Turkish Journal of Agriculture - Food Science and Technology, 10(8): 1334-1341, 2022
DOI: https://doi.org/10.24925/turjaf.v10i8.1334-1341.5318

Turkish Journal of Agriculture - Food Science and Technology

Available online, ISSN: 2148-127X | www.agrifoodscience.com | Turkish Science and Technology Publishing (TURSTEP)

Isolation of Biopolymers from Sustainable Sources and Purification Steps for Biomaterial Applications

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ARTICLE INFO

Research Article

Received : 10/06/2022
Accepted : 13/07/2022

Keywords:
Silk fibroin
Collagen
Sodium Alginate
Aloe Vera
Sustainable polymers

ABSTRACT

In the study carried out, obtaining environmentally friendly biopolymers from sustainable sources and their usability as biomaterials were investigated. For this purpose, collagen from bovine achilles tendon, fibroin from silkworm cocoon, sodium alginate from brown sea algae and bioactive components from gel of aloe vera were isolated and purified. Product efficiency were calculated as 79.8% (w/w), 69,49% (w/w from cocoons), 35.1% (w/w) and 1% (w/v dry weight in gel) for collagen, fibroin, sodium alginate and aloe vera, respectively. Tissue scaffolds were prepared from these biomolecules by freeze drying method. However, aloe vera gel could not maintain the structural integrity in solid form and could not form a 3-dimensional scaffold. FTIR analyzes of fibroin, collagen and sodium alginate scaffolds showed that the products were obtained pure and the chemical structure was preserved during lyophilization. Surface analyzes with SEM, on the other hand, supported that the scaffolds are suitable for tissue engineering applications. As a result, it was determined that bioactive polymers were obtained from sustainable sources, generally at room conditions, with high yield, instead of petroleum-derived polymers, and they could be used as biomaterials. Obtaining biomolecules from sustainable sources in this way has significant potential in solving both the raw material problem and the environmental pollution caused by polymers.

Türk Tarım – Gıda Bilim ve Teknoloji Dergisi, 10(8): 1334-1341, 2022

Biyomalzeme Uygulamaları İçin Sürdürülebilir Kaynaklardan Biyopolimerlerin İzolasyonu ve Saflaştırma Adımları

MAKALE BİLGİSİ

Araştırma Makalesi

Geliş : 10/06/2022
Kabul : 13/07/2022

Anahtar Kelimeler:
İpek fibroin
Kollajen
Sodyum Aljinat
Aloe Vera
Sürdürülebilir polimerler

ÖZ

Yürütülen çalışmada sürdürülebilir kaynaklardan çevre dostu biyopolimerlerin elde edilmesi ve biyomalzeme olarak kullanılabilirliği araştırılmıştır. Bu amaçla, sığır aşil tendonundan kollajen, ipek böceği kozasından fibroin, kahverengi deniz alglerinden sodyum alginat ve aloe vera jelinden biyoaktif bileşenler izole edilerek saflaştırılmıştır. Ürün verimi kollajen, fibroin, sodium alginat ve aloe vera için sırasıyla %79.8 (w/w), %69.49 (w/w kozadan), %35.1 (w/w) ve %1 (w/v jeldeki kuru miktar) oranında hesaplanmıştır. Bu biyomoleküllerden dondurarak kurutma yöntemi ile doku iskeleleri hazırlanmıştır. Ancak, aloe vera jeli katı formada yapı bütünlüğünü koruyamayarak 3-boyutlu iskele yapı oluşturamamıştır. Fibroin, kollajen ve sodyum alginat iskelelerin FTIR analizleri ürünlerin saf olarak elde edildiğini, liyoflizasyon sırasında kimyasal yapının korunduğunu göstermiştir. SEM ile yüzey analizleri ise iskelelerin doku mühendisliği uygulamaları için uygun olduğunu desteklemiştir. Sonuç olarak, petrol kaynaklı polimer yerine sürdürülebilir kaynaklardan, genel olarak oda şartlarında bioaktif polimerler yüksek verimle elde edilmiş ve biyomalzeme olarak kullanılabilecekleri belirlenmiştir. Biyomoleküllerin bu şekilde sürdürülebilir kaynaklardan elde edilmesi hem hammadde sorununun hem de polimer kaynaklı çevresel kirliliğin çözümünde önemli potansiyele sahiptir.

