The Microstructure and Potential of Chondroitin Sulfate in Shark Cartilage Extract

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Abstract

Shark (*Prionace glauca*) cartilage is an industrial waste of the frozen fish industry in Sidoarjo city. Previous studies indicate that the cartilage contains bioactive compounds, glucosamine, and chondroitin, but the extracted product is unstable (easily melted during storage) and smelly ammonia. This study aims to obtain a stable extract product without the smell of ammonia by adding different absorbers. The experiment used a Complete Randomized Design on applying absorbers, HPMC, Cab-Osil, and Avicel PH 101. The parameters measured were the yield and the organoleptic of the shark's extract product. The microstructural observations used a Scanning Electron Microscope (SEM), while the chondroitin sulfate content analyses used high-performance liquid chromatography(HPLC). Results showed that adding different absorbers significantly influenced the yield and organoleptic of the shark's cartilage extract. The use of Avicel PH 101 (90%), Cab-Osil (9%), and Hydroxy Propyl Methyl Cellulose (HPMC) (1%) gave the highest amount of extract, as much as 5.02 g (16.73%). The organoleptic was without smelling salts, whitish beige color**,** and dry structure (stable). The microstructure of the shark's cartilage extract product at this treatment had a dense structure with a soft surface. In contrast, the extract without an absorber addition had a sharp, needle-like microstructure. The chondroitin sulfate content of the shark's cartilage extract product was the highest at this treatment.

Keywords: extraction, chondroitin sulfate, absorber, SEM, HPLC

Introduction

Sharks are one of the cartilaginous fishes that are recently highly exploited for their fins to be a healthy food menu, but the meat and bone are often discarded in the sea. The fish freezing industry in Sidoarjo processes the shark's meat as fish-based meatballs, sausages, and nuggets or sells to the catering services around Surabaya city. The shark's cartilage from the frozen fish company is not economically valuable and becomes waste. The unique history and life of sharks in the ocean have encouraged researchers to obtain beneficial bioactive compounds for humans. Tanna et al. (2020) found that shark cartilage holds substances that can inhibit the growth of new blood vessels needed for cancer to grow. It can also cure various diseases, such as antiangiogenic, immune-stimulator, and anti-inflammation (Safari & Hassan, 2020). Bio-compounds have directly or indirectly been marketed as therapy and prevention for various diseases, such as cancer and rheumatoid arthritis (RA).

The shark's cartilage contains several chemicals, such as protein (troponin-I, tetranectin protein), collagenase, cartilage-derived inhibitor (CDI), tissue inhibitor of metalloproteinases (TIMPs), glycoprotein, and glycosaminoglycan consisting of chondroitin sulphate-D, chondroitin-6-sulfate, and keratosulfate (Molaie et al., 2019). Glucosamine sulfate and chondroitin sulfate are promising therapeutic approaches for osteoarthritis (Jerosch, 2011). Ajeeshkumar et al. (2019) succeed in isolating and purifying proteoglycan from bramble shark's (*Echinorhinus brucus*) cartilage that has two unique peptides identical to the core-protein and epiphycan (protein-coding gene). This proteoglycan can control the inflammation modulator in monosodium iodoacetate (MIA)-induced OA rat, in which the C-reactive and uric acid levels are lowered. According to Bishnoi et al. (2016), chondroitin sulfate is a glycosaminoglycan compound in the cartilage and extracellular matrix. It is a natural substance composed of sulfated heteropolysaccharides. The sulfate group is covalently

bonded to the glucose molecule of glucuronic acid (GlcA) and N-acetyl-galactosamine (GalNAc). The structure of chondroitin sulfate is different depending on the source, number, and position of the sulfate groups (Li et al., 2016). For instance, the chondroitin sulfate extracted from the shark's cartilage is chondroitin-6-sulfate, while that from cow and pig is chondroitin-4-sulfate. It can treat joint ailments, anti-inflammatory, arthritis, atherosclerosis, cancer, and immunostimulant (Rakhmiyati et al., 2023).

