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Extraction, characterization and antimicrobial activity of sulfated polysaccharides from fish skins

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ABSTRACT

Sulfated polysaccharides were extracted from gray triggerfish (GTSP) and smooth hound (SHSP) skins. Their chemical and physical characteristics were determined using X-ray diffraction and Infrared spectroscopic analysis. The antibacterial activities of GTSP and SHSP against *Listeria monocytogenes* (ATCC 43251), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Salmonella enterica* (ATCC 43972) and *Enterobacter sp* were evaluated by determining clear growth inhibition zone diameters and the minimum inhibitory concentration (MIC) values and by essays in liquid media. GTSP and SHSP were fractionated by a Diethylaminoethyl-cellulose chromatography. Fraction F_{GII}, from GTSP, and fraction F_{SII}, from SHSP, showed the most important inhibitory effects against the tested bacterial species. The sulfated polysaccharides from fish skins did not show hemolytic activity towards bovine erythrocytes. Overall, the results suggested that those polysaccharides could offer promising sources of polysaccharides for future application as dietary ingredients in the nutraceutical industry.

1. Introduction

Food spoilage and poisoning are two of the most troublesome problems in the food industry [1]. In fact, food borne diseases have become a major concern throughout the world, even in developed countries. Several pathogenic microorganisms, including *Staphylococcus aureus*, *Salmonella enterica*, *Listeria monocytogenes* and *Candida utilis*, have been identified as the causal agents of food spoilage and food borne diseases [2]. Recent estimates indicate that about one-third of the world's food production is thrown away every year due to microbial spoilage. So far, several technologies, including freezing, lactic acid bacteria, thermal processing, acidified sodium chlorite, antimicrobial ice, irradiation, and high pressure, have been investigated for their protective abilities against the formation and growth of pathogenic microorganisms in food [3–6]. Most of those methods are, however, not amenable to application in food products, particularly for the negative effects they might bring to food quality. Researchers have, therefore, expressed a clear need for natural, efficient, and cost-effective food preservatives that can help maintain quality and safety throughout the shelf life of food products. Moreover, and, in addition to seeking for life-supporting agents, researchers have also become increasingly interested in the molecular modification and structure improvement of biologically active compounds.

Polysaccharides are natural polymers that play a foundational role in sustaining life. Due to their structural variability, polysaccharides offer the potential for carrying biological information and, therefore, support the process of molecular recognition on the surface of cells in higher organisms [7]. Moreover, the chemical modification of polysaccharides, such as selenylation [8] and sulphonation [9] has often been reported to lead to the formation of new pharmacological agents with attractive therapeutic applications. Sulphonation is a particularly simple, powerful and versatile tool for the enhancement of polysaccharides' biological activities.

Sulfated polysaccharides are a complex class of structural polysaccharides with a sulfate group attached to their hydroxyl group. They have a wide range of stronger biological activities compared to non-sulfated polysaccharides, including powerful anti-coagulation [10], anti-virus [11–13], immune enhancing, hypoglycemic [14], antitumor [15–17], and antioxidant [18] activities. Of particular interest, marine sulfated polysaccharides have recently been demonstrated to have a broad range of antibacterial activity [19,20]. To the authors' knowledge, however, no studies have so far been performed to explore the antibacterial activity of

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sulfated polysaccharides from fish skins. Accordingly, the present study was undertaken to investigate the antibmicrobial activity and partial purification of sulfated polysaccharides extracted from the skins of gray triggerfish (GTSP) and smooth hound (SHSP).

2. Materials and methods

2.1. Reagents

All solvents and chemical reagents were obtained from commercial sources and used at analytical grade or highest level of purity available. Alcalase® 2.4 L serine-protease from *Bacillus licheniformis* was supplied by Novozymes® (Bagsvaerd, Denmark). Diethylaminoethyl (DEAE)-cellulose was purchased from Pharmacia (Uppsala, Sweden). All other chemicals and reagents used were of analytical grade. All solutions were freshly prepared in distilled water.

2.2. Preparation of fish skin

Fish by-products were obtained from the local fish market of Sfax, Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w), and transported to the research laboratory within 30 min. Upon arrival, the samples were washed twice with water and separated. Only the fish outer skin was collected and then stored in sealed plastic bags at −20 °C until further use for the extraction and analysis of sulfated polysaccharides.