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Introduction

The first examples of polymer products, which have an important place in our daily life, were produced from petroleum. The first samples synthesized from petroleum derivatives formed by fractionation of petroleum are called synthetic polymers. The ease of processability, the ability to be prepared in many different forms, and the adjustable properties of polymers have made the use of polymers widespread. However, in the following years, the accumulation of petroleum-derived synthetic polymers without degradation in nature has become an important environmental problem. For this reason, biodegradable polymers have started to be produced, especially with the studies initiated for the development of environmentally friendly products at the end of the 1990s. However, although environmental problems were tried to be overcome with modified polymers, this time, the supply of polymer raw materials became difficult with the decrease in world oil reserves. As a result, there are also cost increases. On the other hand, studies on the medical use of polymers have gained momentum since the 1950s. Significant successes have been achieved in increasing the biocompatibility and mechanical fixation of permanent implants. However, polymers that degrade slowly or not at all in temporary implant applications have failed in tissue regeneration treatments. Therefore, the focus has been on biopolymers of natural origin to increase biocompatibility and obtain biodegradable products.

In recent years, organic compounds found in only natural sources were known as biopolymers, which are the most common macromolecules made up of many repeating units (Prochon & Dzeikala, 2021). They include carbohydrates, proteins, nucleic acids, lipids and etc. The molecular backbones of saccharides, amino acids, fatty acids and nucleic acids have some chemical side groups that promote to activities of the molecules in addition to repeating units (Dass et al., 2022; Ezeoha & Ezenwanne, 2013; Jenkins et al., 1996). Understanding the basic and practical aspects of biopolymers is crucial for solving health and well-being concerns, which requires multidisciplinary research (Reddy et al., 2021). Biopolymers have been discovered to be biodegradable and non-toxic, making them useful in a variety of applications, including edible coverings and packaging materials in the food industry, drug carrier, wound dressing materials, medical implants and tissue scaffolds in the pharmaceutical industry (Udayakumar et al., 2021). Biopolymers, particularly those of natural origin, have been discovered to be very promising for biomedical use in a variety of forms, due to their low toxicity, biodegradability, and stability, as well as being renewable (Yadav et al., 2015).

Increasing use of ecological methods to reduce the environmental damage caused by petroleum plastics is contributing to develop of more sustainable plastics. In this way, consumption of the non-renewable resources for plastic production is limited (Atiwesh et al., 2021; Balart et al., 2021). Thus, the polymers extracted directly from sustainable biological sources have get important. In addition, these plastics may present the same advantages of petroleum plastics in terms of performance. (Balart et al., 2021). To reduce environmental effects and dependency on fossil fuels, synthetic polymers have been replaced with biodegradable polymers, especially created from sustainable resources. For this reason, in recent years polymers that can be degraded in nature are generally called biopolymers. Experts have developed several natural or synthetic biopolymers to meet growing demand (Saini et al., 2021). Biopolymers are naturally occurring compounds produced by green plants, animals, bacteria, and fungus throughout their life cycles. Protein-based biopolymers such as wool, silk, gelatin, and collagen, as well as plant-based polysaccharides such as cellulose, starch, and carbohydrate polymers made by bacteria and fungus, are examples of biopolymers (Yadav et al., 2015).

In general, the exact same results cannot always be obtained by using the methods given in the standard protocols in the literature. For this reason, some procedures from the literature are often in need of re-optimization. In this study, isolation of biopolymers was carried out with high efficiency by using methods developed and optimized according to the literature. These methods, which are revealed as their differences from the common standard methods in the literature, are explained in detail. To scope of the study, We showed how to isolate and purify some of the most often utilized biopolymers from different natural sources and their usage as biomaterials for the tissue scaffolds.

Materials and Methods

Fibroin Production from Bombyx Mori Silkworm Cocoons

Fibroin is an amphoteric protein composed of raw silk fiber (Silk fibroin (SF), a natural polymer generally produced from silkworm cocoons has superior properties such as biocompatibility, mechanical strength, biodegradation, low cost etc. SF may be produced in a variety of forms that can be used in medical, pharmaceutical, textile and food industry applications (Qi et al., 2017). The preparation method of fibroin solution from silk cocoons was developed from Sah & Pramanik (Sah & Pramanik, 2010). Bombyx mori silk cocoons were cut into small pieces and soaked in 0.2 M sodium carbonate boiling aqueous solution for 20 minutes on magnetic stirrer (Figure 1). Then, fibers were washed three times with distilled water to remove the basic salts and solute sericin. After drying the raw fibroin fibers, a ratio of 1:7 (1 g to 7 ml) silk was dissolved in 20.4 M LiBr at 60°C for one hour. At this stage, the LiBr solution is slowly added to the silk fibers that was placed in a small glass container and the fibers are completely wetted with the help of a glass baguette. After sealing the container, it is incubated at 60°C for 1 hours to dissolve the fibroin. The obtained fibroin solution was placed on cellulose based dialysis membrane and dialysis was performed in ultrapure water for 3 days. The water was changed every 3 hours for the first 12 hours, then every 12 hours thereafter. After dialysis, the silk fibroin solution was centrifuged at 4500 rpm for 30 minutes at 4°C to remove the impurities. The obtained fibroin solution was stored at 4°C. Fibroin solution may gel over time, depending on temperature. Therefore, it does not have a long storage time. It should be used within a short time after preparation.