Hanindika et al. (2014), who developed the extraction method to obtain the chondroitin sulfate from several cartilages, found 2.37% from sharks and 1.57% from rays, while squids do not contain chondroitin sulfate. Agustin et al. (2016) extract glucosamine from shark cartilage in 2M ammonium carbonate buffer solution, then freeze-dry, and obtain the extract flakes of 14.92%. The chondroitin sulfate extracted from the shark's cartilage in the acetic acid of pH 4.5 at 37°C for 7 hours, then dry-frozen, yielded 7.95% flakes. Glucosamine and chondroitin are unstable during storage, with a strong ammonia odor. Solihah et al. (2019) found the best extract at the application of 90% Avicel PH 101, 12.5% Cab Osil, and 1% HPMC with a yield of 67.32%, but the chondroitin sulfate of this extract product has not been tested yet and difficult to refine to be medicinal powder. The extract was also tricky to scrap from the freeze dryer because the use of the absorber concentration has not been optimal yet. The present study aims to obtain a stable extract product of the shark's cartilage, with no ammonia odor and high chondroitin sulfate content, by adding different absorbers.

Material and Methods

Material

The cartilage was taken from the blue shark *Prionoce glauca*, the most abundant shark species in the international trade (Druon et al., 2022) and has not been included in the IUCN Red List of Threatened Species. This species has faster reproduction than other sharks, with several 30-130 newborns (Nakano & Steven, 2008). The cartilage was collected from the fish processing company in Sidoarjo in frozen condition. It was brought to the Fisheries Processing Laboratory, Hang Tuah University, Surabaya, in a cool box. It was then thawed in running water and cleansed from meat remnants. The shark's cartilage was then stored at -20°C until extraction. The chemicals used to stabilize the extracted product were n-hexane (Merck, Germany) and absorbers, Avicel PH 101 (FMC Biopolymer, Philippine), Cab O Sil ® M-5 (Acros Organics, US), and HPMC 2910 (HPMC) 3 cps from ShinEtsu, Japan. All chemicals used in this study were pro-analyst quality.

Methods

Sample Preparation

Shark's cartilage preparation followed Solihah et al. (2019), in which the frozen cartilage was thawed in running water, cleaned of meat attached to the cartilage, and cut into small and thin pieces for easy drying. It was washed and drained for about 30 min., then dried at 50°C ± 2°C for six hours a day for two days. The dry cartilage was then blended and sieved through an 80-mesh sieve.

Before extraction, the cartilage was washed in n-hexane (1:10 b/v) for 1 hour while continuously stirred on a magnetic stirrer. The cartilage was then filtered and wind-dried at room temperature for a night. Extraction was done by dissolving the hexane-shark cartilage meal in the distilled water at the ratio of 1:10 (b/v) at 45°C ± 2°C at a continuous stirring for 8 hours on a hot plate magnetic stirrer and centrifuged at 4,000 rpm for 10 min. The supernatant was kept in an Erlenmeyer.

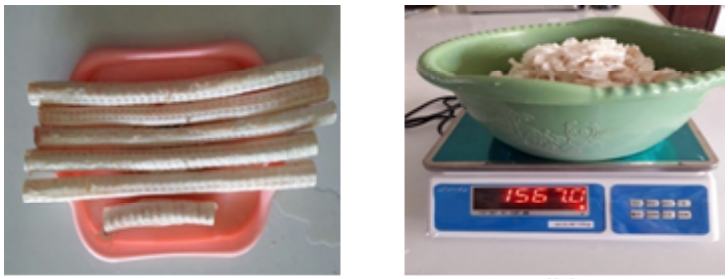
The supernatant was inserted into four freeze-dryer flasks, each of which contained 30 ml. Flask A was not added with an absorber, B was added with avicel PH 101 (90%), C was added with avicel PH 101 (90%) and Cab Osil 10%, and D was added with avicel PH 101 (90%), Cab Osil 9%, and HPMC 1%, then freeze-dried for 12 hours. The extract yield was calculated following the Health Department of Indonesia (2020):

$$\% \text{ yield} = \frac{\text{Extract weight (g)}}{\text{Sample weight (g)}} \times 100$$

The product with the highest yield and the best organoleptic was continued with chondroitin sulfate analysis using Shimadzu HPLC column C-18 (dimensions 4.6 x 250 mm, size of diameter pore 5 μm). The microstructure of shark cartilage extract product was observed using an FEI Inspect F50 Scanning Electron Microscope (SEM). The shark cartilage prepared for drying is given in Figure 1, and the cartilage meal in Figure 2.

Experimental Design

This study applied a Complete Randomized Design with four treatments, A without absorber), B (addition



(a) (b)

Figure 1. Original shark cartilage and small pieces of cartilage (a) Shark's cartilage is cleaned of meat attached to the cartilage, (b) Small pieces of shark cartilage before drying.

