

EXTRACTION OF HYALURONIC ACID FROM *ALOE BARBADENSIS* (ALOE VERA)

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ABSTRACT

Hyaluronic acid have a high moisture preservation and biocompatibility characteristic, thus allowing various usage of this substance in pharmaceutical, medicinal, and skin care products. Present manufacturing process of hyaluronic acid requires extraction of animal cells or through other methods utilizing bacteria. The aim of this research is to investigate an alternative source of hyaluronic acid extraction using plant based which is *Aloe barbadensis* (aloe vera). Three main parts of aloe vera (rind, mesophyll and gel) underwent several steps of extraction process and the result was compared to the sample of hyaluronic acid from goat brain. The evaluation including comparison of total carbohydrates, reducing sugars and degradation using heat treatment. The results of extraction were analyzed using UV-Spectrophotometer at 230 nm and compare to the extraction result of goat brain to ensure the presence of hyaluronic acid. It was found out that the rind part of aloe vera have the highest potential of high content of hyaluronic acid.

Keywords: *Aloe vera*; *extraction*; *hyaluronic acid*.

ABSTRAK

Asam hialuronat memiliki kemampuan untuk mempertahankan kelembapan serta biokompatibilitas yang tinggi, hal ini menjadi alasan asam hialuronat banyak digunakan dalam produk farmasi baik yang berhubungan dengan obat maupun perawatan kulit. Pada proses pembuatan asam hialuronat, ekstraksi dari sel hewan masih merupakan sumber utama disamping penggunaan metode alternatif menggunakan beberapa jenis bakteri. Tujuan dari penelitian ini adalah untuk menginvestigasi sumber alternatif pengekstrasian asam hialuronat menggunakan bahan dasar tumbuhan yaitu *Aloe barbadensis* (lidah buaya). Terdapat tiga bagian dari lidah buaya yang melewati beberapa tahap ekstraksi (kulit, mesofil, dan jel), hasil dari ekstraksi kemudian dibandingkan dengan sampel asam hialuronat dari otak kambing. Evaluasi mencakup perbandingan karbohidrat total, penurunan kadar gula, dan degradasi molekul menggunakan panas. Analisis terakhir menggunakan UV-Spektrofotometer di panjang gelombang 230 nm dan dibandingkan dengan hasil ekstraksi dari otak kambing untuk memastikan keberadaan asam hialuronat. Hasil analisis menunjukkan bahwa kulit lidah buaya memiliki potensi mengandung asam hialuronat yang cukup tinggi.

Kata kunci: *Asam hialuronat*; *ekstraksi*; *lidah buaya*.

Article Information

Article Type: Research Article
Journal Type: Open Access
Volume: 1 Issue 2

Manuscript ID
v1n2286-1

Received Date
10 February 2020

Accepted Date
17 February 2020

Published Date
29 February 2020

DOI:
10.33555/jffn.v1i2.33

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Citation:

Putri, R.R, Nugraha, T., Christy, S. 2020. Extraction of Hyaluronic Acid From *Aloe barbadensis* (Aloe Vera) J. Functional Food. & Nutraceutical, 1(2), pp.95-102.

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INTRODUCTION

Hyaluronic acid (HA) is a biological occurring polymer which has substantial biological functions in almost every organism (Necas, et.al., 2008). In humans, HA can be found in skin, vitreous of the eye, umbilical cord, and synovial fluid, but it is also present in body's tissues such as skeletal tissues, heart valves, lungs, brain, and many others (Meyer K., Palmer, J.W., 1934). Hyaluronic acid was located predominantly within extracellular and peri cellular matrix, although correspondingly existed on the intracellular cell (Balazs, et al., 1986).

Resources to gain hyaluronic acid were commonly taken from various animal tissues such as human umbilical cords, rooster combs, bovine vitreous humor, and bovine synovial fluid (Liu, et. Al., 2011). At present day, even though production through animal-based tissues still remain unshaken to be the major pathway for large HA production, another possibility of production systems have been demanded because of some disadvantages of the existing process. Due to the grinding procedure and several repetition of using acid and organic solvents, both practical and mechanical issues will always happened in animal extraction in terms of cost and safety (Widner, et. al., 2005).