Figure 1. Production steps of silk fibroin. A. Bombyx mori cocoons B. Dissections C. Fibroin fibers after removing the sericin D. Dialysis in the bi-distilled water after solving in LiBr solution E. Pure fibroin solution.

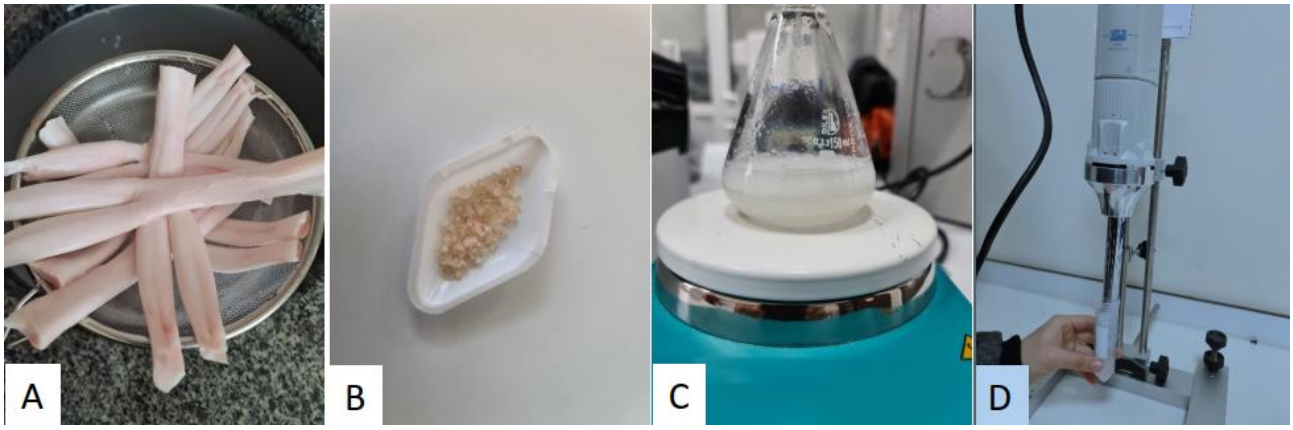

Figure 2. Preparation of collagen solution. A. Bovine tendons, B. Dissection of the tendons, C. Solving in the 0.2% acetic acid solution, D. Homogenization of the solution

The solution concentration was determined gravimetrically. For this, 500 µl of solution was weighed by placing it in a weighing cup. Then it was dried at 37°C and weighed again. This process was repeated 3 times and the average of the dry masses were taken. According to Equation 1, its concentration was calculated as %(w/v). The concentration can then be adjusted to different ratio (w/v) by repeating dialysis in ultrapure water for dilution or in glycerol for concentrating. The concentration, which was obtained between 3.64% (w/v) in 60 ml final solution prepared from 2.2 g silk in our practice, was adjusted to 3% by dialysis in distilled water to prepare biomaterials.

$$\text{Concentration \% (w/v)} = \frac{m_{av\ g}}{0{,}500\ ml} \times 100 \qquad (1)$$

Preparation of Collagen Solution

Collagen is the structural protein of mammals and is the main component of the extracellular matrix. Therefore, it has advantages such as superior biocompatibility, ease of cell attachment, and availability of sustainable resources in its use as a biomaterial. On the other hand, the most important disadvantages are the changes in the chemical composition and the ability to create an immune response in the host, depending on the source from which it is obtained. Collagen is mostly obtained from cattle used in slaughterhouses. It can also be obtained from the tail tendons of subjects. In our study, bovine achilles tendons were obtained from the slaughterhouse. It was brought to the laboratory in sterile isotonic solution, where it was washed with isotonic solution and cleared of impurities. Then, achilles tendons were cut into very small pieces by dissection. The 1,5 g of dried tendons were weighed and placed in 50 ml of 0.2% (v/v) acetic acid solution and kept on a magnetic stirrer overnight. At the end of the time, the solution was mixed with a homogenizer (Figure 2). Subsequently, the insoluble particles were separated from the medium by filtration. The obtained solution was stored at 4°C. Before the grafting reaction on the fibroin fibers, horseradish peroxidase (HRP) was added into the collagen solution at the 2% final concentration. Then, the blend was incubated for 24 hour at 37°C.