2.3. Sulfated polysaccharides extraction

Sulfated polysaccharides were extracted according to a slightly modified version of the method described by Ben Mansour et al. [21]. In brief, the fish skins were cut into small pieces and homogenized using a Moulinex R62 homogenizer (Organotechnie, Courneuve, France). An amount of 5 g of sample was dissolved in 250 mL sodium acetate (0.1 M), EDTA (5 mM), cystein (5 mM) pH 6. Alacalase® was added, and the mixture was kept for 24 h at 50 °C. The mixture was then left to cool down at room temperature and then filtered. The residue was washed with distilled water and filtered again. The filtrates were mixed, and polysaccharides were precipitated with cetylpyridinium chloride 10% (w/v). The mixture was kept for 24 h at room temperature and centrifuged for 30 min at 5000 tr/min and 4 °C using a refrigerated centrifuge (Hettich Zentrifugen, ROTINA 380R, Tuttlingen, Germany). The pellet was washed with cetylpyridinium chloride 0.05% (w/v) and then dissolved in 200 mL NaCl solution in ethanol (100:15, v/v). An amount of 700 mL ethanol was added. The polysaccharide containing solution was left for 24 h at 4 °C and then centrifuged for 30 min at 5000 tr/min and 4 °C. The pellet was washed twice with ethanol 80% and then once with absolute ethanol. After that, the pellet was redissolved in desionised water and lyophilized in a freeze dryer (CHRIST, ALPHA 1-2 LD plus, Osterode am Harz, Germany). The dry matter was referred to as “Sulfated polysaccharides”.

2.4. Determination of chemical composition

The moisture and ash content were determined in accordance with the AOAC standard methods 930.15 and 942.05, respectively [22]. Total nitrogen content was estimated by the Kjeldahl method. Crude protein was measured by multiplying the total nitrogen content by the factor of 6.25. Crude fat was determined gravimetrically after Soxhlet extraction of dried samples with hexane. Total carbohydrates were determined by the phenol-sulphuric acid method [23]. Total uronic acid content was quantified colorimetrically following the method described by Bitter and Muir [24] using galacturonic acid as a standard. Water activity was measured by a NOVASINA aw Sprint TH-500 apparatus (Novasina, Pfäffikon, Switzerland) at 25 °C. All measurements were performed in triplicates.

2.5. Determination of sulfate content in polysaccharides

The determination of the sulfate content in the polysaccharides was performed the National School of Engineering of Sfax (Tunisia) by liquid-Ion Chromatography (HPLC) on a Metrohm chromatograph equipped with columns CI SUPER-SEP using acetonitric and phthalic acid as eluent. The test precision of the instrument was about ±2%.

2.6. Determination of colour

The samples were placed between two steel dishes with a hole of 5.7 cm diameter. The colour of the films was determined by a tristimulus colorimeter (CHROMA METER CR-400/410. KONICA MINOLTA, Japan) using the CIE Lab scale (C/2°), where L^*, a^* and b^* refer to the parameters measuring lightness, redness, and yellowness, respectively. A standard white plate was used as a reference. The results were the average of five measurements taken at ambient temperature at different points on the samples.

2.7. Infra-red spectroscopic analysis

The absorption spectra of the samples were obtained using FTIR spectroscopy (Analect Instruments fx-6 160). The FTIR spectra of the prepared materials were recorded between 450 and 4000 cm^{-1} in a NICOET spectrometer. The transmission spectra of the samples were recorded by using the KBr pallet containing 0.1% of sample.

2.8. X-ray diffraction (XRD) of polysaccharides

The X-ray diffraction pattern of sulfated polysaccharides was recorded at room temperature on an X-ray diffractometer (D8 advance, Bruker, Germany). The data were collected in the 2θ range 2–60° with a step size of 0.02° and a counting time of 5 s/step.

2.9. Antimicrobial activity

2.9.1. Microbial strains

The antibacterial activities of the sulfated polysaccharides extracted from fish skin were tested against six strains of bacteria: three Gram-negative (*Salmonella enterica* ATCC 43972, *Escherichia coli* ATCC 25922, and *Enterobacter sp*) and three Gram-positive (*Listeria monocytogenes* ATCC 43251, *Staphylococcus aureus* ATCC 25923, and *Enterococcus faecalis* ATCC 29212). Antifungal activities were evaluated using *Fusarium solani*, *Botrytis cinerea*, and *Alternaria solani*.

2.9.2. Agar diffusion method

Antimicrobial activity was determined by the method of Berghe and Vlietinck [25]. A culture suspension (200 μL) of the tested microorganisms (10^6 colony forming units (cfu)/mL of bacteria cells and 10^8 spores/mL of fungal strains) were spread on Mueller–Hinton broth and potato dextrose agar media, respectively. The sulfated polysaccharides (20 and 50 mg/mL) were dissolved in distilled water and added (20 μL) to wells (5 mm diameter), punched in the agarose layer, allowed to diffuse in the layer, and incubated in a humidified close container for 3 h at 4 °C. At the end of incubation time (24 h at 37 °C for bacteria strains or 72 h at 30 °C for fungal strains), antibacterial activity was measured as the diameter of the clear zone of growth inhibition compared to a positive control, ampicillin, and a negative control (distilled water),