(a) (b)

Figure 2. (a) Shark cartilage meal, (b) Hexaned shark cartilage meal.

of Avicel 90%), C (addition of Avicel 90% and Cab Osil 10%), D (addition of Avicel 90%, Cab Osil 9%, and HPMC 1%), and six replications. The parameters measured were the yield and organoleptic properties of shark cartilage extract product.

The Organoleptic Test

The organoleptic test was done to know the physical characteristics of the extract product based on senses. Syukri (2018) gives a standard terminology of the organoleptic characteristics of the pharmaceutical powder (Table 1).

The shark cartilage extract product which has the best organoleptic properties continued with the analyses for chondroitin sulfate content and its microstructural observation.

Table 1. Terminology to describe the organoleptic properties of pharmaceutical powders

Color	Aroma	Taste	Texture
Pallor white	Sharp	Sour	Soft particle
Cream yellow	Sulfur	Bitter	Coarse particle
Brown	Fruit	Sweet	Dry
Glittering	Aromatic	Hot	Humid
	No smell	No taste	Delicate

Detection of Chondroitin Sulfate

The HPLC (High-Performance Liquid Chromatography) equipment was set covering the stabilizer, pump, reflective index detector (RID), modular, and Central Processing Unit (CPU) on the computer until the chromatogram showed a flat base (Rakhmiyati et al., 2023). The mobile phase used a potassium phosphate buffer solution of pH 3. The buffer solution was made by adding 1.36 g of potassium dihydrogen phosphate powder into 800 ml of distilled water and then added with phosphoric acid (H_3PO_4). The ratio between the potassium phosphate buffer and the acetonitrile was 99.5:0.5. The flow rate was 1 mL min^{-1}. The HPLC column used was C18 4.6×250 mm 5µm, Merck. The temperature in the column used was 28°C.

SEM Analysis

The SEM analysis was used to know the microstructure of the tablet samples, whereas imaging was carried out on a Carl Zeiss AG - SUPRA 35 VP SEM at an accelerating voltage of 1.0 keV (Kotar et al., 2015). Tablets were sliced with a razor blade and

attached to the SEM sample holder with double-sided adhesive carbon tape.

Data Analysis

Data were analyzed using one-way ANOVA followed by Least Significance Difference (LSD) test using IBM SPSS Version 25. One-factor analysis was conducted on the effects of adding absorber on the yield of shark cartilage extract product. The result is considered significant if the p-value is less than 0.05. The yield of shark cartilage extract product is given in mean value and standard deviations (Table 2).

Results and Discussion

Yield

Yield is the ratio of the extract and sample weights (Health Department of Indonesia, 2020). Yield content is closely related to the amount of bioactive compound in the raw material. The higher the yield is, the higher the bioactive compound possibly withdrawn from the raw material (Senduk et al., 2020). The mean yield of the shark cartilage extract product ranges from 7.57% to 16.73%. ANOVA showed that adding an absorber highly significantly affected the cartilage extract yield ($p<0.01$). The Post Hoc Test using the Least Significance Difference (LSD) indicated a highly significant difference between treatments ($p<0.01$).

Table 2 demonstrates that the highest yield is recorded in treatment D, with the addition of 90% Avicel, 9% Cab Osil, and 1% HPMC and the lowest in treatment A (without absorber). It could result that the shark cartilage extract is very hard to dredge from the freeze-drying flask without an absorber. The more types of absorbers are added, the higher the yield. The absorber can help increase the dryness of the extract so that it is easy to remove from the freeze-drying flask. The shark cartilage extract condition after freeze-drying for 18 hours is presented in Figure 3.

Table 2. The mean yield of shark cartilage extract product

Treatment	Mean Yield (g)	Percent
No aborber (A)	2.27 ± 0.23	7.57 ± 0.76a
Absorber Avicel 90% (B)	3.15 ± 0.17	10.50 ± 0.55b
Absorber Avicel 90% and Cab Osil 10% (C)	3.90 ± 0.40	12.99 ± 1.33c
Absorber Avicel 90%, Cab Osil 9% and HPMC 1% (D)	5.02 ± 0.37	16.73 ± 1.24d

Note: Different alphabets indicate significant differences.