Another issue is that HA from animal tissues may remain connected to a HA-specific binding cellular proteins of hyaluronidase (Fraser, et al., 1997). Hyaluronidase is undesirable since it may trigger the risk to prohibit an immune response. Furthermore, transmitter of infectious diseases in form of nucleic acids, prions, and viruses may well increases within extraction procedure (Shiedlin, et al., 2004). Lastly, the procedure are expensive and require a long period of time, labor, and advanced facilities to accommodate processes involved from animal extraction until purification of HA (Shlini, et al., 2017). Hence, it is preferred to generate hyaluronic acid via an animal cell-free system that could reduce contagion of undesirable contaminant and expense of manufacturing (Widner, et al., 2005) and (Yu & Stephanopoulos, 2008). Therefore, this research was arranged to find another pathway of extracting hyaluronic acid from a plant source, which according to (Shlini, et al., 2017) has proved to be successfully done from

sweet potato and tapioca (Sana, et al., 2017). Moreover, aloe vera (*A. barbadensis*) was chosen due to its popularity to the public and considerably easy to be harvested in Indonesia.

In this research *A. barbadensis* is chosen as the potential source of HA due to similarities with HA in compositions and biological activities. Both aloe vera and hyaluronic acid proven to promotes wound healing (including dermatology applications), anti-inflammatory and therapeutic benefits. Moreover, *A. barbadensis* and hyaluronic acid have been used for dermatology purposes due to their abilities to retain water. There are three major parts of *A. barbadensis* used in this research, those are: rind, mesophyll, and gel. Rind is the external surface waxy cuticle which performs as a wall in a contradiction to moisture loss. Rind covers several levels of structures, with slight beneath from the waxy cuticle remains an area where the aloe related bacteria live (Sushruta, et al., 2013). Mesophyll is a liquid yellow-brownish part of aloe vera which holds the xylem and phloem vascular bundles. Mesophyll has the biggest concentration of anthraquinones and chromones of the whole aloe vera. Last part of aloe vera is the gel which located inside the inner parenchyma part of aloe vera. It consist of two components: juice of the gel and fibrous pulp enriched with cellulose.

Commercial manufacturing of hyaluronic acid is built on either animal-based extraction or genetically modified strains of bacterial fermentation. Both of these pathways are commonly applied and proved to manufacture hyaluronic acid products with molecular weights above 10 kDa that was suitable for medicine and dermatology usage (Liu, et al., 2011). Biological properties of hyaluronic acid are connected with its molecular weight, hence there is a great interest in HA degradation and evaluation of the biological behavior of HA fragments. Mechanisms of the HA cleavage into its smaller fragments involve enzymatic, free radical, thermal, ultrasonic, and chemical methods such as acid and alkaline hydrolysis (Soltes, et al., 2007).

MATERIALS AND METHOD

Materials

All aloe vera (*A. barbadensis*) and fresh goat brain were purchased from a market in Tangerang, Indonesia. The chemicals used for this research were acetone (Amresco), chloroform (Merck), methanol (FULLTIME), sodium acetate (CV. Bina Sejahtera), L-cysteine (Merck), acetic acid (Merck), 37% hydrochloric acid (Sigma Aldrich), ethylenediaminetetraacetic acid/EDTA (Disolvin), distilled water, sodium chloride (HiMedia Laboratories), absolute ethanol (FULLTIME), sulfuric acid (J.T Baker), ice cubes, sea salt, sodium carbonate (Merck), anthrone (Merck), sodium hydroxide (Merck), potassium sodium tartrate tetrahydrate (PUDAK Scientific), dinitrosalicylic acid/DNS (Sigma Aldrich).

Equipment

M254A BEL Engineering Weighing balance, water filtration system (Hydro Water Solution PT. Hydro Water Technology), hotplate stirrer (WiseStir MSH-20D), MColorpHast pH-indicator strips, centrifuge (Type 80-2 China), refrigerator (Electrolux), autoclave HG 50 Hirayama, Phillips food processor/grinder, PG Instruments T60 UV-Visible Spectrophotometer, and VWR V-1200 Visible Spectrophotometer.

