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Der Pharmacia Lettre, 2016, 8 (16):76-82
(http://scholarsresearchlibrary.com/archive.html)

Der Pharmacia Lettre
Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

Isolation of Agarose and its Application as medium of Gel Electrophoresis Method for HPV (*Human papillomavirus*) DNA Identification

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ABSTRACT

Agarose had been separated from commercial agar that was extracted from red algae, Gracilaria gigas. Agarose was obtained by dissolving the agar powder with 70 °C preheated ethylene glycol under continuous stirring followed by increasing the temperature to 105 °C to get homogenous solution with concentration of 1, 2, and 3 % b/v agar. Agarose was separated from more soluble agaropectin by cooling process overnight at -10 °C, followed by adding isopropanol at room temperature to induce precipitation of agarose. Agarose precipitate was then filtered with flannel and dried at room temperature until the smell of isopropanol disappeared and the agarose granule was formed. The granule then was grinded to yield purified agarose powder. Purified agaroses were named agarose A, agarose B, and agarose C. Some chemical and physical properties of isolated agarose such as gelling and melting point, gel strength, sulfate content, and EEO were measured and determined utilizing standard measurement method. It was found that the sulfate content of agarose A, B, and C were 0.13%, 0.16, and 0.22 % respectively; electro-endosmosis (EEO) values were 0.18, 0.29, and 0.36 respectively; and gel strength (1.5 %) were 1594, 1406, and 1219 g/cm² respectively. In this study, isolated agarose gel and agarosa gel standard Thermo Scientific (TopVision®) were used for separation and identification of HPV DNA fractions by gel electrophoresis method. The result showed that banding pattern of isolated agarose gel was close to banding pattern of TopVision® agarose gel. It can be concluded that agarose can be separated from agar using ethylene glycol as a solvent and isopropanol to induce precipitation of agarose.

Key words: Agar, Agarose, Gel Electrophoresis, HPV DNA

INTRODUCTION

Gracilaria gigas (Glacilariaeceae) is one of the marine natural resources with vast potential in Indonesia. This red seaweed produces primary metabolites hydrocolloid compound called agar, which has significant economic value in the cosmetics industry, food, and biotechnology [1]. Agarose is a neutral linier galactose polysaccharide polymer obtained from agar. Agar also contains agaropectin, a sulphated polysaccharide polymer considered to be undesirable when present in agarose. Because agarose contains no charged groups, aqueous agarose gels are employed as a medium for electrophoresis [2]. Impure agaropectin in agarose will affect separation process, where negative sulphate charged group of agaropectin will be attracted toward anode of electrophoresis instrument.

The rapid expansion of interest in the identification and separation of biomolecules such as proteins and nucleic acids, the mapping of genes, and DNA sequencing led to the increasing demands of agarose as separation media. Since Indonesia is a tropical country lush with various seaweed species, we should be able to produce agarose from our own natural resources.

Several methods were reported in the literature for recovering agarose from agar such as by using combination aceton –ammonium sulphate, DMSO (6), by shortening isolation time by adding EDTA-water (12), and by using choline based bio-ionic liquids (bio-ILs)- Isopropanol (13). Agarose was recovered from agar according to

Provoncee [2] by dissolving agar in ethylene glycol at elevated temperature and by precipitating agarose with isopropanol where isopropanol was miscible with the ethylene glycol therefore reducing the polarity of the mixture. Neutral agarose fraction will be precipitated and negative charge agaropectin will then be still dissolved in the solution. Ethylene glycol will interact with agarose at relative high temperatures (105°C), so agarose could be precipitated by decreasing the temperature [2]. This method needs to be refined to obtain high purity agarose.

Researchers are interested in isolating the agarose from agar in order to obtain agarose that could be applied as a medium for separation and identification of HPV DNA by gel electrophoresis method.

MATERIALS AND METHODS

Materials

Agar powder extracted from *Gracilaria gigas* (PT. Satelite Sriti agar-agar powder), ethylene glycol (Brataco), isopropanol (Brataco).

Methods

Isolation of agarose

Isolation of agarose from agar was conducted by the modified method as described by Provonchee [2]. Commercially agar powder was carefully dissolved with ethylene glycol at temperature 70 °C and then the temperature was raised to 105 °C to get 1, 2, and 3% [w/v] agar solution. The mixture was heated to 105°C and maintained at this temperature for one hour.

