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Macroalga *Padina pavonica* water extracts obtained by pressurized liquid extraction and microwave-assisted extraction inhibit hyaluronidase activity as shown by capillary electrophoresis☆

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ABSTRACT

Hyaluronidase degrades hyaluronic acid, the principal component of the extracellular matrix. Inhibition of this enzyme is thus expected to hinder skin aging. Brown alga *Padina pavonica* activity toward hyaluronidase was evaluated using capillary electrophoresis (CE)-based enzymatic assays. This green technique allows evaluation of the biological activity of the natural material in an economic manner. Pressurized liquid extraction (PLE), microwave assisted extraction (MAE), supercritical fluid extraction and electroporation extraction techniques were used. Extraction conditions were optimized to obtain cosmetically acceptable *Padina pavonica* extracts with the best inhibition activity. CE-based assays were conducted using only a few nanoliters of reactants, a capillary of 60 cm total length and of 50 μm internal diameter, +20 kV voltage for separation in 50 mM ammonium acetate buffer (pH 9.0) and 200 nm wavelength for detection. The reaction mixture was incubated for 1 h and CE analysis time was about 11 min. A novel online CE-assay using transverse diffusion of laminar flow profiles for in-capillary reactant mixing allowed efficient monitoring of hyaluronidase kinetics with K_m and V_{max} equal to 0.46 ± 0.04 mg mL^{-1} and 137.1 ± 0.3 nM s^{-1} ($r^2 = 0.99$; $n = 3$), respectively. These values compared well with literature, which validates the assay. Water extracts obtained by PLE (60 °C; 2 cycles) and MAE (60 °C; 1000 W; 2 min) presented the highest anti-hyaluronidase activity. The half maximal effective concentration (IC_{50}) of water PLE extract was 0.04 ± 0.01 mg mL^{-1} ($r^2 = 0.99$; $n = 3$). This value is comparable to the one obtained for *Einsenia bicyclis* phlorotannin fractions ($IC_{50} = 0.03$ mg mL^{-1}), which makes *Padina pavonica* bioactivity very promising.

1. Introduction

The extracellular matrix of the skin consists of a complex and dynamic network of macromolecules composed of a variety of proteins such as collagen and elastin as well as proteoglycans to which glycosaminoglycan (GAG) chains are attached. The most common GAG in the dermis is hyaluronic acid (HA) or hyaluronan [1,2]. Since it is extremely hydrophilic, HA has a high hydration capacity contributing to the viscoelastic properties of the skin. Hyaluronidase is the endoglycosidase enzyme that randomly cleaves internal β-*N*-acetyl-hexosamine and glucosidic linkages in hyaluronic acid (HA) [3,4]. In addition to its important role in skin aging, hyaluronidase has been successfully utilized in ophthalmic surgery and is now being implemented in dermatosurgery and other surgical disciplines [5]. Finding new effectors of this enzyme is of great interest. Shibata et al. [6] evaluated the effect of *Eisenia bicyclis* brown alga phlorotannins on hyaluronidase by using an *in vitro* assay. The 8,8′-bieckol, an eckol-type phlorotannin, was shown to have the strongest inhibitory effect with an IC_{50} of 0.03 mg mL^{-1}. Valentao et al. [7] also found that the phlorotannin extracts from four brown seaweeds, particularly from *Fucus spiralis*, had anti-hyaluronidase activity with an IC_{50} equal to 0.73 mg mL^{-1}. Moreover, Ratnasooriya et al. [8] showed that Sri Lankan black

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tea (*Camellia sinensis L.*) brew had a moderate anti-hyaluronidase activity (IC_{50} = 1.09 ± 0.12 mg mL^{-1}).

Algae are rich in bioactive compounds which increase their importance nowadays in many fields [9–11]. In this work, *Padina pavonica* alga was studied for its inhibition activity toward hyaluronidase. This brown alga is commonly known as Peacocks tail and is found in the Atlantic Ocean and in the Mediterranean Sea. *Padina pavonica* alga is known for its capacity of restoring the calcium metabolism in the epidermis and maintaining a youthful aspect of the skin by promoting the renewal of GAGs, thereby boosting their protective action [12]. Moreover, Ktari and Guyot [13] evaluated the cytotoxic activity of *Padina pavonica* dichloromethane extracts against human buccal epidermal carcinoma cells and found that 0.01 mg mL^{-1} dose was sufficient to obtain 100% inhibition of tumor cell survival. The antioxidant and antifungal activities of this alga were studied by Chbani et al. [14] after maceration and soxhlet extractions with methanol. Ethyl acetate fraction was adequately prepared and showed the best antifungal activity toward *Candida glabrata* and *Candida krusei* species. This activity was referred to phenolic compounds. Mohamed et al. [15] studied the antiviral activity of *Padina pavonica* against *herpes simplex virus* (HSV) and *hepatitis A virus* (HAV). The water extract of sulfated heteropolysaccharide fraction showed an important antiviral activity (72–73%) at 0.02 mg mL^{-1}.

