Chondroitin sulfate and hydroxyapatite from *Prionace glauca* shark jaw: Physicochemical and structural characterization

Miriam López-Álvarez ^a, Pío González ^a, Julia Serra ^{a,*}, Javier Fraguas ^b, Jesús Valcarcel ^b, José Antonio Vázquez ^b

^a New Materials Group, Department of Applied Physics, University of Vigo, IISGS, MTI, Campus Lagoas-Marcosende, Vigo 36310, Spain

^b Group of Recycling and Valorisation of Waste Materials (REVAL), Marine Research Institute (IIM-CSIC), r/Eduardo Cabello, 6, Vigo 36208, Galicia, Spain

Abstract

In the present work, the potential of the *Prionace glauca* jaw as a source of both chondroitin sulfate and bioapatite is explored. The sandwich-type structure in cross section of the jaw based on alternate layers with prevalence in organic tissue or mineralized is shown and these bands respectively confirmed as CS or hydroxyapatite - enriched zones. As result of this, an optimized process in sequential steps for the recovery of both biomaterials and their purification process is proposed, by combining enzymatic proteolysis, chemical precipitation and separation using ultrafiltration membrane for CS production together with controlled thermal treatment for hydroxyapatite obtaining. The purified CS was characterized by Gel Permeation Chromatography, Nuclear Magnetic Resonance and Strong Anion Exchange Chromatography, revealing a polymeric material with a molecular weight of 67 kDa, and prevalent 6S-GalNAc sulfation (68%), followed by 4S-GalNAc (13%), a significant proportion of disulfated disaccharides (12%) and only 7% of non-sulfated units. In the case of the bioapatite a purified biphasic 60:40 porous calcium phosphate of hydroxyapatite: whitlockite/ β -TCP was confirmed. Hydroxyapatite as major component (85%) was also obtained for jaws directly subjected to the thermal treatment. This proved the influence of the enzymatic hydrolysis and centrifugation on the composition of the mineral fraction.

1. Introduction

The marine environment applications [1,2,3]. Some of these compounds may occur in rare and unexploited organisms, but many marine biomaterials originate from commonly fished species. This is the case of blue shark (*Prionace glauca*), a wide-ranging oceanic chondrichthyan dispersed across temperate and tropical waters. This

species represents a relatively abundant source of fish by-products, mostly heads and skeletons, amenable to be processed to obtain valuable biomaterials [4].

Apatites and, in particular, hydroxyapatite (HA) is a bioactive bioceramic which belongs to the family of calcium phosphates and fits the ideal formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. This basic composition enriched by groups and elements in trace concentrations, as CO_3^{2-} , HPO_4^{2-} , Na^+ , Mg^{2+} , Sr^{2+} , K^+ , Cl^- and F^- , constitute the mineral fraction of bone tissue. The rest of the tissue is formed by collagen with other proteins and water. This composition gives the bone tissue a great consistency, hardness, elasticity and resistance to compression [5,6,7]. Moreover, as a bioactive material, HA has the ability to induce its biological integration in living tissues, so that it interacts chemically with the organism, giving rise to a layer rich in phosphorus and calcium on its surface in the form of bioapatite [6,8]. It is, therefore, a property of great interest to promote the activity of bone tissue forming cells, since the presence of hydroxyapatite and the bioactivity generated activate the adhesion, proliferation and osteoblast activity of osteoblasts.

In recent years HA-based materials of biological origin as bone substitutes have attracted greater interest than those of synthetic origin, given that their behaviour is biologically more active compared with synthetic equivalents [9,10,11,12,13]. Moreover, in contrast to commercial synthetic HA the biological version (mainly bovine, porcine origin) is enriched, as in human bones, with essential elements (such as Mg^{2+} , K^+ , Na^+ , etc.) that have specific roles of relevance in bone defect healing, and it has lower crystallinity with disordered nanostructures providing unique chemical and physical properties [10,11,14]. New biological sources are being investigated seeking to guarantee sustainability. One such approach is exploring the potential of the marine environment, especially discards from the fishing industry.

