CHARACTERIZATION OF COMMERCIAL AND ALGAE (*Undaria pinnatifida*) EXTRACTED SODIUM ALGINATE FOR FUTURE APPLICATION IN BONE TISSUE ENGINEERING

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ABSTRACT

*Alginate is a natural, water-soluble, polysaccharide obtained from brown algae. Due to many attractive features such as good biocompatibility, low toxicity as well as ease of hydrogel formation with divalent cations, it has been widely used in a variety of biomedical application. The brown algae, *Undaria pinnatifida*, native from Japan, China and Korea is a powerful invasive species which was introduced worldwide in different coasts, including the one of Puerto Madryn (Argentina). Sodium alginate was obtained from this seaweed and the first aim of the extraction process was to convert the insoluble calcium salt into sodium alginate. In this study we compare extracted sodium alginate from *U. pinnatifida* from two different parts*
of the seaweed: stem and leaf, with the commercial salt. We characterized the samples using a Fourier Transform Infrared Spectroscopy (FTIR), a Thermogravimetric Analisys (TGA), capillary viscosity and a cytotoxicity assay to detect a possible immune response.

Key-words: sodium alginate; undaria pinnatifida, extraction, characterization.

INTRODUCTION

Sodium alginate is a biopolymer widely use in food, textile, pharmaceutical and biomedical industries. Recently, the interest in this polymer has increased due to several attractive features that make it a potential biopolymer suitable for the development of controlled-release systems and tissue engineering scaffolds. The hydration of the alginate matrix forms a gelatinous layer that can act as a drug diffusion barrier, maintain a physiologically moist microenvironment with an adequate biocompatibility and low toxicity (1). It forms an insoluble crosslinked hydrogel when it is mixed with polyvalent cations such as calcium, barium and strontium.

Alginates are natural, water-soluble, polysaccharides that constitute a family of copolymers of 1-4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) (Figure 1) of widely varying sequence and composition that depends on the organism and tissue they are isolated from. The M and G monomers are sequentially assembled in either homopolymeric (MM- or GG-blocks) or heteropolymeric sequences (MG- or GM-blocks) (2).
Brown seaweeds are a potential source of this polymer. One of them is *Undaria pinnatifida* native from Japan, China and Korea. It is a powerful invasive species which was introduced worldwide in different coasts, including the one of Puerto Madryn (Patagonia Argentina) (3, 4).

In the present work, sodium alginate was obtained from Undaria pinnatifida algae and the first aim of the extraction process was to convert the insoluble calcium salt into alginic acid by treating the seaweed with dilute mineral acid. Then, in order to convert the alginate to the soluble form of sodium alginate so as to remove it from the algae, an alkaline solution such as sodium carbonate was used. After the alkaline extraction, the dissolved sodium alginate was separated from the alkali-insoluble seaweed tissue and the alginate was recovered in solid form by precipitation as its calcium salt. Calcium alginate was precipitated as a fibrous material that can be readily separated, and then treated with dilute mineral acid to obtain alginic acid in a fibrous form. Because sodium alginate is more stable, this is converted from the Alginic acid by neutralization with an alkali.

In this study we compare extracted sodium alginate from two different parts of *U. pinnatifida*: stem and leaf, with the commercial salt (Sigma Aldrich, AC). We characterized the samples using a Fourier Transform Infrared Spectroscopy (FTIR), a Thermogravimetric Analisys (TGA), capillary viscosity and a cytotoxicity assay using RAW 264.7 macrophages to detect a possible immune response. An important aspect in the development of a bone scaffold is the possible...
inflammatory response that the material could induce in an in vivo application. A first approach to this aspect may be reached by investigating the in vitro response of macrophages in culture, such as murine RAW 264.7 cells. This cell line is highly sensitive to cytotoxic agents and responds by sharply increasing pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-\(\alpha\)) as well as the production of nitric oxide (NO). Due to these features, macrophages in culture constitute an excellent model for cytotoxicity studies with various substances in biological systems (5).

