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Pectin functionalized with natural fatty acids as antimicrobial agent

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ABSTRACT

Several pectin derivatives were prepared by chemical modifications of the polysaccharide with natural fatty acids. The obtained biodegradable pectin-based materials, pectin-linoleate, pectin-oleate and pectin-palmitate, were investigated for their antimicrobial activity against several bacterial strains, *Staphylococcus aureus* and *Escherichia coli*. Good results were obtained for pectin-oleate and pectin-linoleate, which inhibit the growth of the selected microorganisms by 50–70%. They exert the better antimicrobial activity against *S. aureus*.

Subsequently, the pectin-oleate and the pectin-linoleate samples were coated on polyethylene films and were assessed for their capacity to capture the oxygen molecules, reducing its penetration into the polymeric support. These results confirmed a possible application of the new materials in the field of active food packaging.

1. Introduction

During the past decade, an increasing interest has been devoted to the development of antimicrobial systems to be used as biocides in various areas. In particular, recyclability and biodegradability are nowadays considered important issues when new products with antimicrobial properties are introduced for various applications in medical, pharmaceutical, agriculture, and packaging fields [1–3].

Free fatty acids (FFA) can be considered natural products, since they are usually provided by natural resources, like triglycerides or phospholipids, and for this reason can be employed with the great advantage of low environmental impact. Within their broad spectrum of biological activities, free fatty acids are able to kill or inhibit the growth of several pathogenic bacteria [4,5]. Whilst their antibacterial mode of action is still unclear, fatty acids have as prime target the bacterial cell membrane and the various essential processes that occur within and at the membrane. Other processes that may contribute to bacterial growth inhibition or death include cell lysis, inhibition of enzyme activity, impairment of nutrient uptake and the generation of toxic peroxidation and autooxidation product [6]. However, the usage of FFA as antibacterial agents suffer of several limitations, since some FFAs have an unpleasant taste, while others can be unstable and also have a tendency to bind nonspecifically to proteins. An alternative route for exploring the commercial potential of FFA as antimicrobials could be the conjugation of them to specific carriers, like biodegradable material, in order to modulate their delivery. In this regard, biodegradable polymers, produced from natural, renewable resources, can represent ideal candidates, since they readily decompose thus reducing the negative environment impact [7–9]. We have recently developed a synthetic process to chemically modify the pectin from apple via acylation of its alcoholic functions with several natural fatty acids [10–12]. The aim was to generate new materials by using renewable resources and limiting the number of chemicals and reaction steps. Indeed, natural polymers suffer of low water resistance and poor mechanical properties (tensile strength and elongation to break), and these drawbacks are limiting factors for their use as manufactured materials. The chemical modification of pectin with fatty acids as a matter of fact improved water resistance and barrier properties of the polysaccharide, thus generating new bio-based material for novel applications. Due to the introduction of fatty acids, these pectin derivatives can be studied for their antimicrobial properties, and eventually considered as coatings to be used for the food packaging.

With these ideas in our mind, we decided to prepare several pectin samples functionalized with different fatty acids (palmitic,

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oleic and linoleic acid) in order to investigate their antimicrobial activity against two foodborne pathogens, *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) [8].

2. Experimental

2.1. Materials

The apple peel Pectin was purchased from Fluka. It is a powder sample with high molecular weight (30 000–100 000 g/mol) and a high degree of esterification (70–75%) on a dry basis. The fatty acids and all solvents were purchased from Sigma–Aldrich.

2.2. Synthesis of pectin derivatives

Synthesis of fatty acid anhydrides was performed as follows. The appropriate fatty acid (10 mmol) was dissolved in dichloromethane (2 mL), the solution was cooled in an ice-water bath and stirred vigorously under argon atmosphere. The dicyclohexylcarbodiimide (5 mmol), previously dissolved in the minimum volume of dichloromethane, was added and stirring was continued at ice bath temperature for 2 h. The white solid *N*,*N*′-dicyclohexylurea was removed by filtration and the solvent was evaporated in vacuo to give the final anhydride [10].

By using an agate mortar, 30 mg of pectin were manually milled with 30 mg of the appropriate fatty acid anhydride in the presence of K_2CO_3 (0.1 equiv.) and few drops of ethanol to obtain the different pectin-derived materials. Reactions were carried out in a domestic microwave oven and irradiated with microwaves (900 W) for two cycles of 3 min each. After cooling at room temperature, the final crude product was washed with ethyl acetate. The obtained solid was dissolved in water, and 0.5 N HCl was added to the final solution until a neutral pH was reached. This solution was then dialyzed (membrane cut off 6000–8000) for 1 day in Milli-Q water and finally lyophilized to give the desired product.