Chondroitin sulfate (CS) is a sulfated polysaccharide of the glycosaminoglycan family formed by alternating β 1-3 and β 1-4 glycosidic bonds between glucuronic acid (GlcA) and N-acetyl galactosamine (GalNAc). The basic building block of the polymer is the disaccharide thus formed, which can be sulfated at different positions on both rings. In animals, CS participates as regulator of functional proteins, either at the cell surface or in the extracellular matrix [15], being consequently involved in important processes such as viral infection, metastasis and neurite outgrowth [16]. As a result, CS has found

applications as pharmaceutical [17] and nutraceutical [18], and is actively researched in tissue engineering [19]

The CS ability to interact with proteins appears to be influenced by charge density [20], mainly driven by sulfate groups [21]. In this regard, CS from cartilaginous fish and terrestrial sources can be considered different, as charge density is generally higher in fish CS. This is due to the presence of disulfated units in fish CS and a lower percentage of unsulfated units than in bovine and porcine CS. Although disaccharide composition varies considerably across fish species and also among tissues within the same species [22,23], prevalent sulfation in CS from cartilaginous fish generally occurs in position 6 of GalNAc, while position 4 is the most common in terrestrial sources [24].

Bioapatite and CS originate from distinct shark tissues. CS is a fundamental constituent of cartilage and is mainly extracted from shark heads [25], while bioapatite occurs in hard tissues such as vertebrae and teeth. The latter are especially valuable, as dentine and enameloid present in teeth are promising biomaterials for bone regeneration [7,26,27]. Their obtention implies, as a first step, removal of the jaws from the head to subsequently extract the teeth. The process leaves the remaining jaw as a by-product, yet this material still holds potential for further valorization. With this aim, in the present work we characterize crude blue shark jaws to identify potential HA and CS as valorization products. Both biomaterials are isolated and purified, to the best of our knowledge, for the first time in shark jaws. The isolation is carried out following sustainability principles to minimize our impact in the marine environment. An optimized process in sequential steps is proposed by combining enzymatic proteolysis, chemical precipitation and membrane purification, together with controlled thermal treatment for hydroxyapatite purification. Enzymatic deproteinization was implemented in an environmental friendly method against corrosive alkali following operation at optima parameters previously defined for blue shark CS [4].

2. Materials and Methods

2.1 Raw material preparation and chemical analysis

Heads by-products of blue shark (*Prionace glauca*) were boiled for 2 h and jaws separated manually, the remaining muscle was used for the production of fish protein hydrolysates [28] and teeth were removed to produce calcium phosphates [5]. The clean jaws were used for structural and chemical characterization as described below. Further samples of jaws were milled and homogenized at ~1-5 mm size using a grinder.

Proximate composition of crude and dried jaws (generated in a laboratory oven at 60 °C for 24 h) was assessed by determination of moisture, organic matter and ash content [29]. Fat concentration, total nitrogen and total protein as total nitrogen $\times$ 6.25 were also determined in various cases [29]. Total carbohydrate content was estimated by subtracting protein, fat, ash and moisture from total sample weight. This value of carbohydrates was confirmed by total sugar determination [30] after exhaustive enzymatic proteolysis of cartilage. Samples from the different purification steps were analysed to quantify total soluble proteins (Pr) [31], and CS concentration as glucuronic acid [32] according to the mathematical modifications reported in [33]. CS purity index (Ip), defined as $Ip (\%) = CS \times 100 / (CS + Pr)$, was also calculated in all purification steps.

2.2 Purification of biomolecule and biomaterial from blue shark jaws

The procedure applied for the valorization of jaws, to recover and purify bioapatite and chondroitin sulfate, followed the previously steps optimized for head cartilages of blue shark [34], but with slight modifications for the present work. The optimized flow diagram is presented in Figure 1, where the route for the obtaining of only purified bioapatite, from the direct thermal processing of the crude jaw, has been also included (named as calcium phosphate 1, CaP1). Going into detail with the procedure, 250-300 g of milled jaws were mixed with 750-900 mL of water and the mixture was hydrolyzed, in a 2L-reactor, for 8 h with Alcalase 2.4L from *Bacillus licheniformis* (Novozyme Nordisk, Bagsvaerd, Denmark) at a concentration of 1 % (v/w of initial substrate), pH 8.2, temperature of 56 °C and agitation of 200 rpm. After jaw digestion, the centrifugation (6000 g / 20 min) of the hydrolysate led to a sediment, non-hydrolysable material (NHM), and a supernatant rich in proteins and chondroitin sulfate (CS) (enzyme hydrolysate (EH) in Figure 1). NHM was washed and filtered (using Whatman filter N°4) and the obtained mineral material was thermally processed as described below to obtain the named CaP2. The supernatant (EH) was treated with a hydroalcoholic-alkaline-saline solution (0.54 M NaOH, 1.17 volumes of EtOH and 2.5 g/L of NaCl) for 2 h for the selective precipitation of CS. The impure CS recovered after centrifugation (6000 g / 20 min) was redissolved in water and neutralized with 6M HCl. Then, CS was purified by ultrafiltration (UF) membrane of 30 kDa employing a first step of concentration (by 2-folds) and a subsequent stage of diafiltration (DF), applying 5 diavolumes of distilled water for the elimination of low proteins and

peptides. Finally, purified solutions of CS obtained from DF retentates were dried by lab oven at 60 °C for 48 h. This complete protocol was carried out in duplicate.

