@uqar.ca (K. Lemarchand).

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are among extractable products originating from marine biomass [13,14,16]. Another class of biomolecules with promising applications are the antimicrobial peptides [17] principally with the presence of d-amino acids which could inhibit the biofilm formation [18]. These AMP are widely available and derived from a variety of organisms such as animals, plants, bacteria, fungi and viruses [19] but also from marine by-products.

Incorporating a bio-sourced antifouling agent in paint is not the sole way to take advantage of its capacity to limit the development of marine biofilms on immerged metallic surfaces. By designing the antifouling as a free water soluble additive, it can be include in a mitigation strategy of fouling growth on inert parts of confined metallic structures (e.g. seawater cooling system, pipes, ship ballast tanks, seawater storage reservoirs), otherwise difficult to reach during maintenance work.

In Canada, the harvesting of snow crab is one of the most successful fisheries, with a landing volume around of 103,000 metric tons per year. As a consequence, year to year, over than 30,000 tons/yr of snow crab by-products (cephalothorax shells, digestive systems, including hepatopancreas and hemolymph) are buried in landfill sites in the province of Québec (Canada) [20]. However, upcoming environmental regulations will forbid landfilling of marine wastes in 2020. This challenges the Canadian fishing industry to diversify their activities on by-products valorisation and on biotechnology to ensure aquatic biomass enhancement [21]. Untapped residues of snow crab transformation could constitute a valuable source of components for antifouling strategies. Whereas several AMP were identified or cited in the literature, no AMP from snow crab (*Chionoecetes opilio*) (SCAMPs) were listed among the 2000 AMP present in the Antimicrobial Peptides Database (http://aps.unmc.edu/AP/main.php). One of our previous studies have shown that SCAMPs inhibit the growth of specific bacteria in pure cultures [22] but, to our knowledge, there is still no information concerning their potential as antifouling agents. The aim of our study was to demonstrate the efficiency of SCAMPs as antifouling agents by limiting the formation of marine biofilms on mild steel plates immerged in seawater.

2. Materials and methods

2.1. Enzymatic hydrolyzed fractions of snow carb by-products

Snow crab hydrolysate fractions were produced at Merinov, the Quebec Fisheries and Aquaculture Innovation Centre (Gaspé, QC, Canada) according to a procedure by Beaulieu et al. [20]. Briefly, 100 kg of grinded snow crab by-products were added to equal amount of demineralized water (w/w), the total volume was heated to 45 °C. Then, 100 g Protamex (Novozymes, Bagsvaerd, Denmark) were added to start the hydrolysis. After 120 min hydrolysis at 45 °C, the tank temperature was increased to 90 °C, to inactivate proteases. The liquid fraction was decanted using a clarifying decanter and then centrifuged at 11,000*g* to separate suspended insoluble matter and lipids from the hydrolysate. The hydrolysate was then ultrafiltered (spiral membrane with cut off of 10 kDa) to separate proteins and peptides according to the molecular mass. Permeate from the 10 kDa membrane at 200 Da was nano-filtered (Model R, GEA filtration, Hudson, WI, USA) to obtain a 10 kDa–200 Da retentate (SCAMPs). Nano-filtration retentate was spray-dried and kept at 4 °C until analyses.

2.2. Amino acid identification

Amino acid determination of fractions was performed according to the method described by Beaulieu et al. [23] using the AccQ-Tag amino acid analysis procedure (Waters, Canada). Briefly, the AccQ-Tag method is a pre-column derivatization technique for amino acids in peptide and protein hydrolysates. The amino acids were separated by reversed-phase high performance liquid chromatography (RP-HPLC) and quantified by fluorescence detection. The HPLC system used was equipped with a Waters Alliance e2695 Separations Module (Waters, Mississauga, ON, Canada) and a Waters 2475 Multi λ Fluorescence Detector. Analyses were performed in duplicate and averages are shown.

2.3. Peptide identification by tandem mass spectrometry

Analyses by mass spectrometry were performed using the proteomics platform from Quebec Genomics Centre (Québec, QC, Canada) following the procedure described by Beaulieu et al. [24]. Briefly, 10 μg of proteins were washed 3 times with 50 mM ammonium bicarbonate buffer and 1 μg of trypsin was added before analysed by electrospray mass spectrometry (ES-MS/MS) (Agilent 1200, AB Sciex, Framingham, MA, USA). All MS/MS peak lists were analysed by Scaffold software (version Scaffold_4.2.0, Proteome Software Inc., Portland, OR, USA). Peptide identifications were accepted if they could established at greater than 85% probability by the Peptide prophet algorithm [25] with Scaffold delta-mass correction.

2.4. Growth conditions and biofilm formation assays

The experiments were designed as part of a larger project on the potential of SCAMPs as inhibitor of corrosion of mild steel [26]. The biofilm development on metallic surface, with and without bioactive peptides, was monitored during 10 days on 36 mild steel coupons (2.5 cm × 4 cm) in natural seawater collected from the St. Lawrence Estuary (Rimouski, QC, Canada). For each treatment the coupons were immerged in a 10 L seawater tank, and kept at a temperature of 20 °C ± 0.01 (Digital temperature controller 1196D, VWR) throughout the experiment. This temperature, close to room temperature, was chosen according to previous results on microbial induced corrosion performed in the laboratory that demonstrated no significant difference between corrosion inhibition at 15 °C and 20 °C [26]. The seawater (containing around $1.8 \times 10^6 \pm 0.6 \times 10^6$ bacteria mL^{-1}) and the first tank containing this seawater was used as control whereas the second was SCAMP-treated (300 mg L^{-1}). In seawater, bacteria were enumerated using an EPICS ALTRATM cell sorting flow cytometer (Beckman-Coulter Inc., Mississauga, Canada) equipped with a laser emitting at 488 nm according to Doiron et al. [27]. The biofilm formation was followed by collecting six plates at 3, 24, 48, 96, 168 and 240 h. At each sampling time, three plates were placed into a 30 mL solution of NaCl 9‰, sonicated three times for 1 min at 20 °C, filtered on polycarbonate membranes (0.2 μm pore size, 25 mm diameter) and the filter was conserved at −80 °C until further analyses of bacterial composition by PCR-DGGE (Denaturing gradient gel electrophoresis) (C.B.S. Scientific Company, CA, USA). The remaining three plates were immediately analyzed for biofilm by confocal laser scanning microscopy LSM700 (CLSM) (Carl Zeiss, Germany).

2.5. Fourier transform infrared spectrometry

Fourier transform infrared spectrometry (FTIR) was used to determine the presence of peptides groups on metallic surfaces. Spectral acquisition were realized with a FTIR (Nicolet 6700, Thermo Scientific, USA), in an infrared medium spectral domain (400 cm^{-1}–4 000 cm^{-1}) with a 40 scans numbers and a 4 cm^{-1} resolution.