Aloe Vera Solution

Isolation of aloe vera gel

To prepare bioactive aloe vera gel, various shredding and filtering steps were followed without heat treatment according the Ahlawat and Khatkar (2011) (Ahlawat and Khatkar, 2011). Aloe Vera leaves were washed with water before harvesting from the plant to remove dirt and impurities. The beneficial components of aloe vera are contained in the greenish transparent gel inside the leaves. Therefore, the yellow sap from the bottom of the harvested leaves was removed with a blotter so that it would not contaminate the bioactive gel. Then, approximately 2.5 cm of the leaf base and leaf edges are cut with the help of a scalpel and discarded. The cellulosic layer on the top and bottom of the leaf is peeled off and the gel inside is taken into a falcon tube (Figure 3). First, gel extract was digested by a homogenizer and then, centrifuged at 4500 rpm for 30 min at 4°C to remove the fiber residues. The concentration was determined as 1% (w/v) gravimetrically according to equation 1. Aloe vera extract was placed in a dark colored container to protect the light sensitive bioactive ingredients and stored at 4°C in order to prevent the bioactive substances from being affected by light (Chandegara and Varshney, 2013).

Preparation of active aloe vera solution

In order for aloe vera (AV) to be cross-linked with the fibroin and collagen composite, the nitro groups in its structure must be reduced. For this purpose, Fe^{2+} ions were used to reduce the nitrobenzene groups in the structure of aloe vera to aniline using Benchamp procedure (Equation 2). Generally, an acid equivalent of about 0.05-0.22 is used. Acids commonly used in the reduction process are

hydrochloric and sulfuric acids. The addition of a water-miscible solvent such as ethyl alcohol, methanol, or pyridine often helps significantly. Fe^{2+} ions were customarily prepared fresh from metallic Fe and used immediately, as it has low stability and can be rapidly oxidized to Fe^{3+} (Figure 3) (Popat and Padhiyar, 2013).

$$4RNO_2 + 9Fe + I6H_2O \rightarrow 4RNH_2 + 3Fe(OH)_2 + 6Fe(OH)_3 \rightarrow 4RNH_2 + 3Fe_3O_4 + 12H_2O \quad (2)$$

To prepare Fe^{2+}, 10 g of iron powder (Fe) is put into the beaker in the hot water bath. 75 ml of concentrated HCl was added to the iron powder and dissolve in a water bath. In order to complete the reaction, it is kept in a water bath at 75°C for 1 hour. Then, the solution is cooled to 0°C, the green colored $FeCl_2$ formed is filtered under vacuum and washed 1-2 times with diethyl ether (Figure 4). To prepare the reduced aloe vera solution, 15 ml of 1% AV solution was mixed using magnetic stirrer with 0.18 g $FeCl_2$, which was freshly prepared. While $FeCl_2$ completely dissolved in the AV solution within seconds, the viscosity of the solution also decreased. Stirring was continued for 5 min at room temperature to complete the reaction. It was stored at +4°C until used.

Sodium Alginate Isolation and Purification

The basic principle in the production of alginate from brown-red marine algae is the coagulation of alginic acid as a calcium salt. To achieve this, sodium bicarbonate or calcium carbonate solutions are used in the extraction stage. However, alginic acid component in algae is in a complex structure with mannitol, protein and other polysaccharides. Therefore, in order to obtain alginic acid without degradation, it is necessary to pre-treat it before the extraction step.

In the pretreatment, algae are treated with low concentrations of formaldehyde and hydrochloric acid solutions. In the meantime, the alginic acid in the structure of the algae material is cross-linked using chemicals. In this way, they are separated from the algae residue without being destroyed during extraction, while mineral salts and other foreign materials are removed by washing (Chee et al., 2011; Dennis, 2003).

Obtaining alginic acid from algae is carried out in 2 stages (Figure 5). First, the algae are treated with acid and made ready for extraction. Then, by steam distillation, alginic acid is obtained in solution and separated by sedimentation as sodium salt. At the first stage, 10 g of dry brown algae were weighed and cut into small pieces by dissection. 50 ml of 2% CH_2O solution was added to the algae and incubated for 24 hours in an oven at 45°C. At the end of this process, the algae were filtered and washed twice with distilled water. Afterwards, 300 ml of 0.2 N HCl solution was added onto the filtrate and incubated at 25°C for 24 hours. At the end of the process, the samples were filtered and washed twice with distilled water. At the second stage, the extraction of the filtrate obtained from the pretreatment was carried out in 1% Na_2CO_3 solution at 100°C for 3 hours. The extract was filtered and centrifuged at 3000 rpm for 30 minutes (Hettich Universal 320 R). Absolute C_2H_6O (ethanol) was added to the pure extract at a ratio of 1:4 (v/v) and incubated at room temperature for 24 hours to transform sodium alginate in hydrogel form. The obtained sodium alginate was washed 2 times with absolute C_3H_6O (acetone). Sodium alginate, which changed from gel consistency to solid form, was left to dry at 60°C. It was then ground into powder and stored at room temperature.