Figure 1. Flowchart of the valorization steps executed for the recovery and purification of valuable biomaterials from jaw of blue shark. SUP: supernatant, SED: sediment, RET: retentate and PER: permeate.

2.3 Structural and chemical characterization of shark jaws

The crude mineral fraction obtained as a solid after subjecting the dried jaws to the enzyme hydrolysis combined with the separation by centrifugation (non-hydrolysable material NHM) was calcined at 950 °C in a controlled thermal treatment for 12 h with heating ramp of 2 °C / min and cooling ramp of 20 °C / min. The same process was directly performed on dried jaws. Unprocessed samples of crude jaw in cross section, the ones obtained after direct thermal treatment (CaP1) and the pyrolyzed powder from the NHM fraction (CaP2) were characterized in depth as follows: The morphology and elemental composition by scanning electron microscopy (SEM, JEOL JSM-6700F) and X-ray analysis by electron dispersive spectroscopy (EDS, Oxford Inca Energy 300). The evaluation of functional groups was performed by an FT-Raman B&WTEK i-Raman-785S instrument equipped with a BAC 100 Probe (785 nm) and structural measurements by X-Ray Diffraction (XRD) on a Siemens D-5000 Diffractometer.

2.4 Molecular weight and sulfation of CS

Molecular weight of CS was determined as previously described by gel permeation chromatography [35]. Briefly, 100 µl of CS at 1 g/L were separated with a set of Suprema columns (PSS, Germany) - 30 Å, 100 Å and Ultrahigh – eluted with 0.1 M NaN₃: 0.01M NaH₂PO₄, pH 6.6 on an Agilent 1260 equipped with refractive index and dual angle light scattering detectors. Refractive index increments (dn/dc) were calculated from the RI detector signal. Chemical composition was initially evaluated by ¹H NMR on a Bruker DPX 600 at 600 MHz and 10 °C at 1 g/L in D₂O. Spectra were processed with MestReNova 10.0.2 software (Mestrelab Research, Spain). Detailed disaccharide composition was determined by strong anion exchange (SAX) chromatography as previously described [35]. Briefly, CS was digested with chondroitinase ABC (Prod. No. C2905, Sigma-Aldrich) at 0.2 U mg⁻¹ of CS in 0.05 M Tris-HCl:0.15M sodium acetate pH 8 at 37 °C for 24 h, the enzyme inactivated, samples centrifugated and the supernatant injected (20 µL) onto an Agilent 1200 HPLC equipped with a Waters Spherisorb SAX column (5 µm, 4.6 x 250 mm). Elution was performed at 1.5 mL min⁻¹ in isocratic mode from 0 to 5 min with 50 mM NaCl pH 4 and then in linear gradient from 5 to 25 min, starting with 50 mM NaCl pH 4 and ending with 76 % 50 mM NaCl pH 4 and 24 % 1.2 M NaCl pH 4. Disaccharides were detected at 232 nm and quantified by external calibration made with unsaturated standards (Grampenz, UK).

3. Results and Discussion

3.1 Shark jawbone: descriptive compositional analysis

Crude blue shark jawbone appearance (including the teeth), once the soft tissue has been removed, is presented in Figure 2(a), together with a cut performed on the jaw structure (b). The last image (b) revealed the sandwich-type structure of the jaw in section formed by alternate layers of two different compositions, named at this work as ZONE1 (the darker) and as ZONE2 (the lighter ones) (b). At the same time it can also be observed as the inner structure of the jawbone acts also as a reservoir of dental tissue (white arrow).

Figure 2. Optical image of entire crude jawbone of *Prionace glauca* (a) and a detail of the jaw in section (b) where a sandwich-type structure with alternate layers (ZONE1 and ZONE2) and the reservoir of dental tissue (white arrow) can be observed. The white square in (a) indicates the area where image (b) was taken.