Treatment A, after freeze-drying, yields a thin material that is difficult to dredge from the flask; treatment B, with an absorber of 90% Avicel, produces a slightly sticky material to the flask. In comparison, treatment (C) with the addition of 90% Avicel and 10% Cab Osil results in a thin material spread over the flask and is difficult to scrape from the flask. Treatment D (addition of 90% avicel, 9% cab osil, and 1% HPMC gives a porous material easily scraped from the flask. Kharisma et al. (2018) stated that Avicel PH 101 could increase the tablet's determination index, water absorbability, hardness, and friability, while Cab O Sil is silica oxide or aerosol silica mixed with water to have a viscous suspension (Cabot Corp, 2020). HPMC (hydroxypropylmethylcellulose) is an excipient used in pharmaceutical products due to its compaction ability to increase the mechanical strength of powder drugs or tablets (Dong et al., 2018). It is a non-ionic cellulose polymer utilized as a binder because it can absorb water (Zarmpi et al., 2020).

In the previous study, Solihah et al. (2019) added 12.5% Cab O Sil and obtained 4.37 g of shark cartilage extract, which is lower than the present study. It indicates that adding more Cab O Sil does not increase the yield because the extract is hard to dredge from the freeze-dryer flask after the freeze-dryer. Cab Osil is an excipient that can absorb water to increase the hardness of the end product (Sunkara & Capece, 2018). It is added in small amounts to the pharmacy product to increase the hardness of the drug tablet (Cabot Corp., 2015).

Organoleptic of Shark Cartilage Extract Product

The organoleptic analysis describes the color, aroma, and texture of the shark cartilage extract product through the senses.

Table 3 demonstrates that treatment D yields the best organoleptic of the shark cartilage extract product with a whitish cream color, neutral aroma, and dry texture. Adding 1% HPMC and 9% Cab O Sil in cartilage extract can increase product dryness. Adding 1% HPMC also eliminated the fishy smell of the product so that it could be an acceptable product. This finding is in agreement with Yan Li (2012) that shark cartilage extract powder has organoleptic properties, such as creamy white color, specific odor and hygroscopic. Larsson et al. (2017) said that HPMC is a polymer

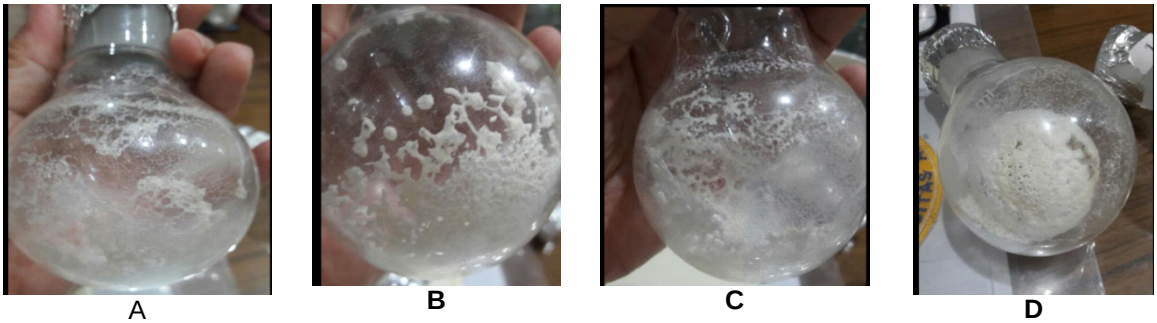


Figure 3. Freeze-drying cartilage. (A) no absorber; (B) absorber Avicel 90%; (C) absorber avicel 90% and cab osil 10%; (D) absorber avicel 90%, cab osil 9% and HPMC 1%.

Table 3. The organoleptic of shark cartilage extract product

Treatment	Color	Aroma	Texture
A	light brown cream	fishy	humid
B	cream	Slightly fishy	Slightly humid
C	cream	neutral	Slightly dry
D	Whitish cream	neutral	dry

with strong binding power, so the addition of HPMC can activate the binding power and the polymer strength, while Cab O Sil is an excipient that can increase the activation and the compactness, and makes the extract be easily scrapped from the freeze dryer (Cabot Corp, 2015). According to Zheng et al. (2021), HPMC is a cellulose polymer often used for food additives as a thickener, emulsifier, and excipient in pharmaceutical preparation to increase solubility and control drug delivery.

Chondroitin Sulfate Content

The chondroitin sulfate content of the shark cartilage was analyzed using HPLC in the Indonesian National Accreditation Committee-standardized commercial laboratory. The selected chondroitin sulfate is indicated by the treatment yielding the highest content (Table 4).