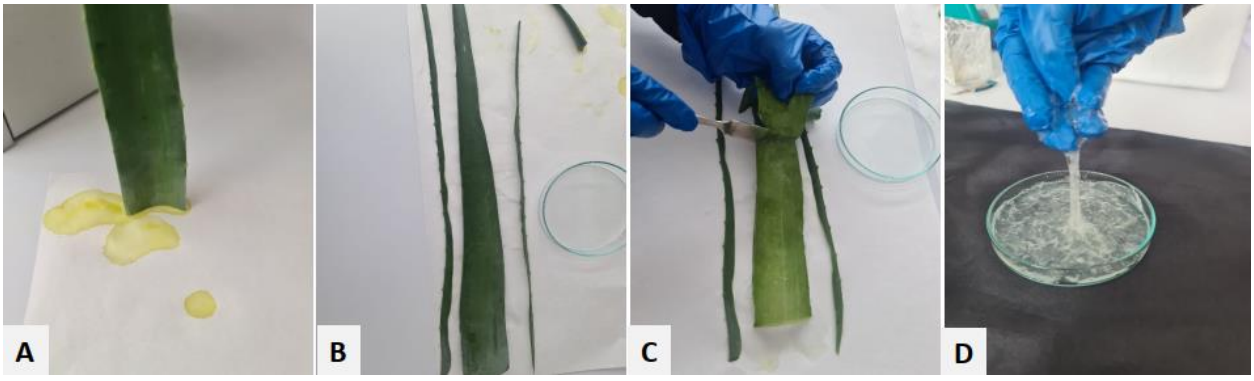

Figure 3. Aloe vera extraction procedures. A. Harvesting of leaves and removing the yellow sap, B. Cutting of leaf base and edges, C. Peeling off the cellulosic out layer, D. Collecting the pure AV gel

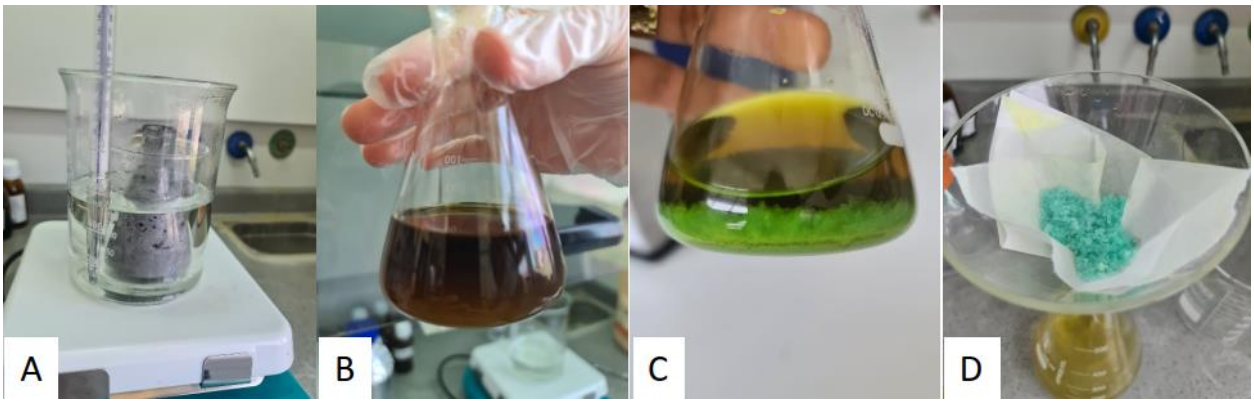

Figure 4. Procedure steps of the $FeCl_2$ synthesis; A. Fe and HCl mixing B. Solving the Fe powder C. Obtained the $FeCl_2$ particles, D. Filtering and washing the products

Figure 5. Sodium alginate production process from brown algae.