These jawbone samples have been subjected to SEM and EDS analysis to elucidate the elemental composition of each layer (ZONE1, ZONE2). At Figure 3 the backscattered electrons (BSE) detector was used to obtain a micrograph of the jawbone cross section. Using this mode the obtained micrograph showed the variations in atomic number of the elements, with lighter tones for ZONE2 (heavier elements) and darker for ZONE 1. This qualitative indication of the different composition of the two layers (ZONE1, ZONE2) was confirmed with the elemental composition by EDS corresponding measurements (shown in Figure 3) with: a clear prevalence in mineralized composition for ZONE2 (measured on the whitest area) based in Ca and P (calcium phosphate), with presence of C of organic material and low relative intensity of Na and Cl from marine salts; in the

case of ZONE1 the major composition is constituted by C with the presence in relevant relative intensity of S, and a lower presence of Ca and P with, again, marine salts. In both ZONES the presence of O was detected (as it forms part of both organic and inorganic contributions) and Mg in a very lower proportion.

Figure 3. Electron scanning microscopy (backscattered electrons mode) of the jaw section in the sandwich type morphology where ZONE1 and ZONE2 are clearly delimited (left). EDS spectra of both zones are also presented (right).

The organic and inorganic contribution was globally quantified with the determination of moisture, organic matter and ash content for the entire crude jaws, obtaining an average ($\pm$ confidence interval for $\alpha = 0.05$) of 98.04 ± 2.12 %, 1.48 ± 0.22 % and 0.47 ± 0.04 % for moisture, organic matter and ashes, respectively. Dried jaws (in oven at 60 °C) reached levels of 14.10 ± 1.01 % for moisture, 63.33 ± 1.15 % for organic material and 22.56 ± 2.01 % for ashes (inorganic material). Chemical composition of jaws, calculated as % of dry weight, results in 39.1 ± 1.6 % ash, 50.8 ± 2.1 % protein, 0.5 ± 0.0 % fat and 9.6 ± 0.8 % carbohydrates. Cartilage from other chondrichthyes fish has shown similar composition [36].

To deepen on this hybrid organic-inorganic composition, the crude unaltered jawbone was subjected to a thermal treatment (950 °C) to check the expected removal of the organic layers. The morphology of the original sandwich-type section after the thermal treatment is presented in Figure 4, by both scanning electron (a, b) and back scattered electron (c) microscopies. The elemental composition by EDS is also shown (d). At the

SEM micrograph the calcined section (a) can be clearly differentiated from the adhesive of carbon, the metallic holder and the silver drop routinely used for the SEM analysis of samples. In higher magnification (b) a globular morphology of the material, typical from a calcium phosphate, can be observed. The section observed by backscattered electrons revealed a uniform composition for the material in a unique layer, in a light tone (lighter than the adhesive of carbon, the aluminum holder and the silver drop) which corresponds to heavier elements. The EDS analysis (d) confirmed the major contribution of Ca and P, a higher relative intensity of Na and Mg (given the removal of the organic material), and the retention of part of sulfur. This make us to conclude that the ZONE1, with most organic material, has been removed with the thermal processing (as expected) and that certain amount of S from the organic material has been retained in the mineralized part, together with the Na and Mg.

Figure 4. Electron scanning microscopy of the pyrolyzed jaw in the same sandwich type morphology section (a), in higher magnification (b), the same section using backscattered electron mode (c) and EDS spectra of the pyrolyzed sample (d).

The previous EDS spectra were reinforced, for both the crude jawbone (in ZONE1 and ZONE2) and the pyrolyzed one, by the analysis of bond configuration through FT-Raman spectroscopy. Figure 5 shows the three spectra obtained, with presence of peaks

in ZONE1 at 1663, 1450 and 1268 cm^{-1} attributed to the organic material-related bonds of amide I, $\text{CH}_{2(\text{def})}$ and amide III respectively [37]. The same peaks were detected in ZONE2 but with lower intensity. The same occurred with the intense peaks at 757 cm^{-1} and 1003 cm^{-1} attributed to amino acids such as tryptophan and phenylalanine respectively [38]. The peaks at 1240, 1066, 940 cm^{-1} detected in ZONE1 correspond to the bonds SO_3^- , OSO_3^- and C-O-C of the molecule chondroitin sulfate with major prevalence of isomer 6S [24]. These peaks were not detected in ZONE2. Moreover, in ZONE2 spectrum the band of higher intensity was detected at 961 cm^{-1} , which is the attributed to PO_4^{3-} *sym stretch* from hydroxyapatite. A mineral phase contribution can also be present in ZONE1 with the broad band at 940 cm^{-1} being designated as vibrational band at 948 cm^{-1} for PO_4^{3-} *sym stretch* of monophasic β -TCP [5]. Finally, at the thermal processed jawbone spectrum the absence of organic material-related bonds was confirmed. The intense and narrow band at 963 cm^{-1} confirmed the presence of PO_4^{3-} *sym stretch* bonds as hydroxyapatite as well as the peaks at 1079 and 1048 cm^{-1} for PO_4^{3-} *asym stretch*, 433 and 589 cm^{-1} for *sym bending* and at 613 cm^{-1} for *asym bending* [5,39].