Extraction Process

The extraction methodology is based on the studies being performed by (Shlini, et al., 2017) with sweet potato (*Ipomoea batatas*) and (Sana, et al., 2017) with tapioca (*Manihot esculenta*). In this research, aloe vera (*A. barbadensis*) will be taken as the plant source and goat brain as sample of pure hyaluronic acid. The samples were washed thoroughly, parts of aloe vera were separated by knife and each of the four samples were homogenously crushed. 50 g of each sample was submerged in 50 mL of acetone and stirred for an hour. Chloroform and methanol with ratio 2:1 was used to incubate 100 mL sample for 24 hours at 25°C. Followed by digestion buffer (100mM sodium acetate pH 5.0, 5.0mM cysteine and 5.0mM disodium EDTA) that arranged in a ratio 2 mL of buffer to 100mg of

tissue. The sample was hydrated inside the digestion buffer for 44 hours at 5°C before centrifuge at 3200rpm for 30 minutes. The solvents was removed and the solid filtrate was splashed by 3 mL of 2.0M sodium chloride and followed by absolute ethanol. Absolute ethanol was inserted in ratio of 2:1 and kept for 24 hours at -16°C. The next procedure was centrifugation at 3200 rpm for 30 minutes. Sequentially, the supernatant was taken away and the solid filtrate was washed with 80% ethanol. Second centrifugation was done as previous one before supernatant was discarded and the solid filtrate dried for 24 hours at 25°C. The final solid was re-suspended in 5 mL of distilled water and stored inside a test tube.

Total Carbohydrate Analysis using Anthrone's Method (Hodge, et al., 1962)

0.1 g of sample was boiled for 3 hours with 5 mL of 2.5N-HCl, then cooled to room temperature with ice and salt. The sample was neutralized by adding solid sodium carbonate until the effervescence ends. The sample was made up to the volume of 100 mL and centrifuged at 3200 rpm for 15 minutes. The supernatant was collected to prepare 1mL aliquots for analysis. The sample was added by 4mL of fresh anthrone reagent (dissolve 0.2 g of anthrone in 100 mL of ice cold H₂SO₄) and heated for 8 minutes in a boiling water. The sample was rapidly cooled with ice and sea salt and observed at absorbance of 630 nm in a visible spectrophotometer.

Reducing Sugar Analysis using DNS Method (Garriga, et al., 2017)

DNS reagent was prepared by making two mixtures; Solution A (1 g of DNS was dissolved in 20 mL of NaOH 2M) and Solution B (30 g of potassium sodium tartrate tetrahydrate was dissolved in 50 mL of distilled water). Solution A was added into Solution B, heated, and mixed on a hot plate at 300°C and 370 rpm. This new solution was completed to the volume of 100 mL with distilled water and stored in amber bottle at refrigerator (4°C). This solution was named as DNS reagent. 1 mL of each sample was placed into a test tube and added by 1 mL of DNS reagent. The test tube was heated in a boiling water for 5 minutes and cooled

by ice and sea salt to room temperature. The sample was added by 8 mL of distilled water and read at 540 nm in a visible spectrophotometer.

Fragmentation of Hyaluronic Acid (Lowry and Beavers, 1994) and (Botner, et al., 1988)

Degradation of the pre-assumed HA sample and goat brain sample were done through thermal degradation. 10 mL of each sample was taken into small bottle and inserted into the autoclave for 4 hours at 128°C. Sequentially, the sample was observed using UV-spectrophotometer in 230 nm wavelength.

RESULTS AND DISCUSSION

Total Carbohydrate Measurement

Hyaluronic acid is a carbohydrate compound, more specifically a repeated glycosaminoglycan (GAG) which formed of β 4-glucuronic acid and β 3-N-acetylglucosamine (Meyer K, 1934). Hyaluronic acid occurred in a high molecular weight due to the repetition of glucuronic acid and N-acetylglucosamine that able to goes up to a thousand repetition even further as can be seen from figure 1.