Nowadays, different extraction techniques can be used. The conventional one such as soxhlet extraction is very time consuming (several hours) and requires relatively large quantities of solvent which cause environmental problems and high operating costs. Recently, several novel extraction techniques such as pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) as well as ultrasonic- and electroporation – assisted extractions have been developed [16–20]. PLE is an automated extraction technique developed by Richter et al. [21] in which elevated temperatures and pressures are applied to achieve extractions in short periods of time. MAE utilizes microwave energy for heating, and thus increasing the mass transfer rate of the solutes from the sample matrix into the solvent. The first extraction of a natural product by MAE was achieved by Pare in 1994 [22]. These techniques are widely used to extract biologically active molecules [23–25]. SFE is a promising green technology that can potentially displace the use of traditional organic solvents by carbon dioxide (CO_2) for extraction. CO_2 facilitates a safe extraction due to its low toxicity, low flammability and lack of reactivity [18,26]. SFE presents additional advantages such as operating at a low temperature that reduces the thermal degradation of labile compounds and high selectivity in the extraction of target compounds. Recently, a lab-made apparatus using electroporation-assisted extraction was developed in our group to extract amino acids from microalgae cells by applying a continuous voltage of few volts [20]. Electroporation is an efficient process for breaking cell membranes that was also used for extracting proteins and small molecules from bacterial and eukaryote cells [27,28].

In this study, *Padina pavonica* extraction was optimized using MAE, SFE, electroporation and PLE. Different parameters were studied to obtain the most efficient anti-hyaluronidase extract adapted for cosmetic use. Miniaturized CE enzymatic assays were chosen to study algae extract bioactivity [29,30]. Our previously developed pre-capillary CE-based hyaluronidase assay [31] and a novel online assay were conducted. The novelty of the online assay consists of studying the degradation of a multifunctional high molecular weight polysaccharide, the hyaluronic acid, into different poly(di)saccharide fragments. These fragments will be then considered as novel substrates of hyaluronidase inside the capillary [4]. Moreover, algae extracts must be homogeneously mixed with the enzyme and its substrate(s) in the capillary for online evaluation of the bioactivity of this vegetal. IC_{50} value of the most efficient extracts was determined.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade and used as received without further purification. Ammonium acetate ($C_2H_3O_2NH_4$, purity ≥ 98%), hyaluronidase type I-S from bovine testes (BTH, 400–1000 units mg^{-1} solid), sodium acetate (CH_3COONa, purity ≥ 99%), sodium hydroxide (NaOH, purity ≥ 98%) and oligohyaluronic acid 4 (oligo-HA4 or tetrasaccharide (Tet), $C_{28}H_{44}N_2O_{23}$) were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Hyaluronic acid sodium salt (HA) from *streptococcus pyrogenes* was purchased from Merck Millipore (Molsheim, France). Glacial acetic acid (CH_3CO_2H), ammonia (NH_3, purity 28%), diethyl ether (Et_2O), ethanol (EtOH), ethyl acetate ($C_4H_8O_2$) and sulfuric acid (H_2SO_4, purity 96%) were purchased from VWR International (Fontenay-sous-Bois, France). Petroleum ether was purchased from Carlo Erba (Val-de-Reuil, France). The CO_2 was supplied by Linde (Munich, Germany). Ultra-pure water (18 MΩ cm) was produced from an Elga apparatus (Elga, Villeurbanne, France). Syringes and hydrophilic polyvinylidenedifluoride (PVDF) Millex-HV Syringe Filters, pore size 0.45 μm, were purchased from Millipore (Molsheim, France).

2.2. Solutions

Solutions were filtered through PVDF Millex-HV Syringe Filter before use and stored at 4 °C. The pH of the buffers was measured with a MeterLab PHM201 Portable pH-Meter (Radiometer Analytical, Villeurbanne, France). The different buffers were prepared each day and their parameters were given by Phoebus software (Analis, Namur, Belgium).

Sodium acetate at 2 mM was used as the incubation buffer (IB). Its pH was fixed at 4.0 by adding glacial acetic acid. The background electrolyte (BGE) was ammonium acetate 50 mM with a pH of 9.0 adjusted with ammonia.

Stock solutions of BTH, HA and Tet were prepared in the IB at a concentration of 2 mg mL^{-1} and diluted to the appropriate concentrations in the same buffer. The enzyme stock solution was prepared fresh every day.

When obtained, extracts were evaporated under a stream of nitrogen. Stock solutions of 1 mg mL^{-1} were obtained by dissolving the dried extracts in EtOH-water (10:90, v/v) except for water extracts which were dissolved in pure water. Each extract was then diluted in IB to the appropriate concentration and the incubation with hyaluronidase was done according to our previous work [31]. EtOH percentage did not exceed 2% (v/v) in the final reaction mixture.

2.3. Plant materials

Brown seaweed *Padina pavonica* was manually collected from the coast of Berbara (also spelled Al-Barbara) in Byblos city in Lebanon at the end of May 2015. GPS coordinates of Berbara are 34°11′33 N 35°38′31″ E. It has an average elevation of 200 meters above the Mediterranean sea level. The collected samples were transported to the laboratory where they were first cleaned and washed thoroughly with fresh water to remove salts, sand and epiphytes. The algae were lyophilized and ground to powder of about 100 μm size and stocked at ambient temperature.