Figure 5. Raman spectra of both *P. glauca* jaw sandwich-type morphology layers (ZONE1, ZONE2) and the material after thermal treatment (pyrolyzed jaw).

After the descriptive structural and chemical analysis of *P. glauca* jaw, the high potential of this fishing by-product as a source of both chondroitin sulfate (CS) and

calcium phosphate is clear. In the case of the sulfated glycosaminoglycan (GAG) the finding of a new sustainable source of this biomolecule and, moreover, appearing it with prevalence in isomer CS-C (6-sulfation) is of great interest. The obtention of CS with prevalence of 6 sulfation position in substantial yields is rare in common and sustainable sources. This isomeric form has been recently described for fish by-products such as small-spotted catshark (*Scyliorhinus canicula*, wholebody), blue shark (*Prionace glauca*, head), and ray (*Raja clavata*, skeleton) [23,25]. It is the first time that CS has been documented in the *P. glauca* jawbone, concentrated in alternating layers to the calcium phosphate ones. Its interest is related to the proved CS-based hydrogels promotion of 3D chondrogenesis [40] and the providing of biomechanical properties on numerous connective tissues such as cartilage, ligaments and tendons. In fact, and in relation to the isomeric form found at the present work, a significant negative correlation between levels of C6S and age has been described in human tissues as well as a diminished ratio of C6S/C4S in synovial fluid and the cartilage of patients affected by osteoarthritis in advanced and terminal stages compared with early ones [24]. The obtaining of porous calcium phosphates from biological origin is also of interest for bone tissue regeneration purposes, as previously mentioned. The thermal treatment applied of 950 °C for 12 h was proven to be the optimal for the entire removal of organic material (Figure 5) required for the use of these ceramic materials from biological origin. Given the interest of both biomaterials, an extraction method has been applied at the present work for the obtaining of purified CS and calcium phosphate from the *P. glauca* jaw.

3.2 Extraction method for purification of CS and CaP

As previously mentioned at the optimized flow diagram (Figure 1) proposed, the shark jaws were initially subjected to an enzyme hydrolysis combined with the separation by centrifugation. As result of this, a liquid enzymatic hydrolysate with high content in proteins and glycosaminoglycans, mainly CS, and a solid NHM (non-hydrolysable material) used for the extraction of calcium phosphate, were obtained. The amount of fresh NHM recovered was 26.8 ± 2.0 % (w/w of jaw), being the mineral material after washing, filtration and drying of 10.7 ± 0.6 % (w/w of jaw). This material was utilized, as substrate, for the production of CaP. The purity in CS of enzyme hydrolysates (EH) showed an average level of Ip (purity index) = 37.4 ± 2.4 %. From the selective precipitation of CS by chemical treatment this parameter was increased up to Ip = 80.7

± 3.0 %. The final dried sample of CS, after membrane purification, had a chemical composition of 8.7 ± 0.8 %, 97.1 ± 0.4 % and 76.5 ± 0.4 % for moisture, CS purity as Ip value and CS richness, respectively. In view of these percentages, the present CS could be a potential candidate as ingredient of nutraceutical and pharma formulations for joint disease treatments and regeneration of cartilage [16]. In addition, the yields of dried CS isolated after purification steps were of 3.41 ± 0.27 % (w/wet weight jaw). This value was lower than observed for CS from small-spotted catshark (4.8 % w/w of wet head cartilage) and blackmouth catshark (3.7 % w/w of wet head cartilage) but higher for CS recovered from rabbit fish cartilage [34,35,36]. The molecular weight of CS from *P. glauca* jaw was estimated at 66.8 kDa (number average molecular weight) with an associated polydispersity index of 1.224. This value is similar to values previously reported for the whole head (60 kDa) [23] [25] and in hybrid chondroitin sulfate/dermatan sulfates chain in the skin of this shark species (70 kDa) [41].