Table 4. Chondroitin sulfate content

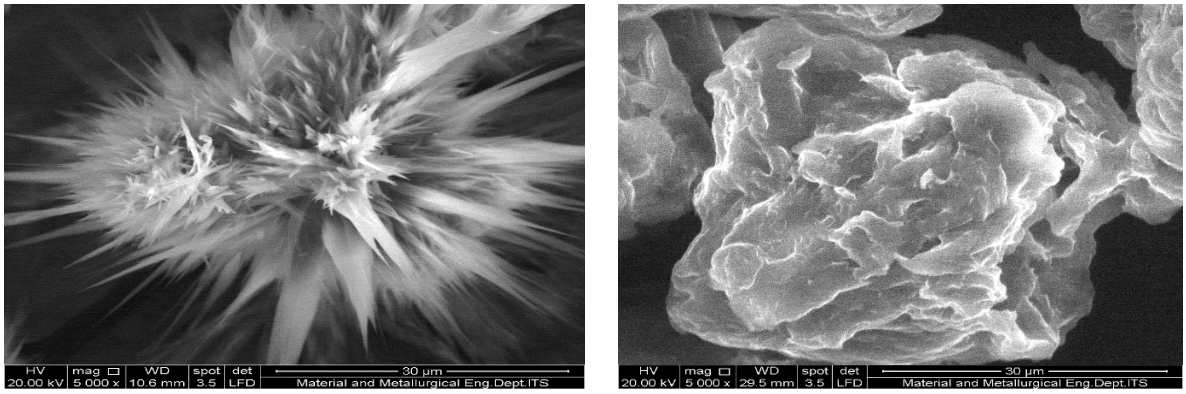
No	Type of samples	Chondroitin sulfate
1	Fresh cartilage	0.49%
2	Cartilage meal	0.34%
3	Water-extracted shark cartilage	0.12%
4	Shark cartilage extract product	2.62%

Table 4 exhibits that the highest chondroitin content is recorded in the cartilage extract added with an absorber and freeze-dried, 2.62%. It is higher than the chondroitin content extracted by Hanindika et al. (2014) using several chemicals. Therefore, adding an absorber and drying could increase the chondroitin content of the shark cartilage. However, Xie et al. (2014) found as much as 88.4% chondroitin sulfate enzymatically extracted from the shark cartilage and purification through column chromatography. It indicates that several extraction procedures are needed to maximize the extract quality.

Shark Cartilage Extract Microstructure

The microstructure characterization using a Scanning Electron Microscope found that the best treatment was recorded in the addition of Avcel PH 101 (90%), Cab Osil (9%), and HPMC (1%). The microstructure of the shark cartilage extract is presented in Figure 4.

Figure 4 shows that the microstructure of shark cartilage extract without an absorber is different from that with an absorber addition. Figure 3b indicates that the extracted product added with an absorber (avicel 90%, Cab Osil 9%, and HPMC 1%) is dense with a smooth surface since the absorber absorbs the water on the extracted product. Figure 3a shows a needle-like sharp microstructure. This condition makes the extracted product unstable and can potentially absorb the surrounding water vapor during storage. According to Beretzky et al. (2002), the flowing ability and deformability of the material are essential in tablet production. The needle-shaped powder has bad compression, while a round-shaped powder can increase the flowing ability of the particle and reduce the porosity. A symmetrically crystal-shaped microstructure has better compaction ability and flow



(a) (b)

Figure 4. Shark cartilage microstructure at 5000 x enlargement. (a) shark cartilage extract without an absorber, and (b) shark cartilage extract product with an absorber.

properties than a needle-shaped one (Kotar et al., 2015). It means that the use of an absorber could improve the extract quality, especially concerning the use of cartilage for drug development.

Conclusions

The combination of absorber avicel 90%, Cab O Sil 9%, and HPMC 1% can increase the yield and quality of shark cartilage extract product. An absorber could increase the chondroitin sulfate content from 0.49% in the fresh cartilage to 2,62% on shark cartilage extract powder. The organoleptic properties of the shark cartilage extract product is categorized as very good, with light cream color, neutral aroma, and dry texture. The microstructure of shark cartilage extract product, with the addition of an absorber, produced a dense structure with a smooth surface so that it is stable during storage.

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Supplementary Materials

Supplementary materials is not available for this article.

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