Biomaterial Uses of Biopolymers: Tissue Scaffolds

Tissue scaffolds were produced for the use of biopolymers and bioactive components produced from natural and sustainable sources as biomaterials. For this purpose, in addition to single component scaffolds, composite scaffolds were produced from biopolymers activated with bioactive components and polymer blends. The freeze-drying method in a lyophilizer, which is an effective method for turning hydrogel systems into solid and porous scaffolds, was preferred. In this method, 4 ml of polymer solutions were added to 12-well petri dishes and allowed to freeze at -20°C for 4 hours. Subsequently, They were kept overnight at -80°C. At the end of the process, the frozen samples were placed in the lyophilizer and dried under vacuum at -55°C for 24 hours. During drying, porosities were formed in the areas where the water molecules were separated, and a sponge-like structure was obtained as a result. For the scaffolding, aloe vera gel was first powdered by lyophilization, and its aqueous solution was prepared prior to use. However, scaffolds that could preserve their 3-dimensional structure could not be obtained from pure aloe vera solution. Because of that SEM analysis could not be performed to solid aloe vera biomaterial.

Chemical and Morphological Analysis

The chemical structure of the samples was examined by Fourier Transform Infrared Spectroscopy (FTIR), and their morphological structures were examined by SEM. The efficiency of bioactive ingredients obtained from natural sources was calculated with equation 3 as the ratio of the obtained product mass (m_p) to the raw material mass (m_r).

$$\text{Efficiency}\% = \frac{m_p}{m_r} \times 100 \quad (3)$$

Results

Extraction Efficiency

Product efficiency of biopolymers are calculated according to equation 3 and given in Table 1. The highest product efficiency was achieved in collagen production. Collagen was obtained with high efficiency due to the high content of collagen (60-85%) in the matrix structure of the tendons. Other tissue components such as cells, glycosaminoglycans, proteoglycans were separated from the structure as impurities (Binder-Markey et al., 2020; Buckley et al., 2013; Kannus, 2000; Liu et al., 1995) The efficiency of alginate obtained from brown algae was calculated as 35.1% of dry weight. Although the amount of alginate in the structure of macroalgae is within normal ranges, it is in the range of 25-37% depending on the method used in the literature (Chee et al., 2011; Dennis, 2003). Silkworm cocoon contains approximately 65-70% fibroin. While sericin is removed by alkaline treatment, some of the fibroin can be dissolved and separated from the structure. In the purification step, fibroins with short chain lengths can pass into the water through the membrane pores during dialysis. Despite that, the concentration of fibroin solution prepared by dissolving in LiBr was calculated as 36,4 mg/ml and this value is much higher than standard methods (Sah and Pramanik, 2010). The fibroin efficiency from Bombyx mori silkworm cocoons was 69.49%, and the fibroin efficiency from sericin-free silk fibers was calculated as 99.28%. Aloe vera gel contains a high amount of water. Approximately 0.5-1% of the dry weight of the gel contains bioactive molecules. In this study, the dry bioactive molecule efficiency of aloe vera was obtained as 1% in accordance with the literature (Bozzi et al., 2007; Hamman, 2008; Ni et al., 2004).

Table 1. Efficiency of final products isolated from natural sources.

Product	Raw Material	Efficiency (%)
Fibroin	Bombyx Mori cocoons	69.49
Fibroin	Silk fiber removing from sericin	99.28
Collagen	Achille tendon	79.8
Bioactive aloe vera components (solid form)	Aloe vera gel	1.0
Sodium alginate	Algae	35.1