The GPC eluogram in Figure 6 shows the polymeric peak of CS at 22.03 min in the refractive index (RI) signal. A secondary small peak at lower elution times appears in the light scattering signals but is not visible in the RI detector. This indicates higher molecular weight species than the main CS peak at low concentration, possibly due to aggregation of CS chains. Such aggregates have been reported in other polyelectrolytes [42,43,44]. Composition of purified CS was initially assessed by ^1H NMR spectroscopy (Figure 6), with characteristic CS signals appearing between 3 and 5 ppm, except the acetyl group of GalNAc, which appears at 2.06 ppm. Additional signals typical of aliphatic (0.5-1.5ppm) and aromatic (7.0-8.5 ppm) aminoacids are largely absent, indicating the suitability of the purification procedure to remove protein. A small quantity of keratan sulfate (KS) might remain as a contaminant in the CS sample, as evidenced by the signal at 4.71 ppm corresponding to the anomeric carbon (H1) of N-acetyl glucosamine [45]. This possible contamination may be estimated at 1 % KS by comparing the intensity of this signal to that of GalNAc 4S (H4) at 4.79 ppm, based on the percentage that sulfation at position 4 of GalNAc represents of the total disaccharides. Such percentage can be calculate by relating the intensity of the signal at 4.79 ppm to that of the acetyl group of GalNAc at 2.06-2.08 ppm [46], resulting in 7.5 % GalNAc 4S. Determination of sulfation in other positions is difficult because of signal overlap, but the information provided by NMR can be complemented by SAX-HPLC after enzymatic hydrolysis. Using this method, sulfation at position 4 of GalNAc

Figure 6. FT-Characterization of CS from shark jaw. Top left: Gel Permeation Chromatography (GPC) eluograms (RALS: right angle light scattering; LALS: lowangle light scattering; RI: refractive index); Top right: SAX-HPLC chromatograms (O: Δ UA-GalNAc; A: Δ UA-GalNAc(4S); C: Δ UA-GalNAc(6S); D: Δ UA(2S)-GalNAc(6S); E: Δ UA-GalNAc(4,6S); B: Δ UA(2S)-GalNAc(4S)); Bottom: ^1H NMR spectrum in D_2O at 10°C .

accounts for 13 % of the total disaccharide units in CS, slightly higher than 7.5 % estimated by NMR. This discrepancy may be partially due to the contribution to the signal at 2.06-2.08 ppm of other GAGs containing GalNAc or GlcNAc in their structures, such as KS. Besides 4S GalNAc, SAX-HPLC analysis reveals predominant

sulfation at position 6 of GalNAc at 68 %, while non-sulfated disaccharides only comprise 7 %. Disulfated units are mainly represented by D-units (GlcA2S-GalNAc6S) at 11 %, as other disulfated disaccharides are only present in minute amounts (close to 1 %). The results are quite similar to those reported for CS isolated from the whole head of *P. glauca* (10 % CS-A, 64 % CS-C, 16 % CS-O, 9 % CS-D). The main difference is the slightly higher percentage of sulfated units in jaw CS and about only half of the unsulfated units found in head CS [25].

In parallel to the CS purification mentioned above, thermal treatment up to 950 °C with controlled heat and cooling ramps was carried out on the solid NHM (non-hydrolysable material) sample for the purification of the mineral part. At Figure 7 the porous microstructure of pyrolyzed jawbone in cross section is showed (a-c) with porosity uniformly distributed for the entire sample (a) in a range of mean sizes of 80-150 μm and with the presence of merged pores up to $\approx 250 \mu\text{m}$ in the longest axis is shown (b). A pore in detail can be observed at micrograph (c). These pore sizes are within the appropriate ranges recommended for biomedical applications LeGeres, 2002; Tatay-Diaz, 2008; Mour, 2010 [45,46,47] [47,48,49]. This innate porosity will be complemented with intergranular pores after the milling of the jaw. Morphology of solid NHM fraction before (d,e) and after thermal treatment (f,g) is also presented. The porosity of the solid NHM fraction was clearly observed before the thermal treatment (e) together with change in color (d) to whiter tones (f) of the fraction after thermal treatment. Finally, the EDS spectrum of crude jaw ZONE1 is included (h) together with the one of the solid NHM before (i) and after thermal treatment (j). After the removal of CS (i), C and O signals were detected at the same levels of Ca and P (h). As it was expected relative intensity of S was also diminished. Once NHM was subjected to the thermal treatment (j), the intensity of C detection was drastically diminished (as desired) as well as O, this latter with lower intensity to levels of Ca and P. The detection of Mg increases at this step as consequence of the purification process, being registered at the same level of Na. Sulfur was detected but in an intensity even lower than before the thermal treatment (i).

Figure 7. SEM micrographs of the pyrolyzed jaw material in cross section at different magnifications (a–c) where porosity of the material can be observed. Micrographs of NHM fraction before (d, e) and after thermal treatment (f, g) are also presented together with EDS spectra of crude jaw in ZONE1 (h), NHM before (i) and after thermal treatment (j).