MATERIALS AND METHODS

Alginate Extraction

Undaria pinnatifida was proportioned by CENPAT-Puerto Madryn. The seaweed was dried and separated into two samples: stem (MB) and leaf (MV). Then, these were milled until a size smaller than 1mm. Alginate was obtained in accordance with Arizu-Higuera et. al 1993 (6).

One part of the seaweed was soaked with 9 parts of formaldehyde 0,1% overnight with constant stirring to eliminate pigments. Distilled water was added (20 parts per part of algae) and the solution was taken to pH= 4 with a 1N HCl under constant stirring during 15 minutes. This procedure was repeated two times.

The extraction was performed adding 25 parts of distilled water, the solution was taken to pH=10 with Na2CO3 10%, letting the samples under agitation for 2h at 80°C. After the alkaline extraction, the dissolved sodium alginate was separated from the alkali-insoluble seaweed tissue by centrifugation. The alginate was recovered in solid form by precipitation with a solution of CaCl\(_2\) 10%. The solid was
suspended with distilled water and 1N HCl until pH=2, the procedure was repeated two times. Finally, acid alginate was precipitated with ethanol at pH=8 using a solution of 10% Na$_2$CO$_3$ and agitation for 1h before been dried at 50°C.

Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) spectra of all sodium alginates were recorded on a Shimadzu IRAffinity-1 between 4000-400 cm$^{-1}$ with a resolution of 4 cm$^{-1}$ and 40 scans of accumulation. In order to reach an adequate spectra, aqueous solutions of 0,5% sodium alginate were prepared to obtain films.

Thermogravimetric analysis

Thermogravimetric analysis (TGA) of the different sodium alginates was performed in order to evaluate the variations in the temperature-mass relationship of the materials. The analysis was carried out using under a nitrogen atmosphere (TGA Q500-TA Instruments), with a gas purge at 90mL/min and tamp from room temperature to 700°C.

Viscosity measurements

The determination of the intrinsic viscosity ([η]) was carried out by dissolving the polymer in 1M NaCl, previously filtered with a 0.22 µm membrane, under mechanical agitation during 24 h at room temperature.
After that, dilutions were prepared from a stock solution such that $0.3 < \eta_{sp} < 0.8$ ($\eta_{sp}$ being the specific viscosity). Dilutions viscosities were measured with Ostwald capillary viscometer at 20°C. The viscometric average molecular weight (Mv) was estimated according to the following Mark-Houwink equation (7):

$$[\eta](\text{mL g}^{-1}) = 9.1 \times 10^{-3} \text{Mv}^{0.87}$$

Citotoxicity studies with RAW 264.7 macrophages

Murine macrophage RAW 264.7 cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) FBS and antibiotics (100 U/mL penicillin and 100 g/mL streptomycin) in a humidified atmosphere of 5% CO₂. For the experiments, alginate hydrogels were made from 2% (w/v) solutions from the three sources (Commercial Alginate, AC; Stem sample, MB and leaf sample, MV). The solutions were prepared with distilled water and sterilized by autoclaving. In order to form alginate hydrogels by an external gelation, 1mL of alginate solution was added in 12-well TCP plates. Sterile 50mM CaCl₂ and was used to crosslink the alginate solution to form the hydrogel discs. The condition of gelation was carried out for each alginate sample using 500 µL of CaCl₂ solution.

After 1h gelation, macrophage cells were seeded over the hydrogels, cultivated with DMEM without phenol red indicator. We used lipopolysaccharide (LPS) for positive control.

Nitric oxide (NO) production was assessed using Griess reaction (8, 9), using sulfanilic acid as the diazotizing agent and N-1-naphthylethylene diamine as the coupling agent. The stable end-product of NO and nitrite released into the culture medium by RAW 264.7 cells was measured after 24, 48 and 96 hours of culture. Briefly, 500µL samples of conditioned media or nitrited standards 0-100 nM were
mixed with 500 µL of Griess reagent (1% sulfanilamide and 0,1% naphthylethylene-diamine in 5% phosphoric acid) and absorbance was measured at 530 nm against a blank prepared with non-conditioned medium.