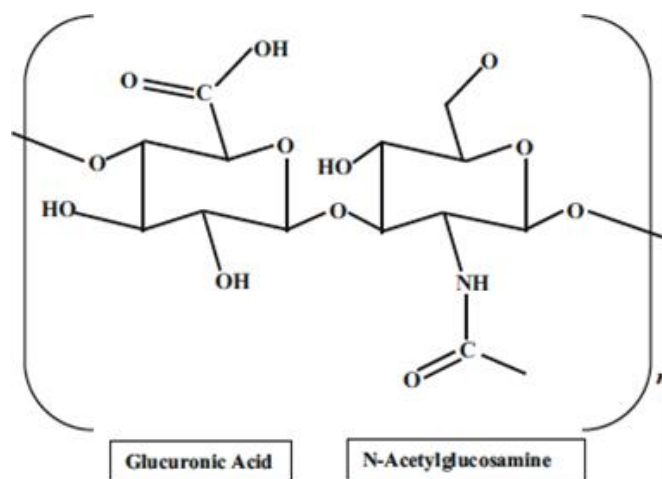


Figure 1. Structure of hyaluronic acid monomer (Cowman & Matsuoka, 2005)

Anthrone's method was used to measure total carbohydrate content from three different part of aloe vera samples (rind, mesophyll and gel) to be compared to total carbohydrate content of hyaluronic acid from natural source, in this case goat brain. This method used as the initial stage to identify hyaluronic acid.

As can be seen from Figure 2, all of aloe vera's parts (rind, mesophyll, and gel) were proved to

show some value of absorbance at 630 nm, which showed that aloe vera does contains carbohydrate.

Goat brain as the hyaluronic acid source showed highest peak with the value of absorbance of 0.034 followed by rind with absorbance of 0.023. From three parts of aloe vera (rind, mesophyll and gel), rind part showed highest and closest absorbance to hyaluronic source from goat brain, but to be certain further analysis through reducing sugar needs to be done.

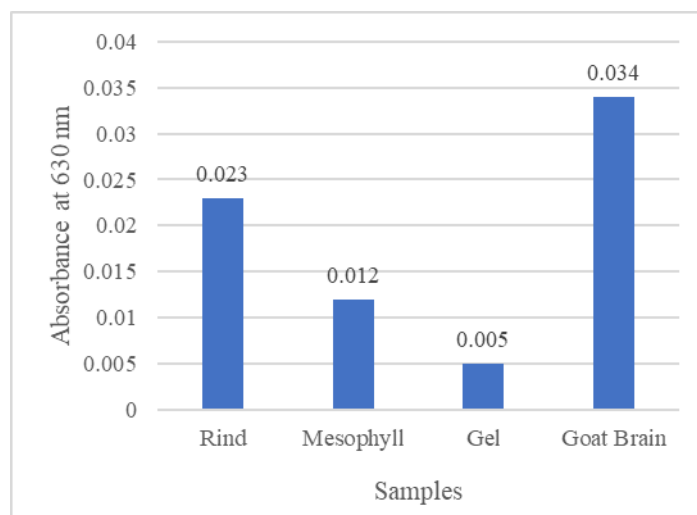


Figure 2. Graph showing comparison of total carbohydrate of aloe parts with goat brain

Reducing Sugar Measurement.

Anthrone method only cover the general picture of finding carbohydrate, hence another method is used to observe more specific compositions of carbohydrate which downgrade the structure from polysaccharides into smaller fragments of

carbohydrates; reducing sugar. Moreover, hyaluronic acid chemical structure is particularly included a form of reducing sugar: β -D-glucose (Gunawardena, 2015), which made the essential on doing DNS is highly proposed.