The bonding configuration of this purified mineral fraction was characterized by Raman spectroscopy (Figure 8), where spectrum obtained for the NHM fraction before thermal treatment is also presented. The NHM spectrum before thermal treatment validates the efficiency of the enzymatic hydrolysis for the separation of inorganic-organic phases as presence peaks at 1663, 1450 and 1268 cm^{-1} respectively attributed to the organic material-related bonds of amide I, $\text{CH}_{2(\text{def})}$ and amide III were not detected or detected in an extremely low intensity together with the ones related to the amino acids such as tryptophan and phenylalanine at 757 cm^{-1} and 1003 cm^{-1} respectively, which intense peaks were detected at the CaP-based ZONE2 layer in crude jaw (Figure 5). As expected peaks at 1240, 1066 and 940 cm^{-1} corresponding to the bonds SO_3^- , OSO_3^- and C-O-C of the molecule chondroitin sulfate are also absent, and an intense peak at 963 attributed to PO_4^{3-} *sym stretch* (ν_1) of calcium phosphates was clearly detected. This vibration appears at the spectrum of the purified mineral fraction (*P. glauca* pyrolyzed CS-free jaw) in position 965 cm^{-1} with a shoulder in 975 cm^{-1} (moved from positions 961 cm^{-1} in crude jaw and 963 cm^{-1} in NHM fraction) as a very intense and sharp band.

Figure 8. Raman spectra of *P. glauca* jaw NHM fraction before and after thermal treatment.

Going into detail within calcium phosphates, this vibration is refereed in literature Kotsopoulos, 2002 [37][39] at 961 cm^{-1} for hydroxyapatite and at 970 cm^{-1} with a shoulder at 961 cm^{-1} in case of tricalcium phosphate. Other authors Aguiar, 2018 [5] [5] assign the vibration $\text{PO}_4^{-3}(\nu_1)$ at 962 cm^{-1} and 956 cm^{-1} for hydroxyapatite and 972 cm^{-1} , $958\text{--}968\text{ cm}^{-1}$ and 948 cm^{-1} for β -TCP. Having into account the displacement of the band, it seems that after the enzyme hydrolysis process and the thermal treatment, the initial hydroxyapatite becomes into a biphasic material with a certain contribution of tricalcium phosphate or other variations such as whitlockite (presence of Mg). The other vibrations attributed to $\text{PO}_4^{-3} \text{ asym stretch } (\nu_3)$ of hydroxyapatite at 1079 , 1050 cm^{-1} and 1044 cm^{-1} , $\text{PO}_4^{-3} \text{ bend } (\nu_4)$ at 611 and 591 cm^{-1} of hydroxyapatite and, finally, $\text{PO}_4^{-3} \text{ bend } (\nu_2)$ of hydroxyapatite at 449 and 433 cm^{-1} are also detected.

The structure of the NHM fraction after thermal treatment was confirmed with XRD analysis (named as CaP2), compared to the one obtained from the direct thermal treatment of the crude jaw (avoiding the enzymatic hydrolysis for the extraction of CS, named as CaP1). Both diffraction patterns are shown in Figure 9 with peaks at CaP2 pattern located at positions 25.91° , 31.86° , 32.23° , 33.01° and 34.10° characteristic of hydroxyapatite $[\text{Ca}_5(\text{PO}_4)_3(\text{OH})]$ corresponding to the diffraction planes (0,0,2),

(1,2,1), (1,1,2), (3,0,0) and (2,0,2) (5Aguiar et al 2018) and a contribution of β -TCP ($\text{Ca}_3\text{O}_8\text{P}_2$) or other similar structures (isomorphisms) such as whitlockite [$\text{Ca}_9\text{Mg}(\text{PO}_3\text{OH})(\text{PO}_4)_6$], given the presence of Mg in the mineral composition as shown in EDS spectrum at Figure 7(j), at positions 28.04, 31.29 and 34.65 attributed to crystal planes (2,1,4) (0,2,10) and (2,2,0) respectively Aguiar, 2018; Tabares, 2013 [5, 48] [5,50]. XRD semi-quantitative estimation attributed the composition of CaP2 for HA in around a 60 % and whitlockite / β -TCP in around a 40 %. Diffraction peaks of CaP1 pattern were mainly detected in typical positions (25.94 °, 31.87 °, 32.26 °, 33.00 °, 34.13 °) for crystal planes of hexagonal hydroxyapatite ((0,0,2), (1,2,1), (1,1,2), (3,0,0) and (2,0,2) respectively) (JCPDS No. 09-0432). A slight contribution of whitlockite/ β -TCP was also detected at position 28.14 ° and 34.65 ° respectively attributed to the planes (2,1,4) and (2,2,0). Potential small contribution of Na as NaCaPO_4 (rhenanite) can be observed in both amplified patterns (Figure 9b) in the range of positions from 30 ° to 35 °, which comes from the incorporation of Na^+ in the HA Hung, 2012 [53] [51]. At this case the XRD semi-quantitative estimation mainly attributed the composition to HA (around an 85 %).