RESULTS AND DISCUSSION

The Fourier transform infrared (FTIR) spectra of commercial sodium alginate, sample extracted from seaweed leaf and sample extracted from algae’s stem (Figure 2) were recorded and compared in order to analyze if the products obtain after the procedure of extraction presented the same signaling as commercial sodium alginate. It can be observed that all three samples present the same characteristic bands. For sodium alginate, this bands can be associated with stretching vibrations of O-H bonds of alginate appeared in the range of 3000-3600 cm$^{-1}$. Stretching vibrations of aliphatic C–H were observed at 2900–2930 cm$^{-1}$. Observed bands in 1597-1609 cm$^{-1}$ and 1414 cm$^{-1}$ were attributed to asymmetric and symmetric stretching vibrations of –COO$^-$, respectively. The bands at 1086-1090 and 1036 cm$^{-1}$ were attributed to the C–O stretching vibration of pyranosyl ring and the C–O stretching with contributions from C–C–H and C–O–H deformation (10,11)
When comparing the TGA curves of the three samples of sodium alginate as illustrated in Figure 3, we observed that the two extracted polymers had mass loss at low temperature (at 58°C and 100°C for MB, 57°C for MV) that corresponds to water desorption (12). The mass loss at higher temperatures (>200°C) is attributed to polysaccharide degradation (13). The results obtained show polymer decomposition in one step at 244°C for AC and MB sample at 240°C for MB, characteristic temperatures for sodium alginate. On the other hand, MV shows a two overlapping steps of degradation at 223-250°C and 448°C under nitrogen atmosphere. This suggests that most of MV sample might be in the form of acid alginic, and the decomposition product around 400°C is carbonaceous material (14).
FIGURE 3. A) TGA curves of AC, MB, and MV in N₂. B) AC TGA (solid)/DTGA (dashed) curves. C) MB TGA (solid)/DTGA (dashed) curves. D) MV TGA (solid)/DTGA (dashed) curves.

Figure 4 shows the viscosity measurements for double logarithmic plots of all three samples. The concentration range of the viscometric determination was 0.3<c<0.8 mg/mL. The intrinsic viscosity, \([\eta]\), was conventionally obtained from double extrapolation of the Huggins and Kraemer equations.

\([\eta]\) was estimated as the average of the two ordinate intercepts from the two extrapolations and the values obtained were: \([\eta]_{AC}=1014.5\) g/mL and \([\eta]_{MB}=481\) g/mL. The viscosity average molecular weight calculated from the \([\eta]\) was of 6.3x10⁵ g/mol for AC and 2.7x10⁵ g/mol for MB. MV sample presented an
anomalous behavior, congruent with the one obtained in TGA, which prevented an appropriate measure.

FIGURE 4. Viscosity of AC and MB at 20ºC: (●) \( \eta_{sp}/c \), (■) \( \ln(\eta)/c \).

The study with macrophage cells did not show conclusive results. Because the low stability of ionically crosslinked hydrogels at non-toxic concentration of \([Ca^{2+}]\), in the culture medium has dissolved a part of alginate being interfering in the Griess reaction. Our previous studies showed that, at high concentrations of the divalent cation, we acquire a better stability of the hydrogels but this concentrations has induced RAW264.7 cell dead.

CONCLUSIONS

The IR spectra from the two extracted samples of the alga Undaria pinnatifida showed absorptions that were consistent with commercial sodium alginate, which indicates that the samples are structurally equals.

TGA curves presented in Figure 3 suggest that MV sample was not totally converted into sodium alginate according to its decomposition profile in two overlapping steps under nitrogen. This result is reaffirmed with data obtain in the study of intrinsic viscosity for the same sample.
On the other hand, stem source sample (MB) showed a similar behavior as commercial sodium alginate.

These results suggest that a modification of the protocol for precipitation of alginic acid into sodium alginate should be performed for MV sample in order to obtain a homogeneous extraction product.

REFERENCES