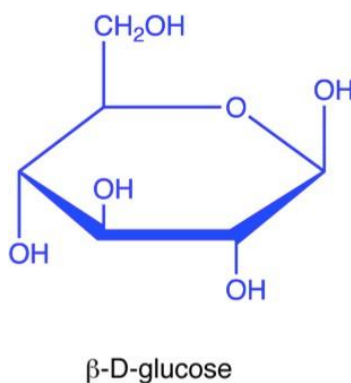


Figure 3. β -D-glucose (Gunawardena, 2015)

DNS method was done as a complement procedure from anthrone's result to specifically qualify any reducing sugars inside the sample. Total carbohydrate analysis through anthrone's methods already showed that rind and goat brain has highest and closest absorbance compared to other part of aloe vera. Figure 4 below showed that all parts of *A. barbadensis* have shown value of absorbance which suggested contains reducing sugar. It should be highlighted that both in anthrone and DNS

method, rind part of aloe vera showed the highest absorbance 0.333 in comparison to mesophyll and gel. In addition, rind have the closest absorbance to goat brain (0.288) that contain high concentration of hyaluronic acid in both anthrone and DNS method thus conforming that rind have a very high chance to contain hyaluronic acid. Based on these findings, rind was chosen to undergo further analysis step which is thermal degradation.

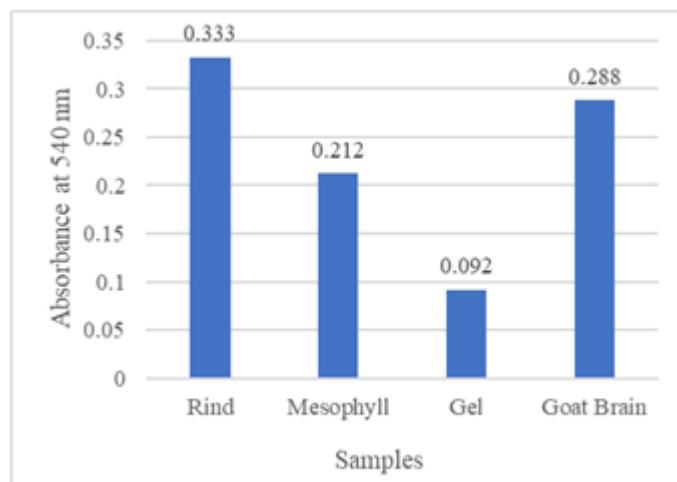


Figure 4. Graph showing comparison of reducing sugar of aloe parts with goat brain

Thermal Degradation

Hyaluronic acid is naturally occurred in a high molecular weight, but since there are various applications which came from different sizes of molecular weight of hyaluronan, it prompted a HA cleavage method to be performed. There were numerous ways to decrease the molecular weight of hyaluronic acid into smaller fragments which engage with enzyme, free radical, heat, ultrasound, and chemicals. Unfortunately, most of those methods will produce unwanted toxic impurities and demand a high cost. Thermal degradation of hyaluronic acid proved to be successfully done by (Botner, et al., 1988) at 128°C in an autoclave.

Based on total carbohydrate and reducing sugar measurement, rind has the highest chance of containing hyaluronic acid, hence thermal degradation analysis was done to conforming the presence of hyaluronic acid in rind compared to goat brain. Hyaluronic acid was proved to be existed on the wavelength of 230 nm based on several studies being done by (Shlini, et al., 2017) and (Sana, et al., 2017). Therefore, the rind sample and goat brain were gone through UV-spectrophotometer before and after thermal degradation to showed the existence of hyaluronic acid.