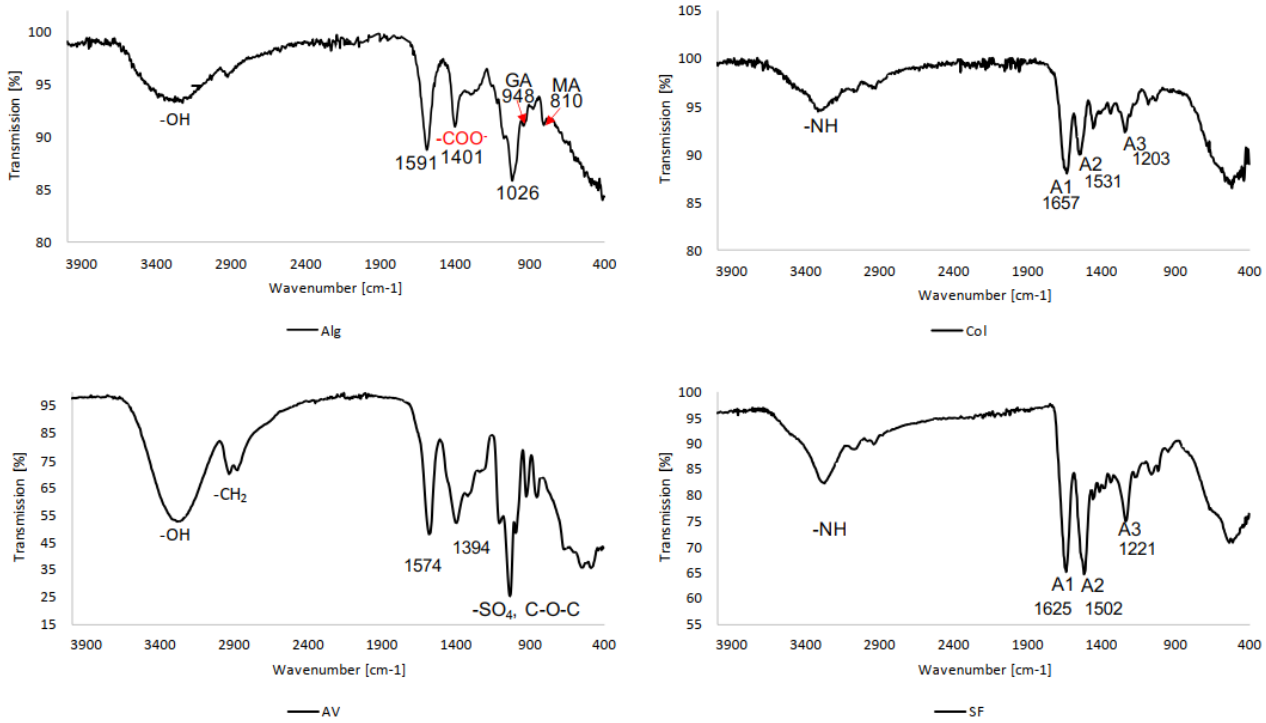

Figure 6. FTIR spectrum of Alginate (left top), Collagen (right top), Aloe vera (left bottom), Silk fibroin (right bottom). A1: Amide I, A2: Amide II, A3: Amide III

FTIR Analysis

Evaluation of the FTIR spectrum of the alginate (Figure 6), the stretching vibrations of the O-H bonds of the alginate were seen around 3283 cm-1 and in the range of 2980-3400. Stretching vibrations of aliphatic C-H were observed at 2970-2850 cm^{-1}. The peaks observed around 1591 and 1400 cm^{-1} show the asymmetric and symmetric stretching vibrations of the carboxylate salt ion of O-C-O stresses, respectively. Subsequent bands are very important and can be used to characterize the alginate structure from its derivatives and ingredients. The bands around 1122, 1073, 1024 and 946 cm^{-1} were evaluated as C-O stretching with the contributions of the C-O stretching vibration of the pyranose ring and the C-C-H and C-O-H deformation. The C-H stretches of mannuronic acid were seen in the range of 810 - 822 cm^{-1}, and the C-H stretches of guluronic acid were in the range of 948-932 cm^{-1}.

Collagen and fibroin are in polypeptide structure and contain amide bonds. Peaks of Amide I, Amid II, Amid III bonds were observed in both structures (Buckley et al., 2013; Jaramillo-Quiceno et al., 2017; Sah and Pramanik, 2010). However, a shift in the peaks given at figure 5 was observed due to differences in secondary structures owing to amino acid content. Peaks (1574, 1394, 1031 cm-1) belonging to -NH, NO2 and SO4 groups were observed in bioactive components isolated from aloe vera gel. Hydrophilic groups specific to the bioactive components indicate the medicinally used peaks of aloe vera. In addition, the peaks observed at 1031 cm^{-1} and around prove the existence of ether structures (Narsih, 2016; Ni et al., 2004; Rani et al., 2021). These peaks observed in the spectrum belong to the components of bioactive components used in pharmaceuticals.

SEM Analysis

The SEM image of the fibroin scaffold is given in Figure 7, and it has been observed that there is a laminar structure similar to the natural structure of fibroin. However, the gaps formed between the layers caused the formation of pore-like structures. In this way, it gained the appearance of a sponge. It can be said that the openness of these pore-like formations is favorable for cell adhesion and migration in cell culture applications.

SEM images of alginate structures are given in Figure 7B. As can be clearly seen from the micrographs, it was determined that the pore sizes decreased compared to fibroin and a scaffold with open porosity was formed. The increased amount of small pores provides a greater surface area for cells to attach and grow in both in vitro and in vivo applications. In this regard, porous scaffolds with dimensions between 50-400 µm are generally preferred for tissue engineering. Collagen scaffolds have a surface between fibroin and alginate scaffolds. While the helical structure of collagen prevents the formation of layers as in

the fibroin scaffold, the closely packed chain structure has given its morphology a more uniform appearance. In this respect, the pore structure resembles alginate scaffold, but has a more regular pore distribution (Hu et al., 2006).