Therefore, as for CS, the mineral phase has been purified in the form of biphasic calcium phosphate of HA and β -TCP / whitlockite. The contribution of β -TCP / whitlockite increases when the crude jaw is previously subjected to an enzymatic hydrolysis, this process causes changes in the material that are reflected at a structural level after the heat treatment. In addition, the presence of sulfur in the mineral fraction directly obtained from the thermal treatment on the crude jaw and the presence of sodium and magnesium in both mineral fractions (essential elements with role in bone metabolism) were also proven, being the latter incorporated into the crystalline network as whitlockite. A range in the mineral fractions can be then obtained to cover clinical requirements of highly stable calcium phosphates (mostly based on HA) or others where the bioceramic must provide a certain degree of resorbability (relevant presence of β -TCP/whitlockite) Tataya-Diaz, 2008;Fernandez-Herandez, 2017; Giannoudis, 2005; Chiarello, 2005; Cook, 2009 [48,51,52,53,54] [48,52,53,54,55].

4. Conclusions

Crude blue shark jawbone was revealed to be organized in a sandwich-type structure in cross section based on alternate layers. These layers presented different compositions

with ones enriched in content of Ca and P with presence of C against the others with prevalence in C content, relevant presence of S and low contribution of Ca and P. In both alternate layers the presence of O was quantified (as it forms part of both organic and inorganic contributions) as well as Mg in trace quantities and marine salts (Na and Cl). The inner cavity of the sandwich-type structure was also observed to act as the reservoir for new dental pieces. FT-Raman spectroscopy confirmed the elemental analysis with the most intense band detected at 961 cm^{-1} attributed to PO_4^{3-} *sym stretch* from hydroxyapatite [$\text{Ca}_5(\text{PO}_4)_3(\text{OH})$] in the mineralized layers (inorganic material globally quantified in a $23 \pm 2\%$ in dried jaw) and more intense bands attributed to the organic material-related bonds at the organic layers ($63 \pm 1\%$ in dried jaw), with peaks at 1663 , 1450 and 1268 cm^{-1} assigned to amide I, $\text{CH}_{2(\text{def})}$ and amide III respectively, and at 757 and 1003 cm^{-1} attributed to amino acids such as tryptophan and phenylalanine respectively. Moreover, at the organic layer specific peaks (absent at the mineral one) were detected at 1240 , 1066 , 940 cm^{-1} attributed to the bonds SO_3^- , OSO_3^- and C-O-C of the molecule chondroitin sulfate with major prevalence of isomer 6S. The integral valorization of both the hydroxyapatite and the chondroitin sulfate, is proposed at the present work and a recovery and purification process was applied in sequential steps combining enzymatic proteolysis, chemical precipitation and membrane purification, together with controlled thermal treatment at $950\text{ }^\circ\text{C}$ for hydroxyapatite purification. A purified biphasic (60:40) porous calcium phosphate with characteristic diffraction peaks of hydroxyapatite and of β -TCP ($\text{Ca}_3\text{O}_8\text{P}_2$) / whitlockite [$\text{Ca}_9\text{Mg}(\text{PO}_3\text{OH})(\text{PO}_4)_6$] was confirmed. A purified chondroitin sulfate with CS purity as Ip value of $97.10 \pm 0.39\%$ and CS richness of $76.5 \pm 0.39\%$, molecular weight of 67 kDa and predominant (68%) sulfation in position 6 of GalNAc was also confirmed. The required enzymatic hydrolysis combined with the separation by centrifugation was proven to promote changes in the crude mineral fraction that were reflected at a structural level after the thermal treatment. In fact, crude jaw directly subjected to the thermal treatment (avoiding the CS recovery) revealed a calcium phosphate with main contribution of hydroxyapatite (85%) and much lower of β -TCP/whitlockite. This finding opens the range in the mineral compositions that can be provided, from highly stable (HA) to resorbable calcium phosphates (relevant presence of β -TCP/whitlockite). On the other hand, the properties of the purified CS obtained situate it as a potential ingredient of nutraceutical and pharma formulations for joint disease treatments.

Conflict of interest

The authors declare no conflict of interest.

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