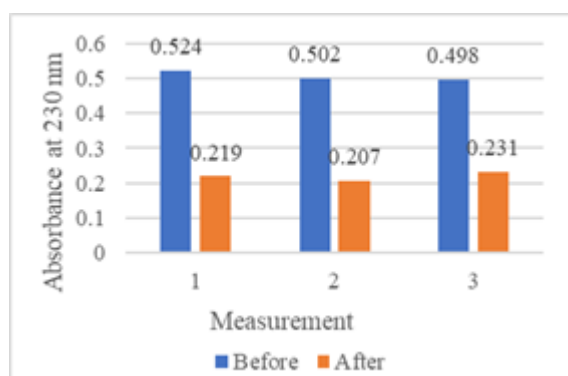


Figure 5. Absorbance of rind before and after thermal degradation

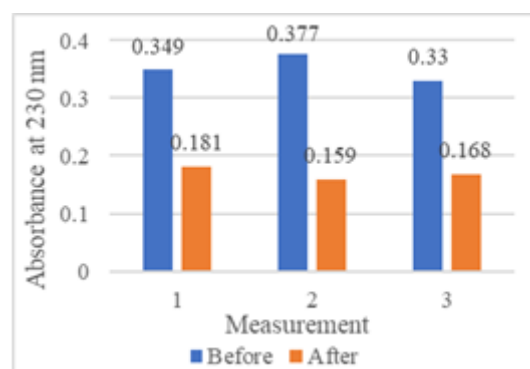


Figure 6. Absorbance of goat brain before and after thermal degradation

Hyaluronic acid is naturally occurred in a high molecular weight, but since there are various applications which came from different sizes of molecular weight of hyaluronan, it prompted a HA cleavage method to be performed. As can be seen from Figure 5 and Figure 6, three repetitions of both samples showed a decrease of absorbance with very similar value, hence showed degradation process using heat treatment to be successful and hyaluronic acid component from both samples was successfully fragmented as the end product. One law that affirm molecular weight of the end product after degradation will decreased is the law of conservation of mass. The law stated that mass is neither created nor destroyed in chemical reactions (Stern, R and Hood, J., 2011). Since thermal degradation was not a chemical reaction, it only shrinks the structure molecules which produced a less bulky compound with smaller weight of mass.

Another supportive evidence to show the declining of its molecular weight is the smaller value of the concentration after degradation procedure. If the chemical structure of HA were cut during thermal degradation, it ends with less bulky chemical compounds which leads to smaller value of concentration. The concentration of the sample was declined after degradation as can be seen in the decreased of absorbance value. This can be explained through the Lambert Beer's Law, expressed through:

$$A = \varepsilon c l \quad (\text{Equation. 1})$$

Whereas A is absorbance, ε is molar absorption coefficient, c is molar concentration and l is optical path length passed by the UV light. Since the value of absorbance after thermal degradation was lower compared from before degradation process, it concluded that concentration after degradation was also dropped due to proportionally equivalent value of absorbance and concentration according to the Equation. 1. It can be clearly seen that the drop of concentrations was constant through three repetitions of sampling using UV-spectrophotometer which referring back to Figure. 5 and Figure. 6.

This result also supported by the fact that rind is highly composed by one of the hyaluronic acid structures; carboxyl group which are richly present in form of oxalic acid inside rind. Moreover, rind has anti-inflammatory property due to chromones which somehow equaled with hyaluronic acid's anti-inflammation property. Chromones also have skin protection effects which matched with one of hyaluronic acid's benefits for skin; protection of water loss to the skin. Lastly, on just below the waxy cuticle of rind, there is an area where aloe correlated bacteria live. Gram-positive microbes (including Group A and group C *Streptococci*) which able to produce hyaluronic acid through bacterial pathway, were only found on the surface of aloe vera (*A. barbadensis*), whereas *coccobacilli* (*streptococcus morbillorum*, *enterococcus faecium*, and other Gram-negative rods) are observed only in gel part.

CONCLUSION

Anthrone method showed that all parts of aloe vera containing carbohydrate with rind has the highest absorbance, just below the absorbance of goat brain. This result was confirmed by DNS method which showed that again rind has the highest absorbance just like the goat brain. Furthermore, thermal degradation process was done to degrade high molecular weight HA into small molecular weight HA. The result of thermal degradation can be seen through UV-Spectrophotometer which showed constant and very similar decrease of absorbance on both rind and goat brain sample, thus showed that rind is containing hyaluronic acid. For further studies, isolation and purification of hyaluronic acid and quantification of its concentration, ion exchange chromatography is preferred due to anionic nature of hyaluronic acid. The elution obtained by ion exchange chromatography can be further purified using gel permeation chromatography and for determination of precise structure of HA, NMR (Nuclear Magnetic Resonance) followed by FT-IR can be used in future research.

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