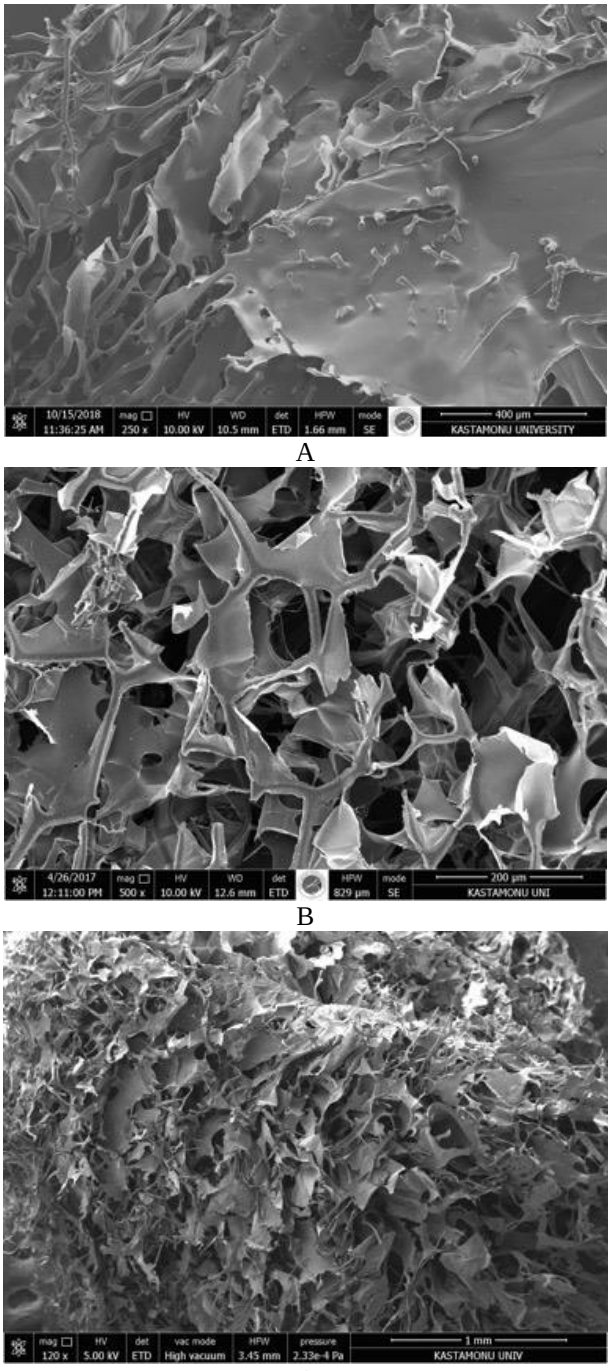

Figure 7. SEM images of A. Fibroin, B. Sodium alginate C. Collagen scaffolds

Conclusion

Natural biopolymers are already present in nature as polymers and after a certain period of time, they completely biodegrade and participate in the environmental oxygen, nitrogen, carbon dioxide cycle. With the decrease in petroleum reserves, natural polymers, which are more environmentally friendly, gain great importance as a sustainable raw material source. In the study carried out, natural biopolymers were isolated and purified from natural sources in a standard laboratory environment with high efficiency. It has been found that the product efficiency of these bioactive macromolecules obtained from sustainable sources is high. In this respect, it has the potential to be an alternative to synthetic polymers, and the inadequacy of its mechanical properties, which is its most important disadvantage, can be improved by surface modifications. In order to determine that the obtained biopolymers can be used as tissue scaffolds, biomaterials were designed by freeze drying method. FTIR analysis confirms the purity of the biopolymers. In addition, the morphology that will promote cell adhesion and migration was supported by SEM photographs. Scaffolding could not be obtained from the obtained macromolecules only from aloe vera gel. However, it is known that these molecules, whose bioactive properties are known, can be used in the surface modification of other polymers. Within the scope of the study, it has been shown how the chemical activation of the molecules obtained from the aloe vera gel will increase the reaction affinity. As a result, natural polymers can be produced from sustainable sources without seasonal limitations. As a biomaterial, its properties can be improved by using in the form of different polymer blends and chemical modifications.

Acknowledgment

This study was carried out in Kastamonu University, Faculty of Engineering and Architecture, Tissue Culture Laboratory. Sodium alginate was produced by using brown sea algae collected from the coast of Inebolu district of Kastamonu province, with the permission of the Republic of Türkiye Ministry of Agriculture And Forestry General Directorate of Nature Conservation And National Parks, with the number 21264211-288.04-E.3028336 and dated 04.10.2019, within the scope of the TÜBİTAK ARDEB project numbered 119O558.

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