2.3. Characterization

All modified pectin samples were analyzed by FT-IR spectroscopy. The FT-IR spectra were recorded on a Jasco spectrometer. Samples were ground into a fine powder using an agate mortar before being compressed into KBr discs. The characteristic peaks of IR transmission spectra were recorded at a resolution of 4 cm^{-1} over a wavenumber region of 400–4000 cm^{-1}. The bands relevant for the structural organization are: pectin-linoleate (1): FT-IR (cm^{-1}): 3425 ν (O—H), 2926 and 2845 ν (C—H), 1741 ν (C=O methyl ester), 1722–1704 ν (C=O fatty acid ester), 1629 ν_{as} (COO^-), 1439 ν_s (COO^-), 1207 and 1133 ν (C—O); pectin-oleate (2): FT-IR (cm^{-1}): 3424 ν (O—H), 2925 and 2855 ν (C—H), 1747 ν (C=O ester), 1721–1704 ν (C=O fatty acid ester), 1639 ν_{as} (COO^-), 1443 ν_s (COO^-), 1207 and 1142 ν (C—O); and pectin-palmitate (3): FT-IR (cm^{-1}): 3445 ν (O—H), 2923 and 2854 ν (C—H), 1749 ν (C=O ester), 1723–1705 ν (C=O fatty acid ester), 1635 ν_{as} (COO^-), 1436 ν_s (COO^-), 1207 and 1133 ν (C—O).

Thermal analysis (TGA) was carried out in air atmosphere with a Mettler TC-10 Thermobalance (Novate Milanese, Italy) from room temperature to 1000 °C at a heating rate of 10 °C/min on 10 mg samples in duplicate.

2.4. Antimicrobial assessment

2.4.1. Antimicrobial test

The strains used for the antimicrobial assays were the following: the Gram-positive bacterium *S. aureus* ATCC 6538 and the Gram-negative bacteria *E. coli* ATCC11219. To standardize the bacterial cell suspension for antibacterial activity assay, some colonies of each strain grown overnight on MHA plates were resuspended in MHB and incubated at 37 °C until visible turbidity. This log-phase inoculum was resuspended in 0.9% sterile saline to reach an appropriate OD600 value (with a Bio-Rad Microplate Reader – Bio-Rad Laboratories, Hercules, CA, USA) corresponding to a concentration of about 1×10^8 CFU/mL. This standardized inoculum was diluted 1:10 in MHB and the inoculum size was confirmed by colony counting.

2.4.2. Antimicrobial activity assay

Susceptibility testing was performed using the broth microdilution method outlined by the Clinical and Laboratory Standards Institute using sterile 96-well microtiter plates (Falcon, NJ, USA). For the quantitative assay of the antimicrobial activity of our compounds by the microdilution method in liquid medium distributed in 96-well plates, binary serial dilutions of the tested compounds solutions were performed.

Each well was then inoculated with 5 μl of the standardized bacterial inoculum, corresponding to a final test concentration of about 5×10^5 CFU/mL. Antimicrobial activities were expressed as the percentage value of colony (formation) reduction observed after 24 h of incubation at 37 °C.

2.4.3. Eukaryotic cells cytotoxicity

Vero cells were exposed to increasing concentrations of compounds, and the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay that is based on the reduction of the yellowish MTT to the insoluble and dark blue formazan by viable and metabolically active cells. Vero cells were subcultured in 96-well plates at a seeding density of 2×10^4 cells/well and treated with compounds for 3, 10 and 24 h. The medium was then gently aspirated, MTT solution (5 mg/mL) was added to each well, and cells were incubated for a further 3 h at 37 °C. The medium with MTT solution was removed, and the formazan crystals were dissolved with dimethyl sulfoxide. The absorption values were measured at 570 nm using a Bio-Rad Microplate Reader. The viability of Vero cells in each well was presented as a percentage of control cells.

2.5. Coating preparation and SEM analysis

The polyethylene (PE) film, 80 μm thick and 5 cm diameter, was used as polymeric substrate for the coating. The coating solutions were prepared by dissolving 15 mg of pectin sample (pectin-palmitate, pectin-oleate, pectin-linoleate) in 20 ml of water, the mixture was heated at 150 °C for 1 h. Both sides of PE film were coated by evaporation of the water from pectin, pectin-palmitic, pectin-oleic and pectin-linoleic solution, giving a layer of coating of 0.7–1 μm. PE film was used as control and the coatings resulted mildly adhesive.

The morphology of the films was analyzed by scanning electronic microscopy (SEM).

Pieces of film (10 mm × 10 mm) from each samples were cut and fixed in a little support (stub). All samples were sputter coated with gold (Agar Automatic Sputter Coater Mod. B7341, Stansted, UK) at 30 mA for 180 s and micrographs were collected by a FEI Quanta 200 FEG scanning electronic microscope (Eindhoven, The Netherlands).

2.6. UV irradiation test

Accelerate Weathering tests were carried out using QUV/Spray accelerated weathering tester supplied by Q Panel lab products-USA, at an irradiance of 0.78 W/m^2 at 340 nm and at temperature of 45 °C. This is considered a good match with noon summer sunlight. The QUV/Spray tester uses fluorescent UVA-340 lamps that gives a good simulation of sunlight in the critical short wavelength