The solution mixture was removed from hot plate and allowed to cool down. When the temperature decreased to 70°C, the slurry of mixture was placed in freezer at -10ºC overnight. Next day, one litre of isopropanol was added to the slurry at room temperature, the mixture was stirred for two hours and returned to the freezer overnight. The following day, the slurry was resuspended by stirring and then allowed to cool at room temperature to induce formation of an agarose precipitate. The precipitate was filtered with flannel and washed twice with isopropanol before drying at room temperature until the smell of isopropanol was disappeared and agarose granule was formed. Finally, agarose granule were grinded with mortar to get agarose powder for further experiments.

Assay of sulphate content

The sulphate content of agarose was measured from specific infrared absorbance band. The absorbance at 2920 cm^{-1} attributed to C-H was used as an index for total sugar content. Sulfate was determined by Nicolet™ IS™ 10 FT-IR Spectrometer, using a KBr pressing sheet, from the absorbance ratios at certain wave number: total sulphate at 1250/2920, galactose-4-sulfate at 930/2920, and 3,6 anhydrogalactose-2-sulfate at 805/2920. Sulphate obtained from characteristic infrared absorbance bands and determined by base line method [3].

Determination of gel strength

Gel strength of agarose was determined by CT3 Texture Analyzer Brookfield Instrument. The gel strength was measured as the force required by 6 mm diameter probe to penetrate 25 mm thick slandered agarose gel (1.5% w/v) at room temperature. Gel strength was expressed as unit of g/cm².

Measurement of gelling temperature and melting temperature

Gelling temperature and melting temperature were determined by the modified method as described by Santos [4]. Agarose solution (1,5% w/v) was prepared by a method where 5 ml of solution was poured into 18x150 mm test tubes. Tubes then were placed into a rack in 60 °C water bath. When the temperature of the tubes was the same as the bath, cold water was passed through a copper coil in the bath. When temperature in the tubes reached 50°C, flow rate was adjusted with cold water so that the temperature dropped about 0.3-0.5 per minute. The gelling temperature was recorded when the agarose solution did not flow back anymore after it from a normal meniscus.

Agarose solution (1,5% w/v) was prepared as above and solution then allowed to gel for one hour at 20°C. The tubes then was placed into a rack in 60°C water bath. On the surface of each gel sample, a lead shot (25 mg) was dropped. Afterwards the temperature of the bath was raised gradually at 0,3-0,5°C per minute. The melting temperature was then recorded when the gel melted and the lead shots dropped to the bottom of the tube.

HPV DNA identification by isolated agarose

Sample Preparation

10 samples of cervical scrapes and some paraffin-embedded biopsy tissues submitted for HPV assessment were obtained from HPV positive patients. These specimens were derived from women participating in a cervical cancer

screening program and from women attending a gynaecologic assessment at a hospital in Padang. DNA extraction of samples was performed using the gSYNC™ DNA Extraction Kit, following manufacturer's instructions.

DNA Amplification by PCR and HPV identification by electrophoresis

DNA samples were amplified for the presence of HPV using the standard PCR (ThermoCycler®) approach consisting of the GP5+/6+ primer set with 150 bp length amplicon (GP 5+5'-TTT GTT ACT GTG GTA GAT ACT AC-3' and GP 6+ 5'- GAA AAA TAA ACT GTA AAT CAT ATT C-3') as described by Sotlar et al. [7].

Platinum® PCR (Invitrogen®) super mix reagen was used in all Multiplex PCR reactions. The manufacturer's protocol was followed with certain modifications: 22.5 µL Platinum® PCR super mix, 1.5 µL DNA template, 1 µL primer (0.5 µL forward primer and 0.5 µL reverse primer). PCR was carried out in a DNA thermal cycler with the following conditions: 30 cycles with initial denaturing step at 94°C for 5 min, denaturation step at 94°C for 30 s, annealing step at 50°C for 60 s, extension step 72°C for 60 s, and final extension at 72°C for 10 min. DNA of PCR products were analyzed by electrophoresis instrument (mupid-EXU®) on a 1% agarose gel (TopVision®), stained with SYBR® Safe DNA Gel Stain, band sizes were estimated by comparison with a DNA ladder 100 bp (Pure Extreme®), and gels were photographed by GelDoc XR BIO- RAD®. HPV type was assigned based on their bonding patterns.

Electro-endosmosis (relative mobility (—mr))

Electro endosmosis was determined by the modified method as described by Rugui [5] by analyzing the bonding patterns of electrophoresis DNA fractions. EEO was calculated by dividing the distance (OA) between the original sites where the sample was added to purple elliptical speckle around the anode over the distance (OD) between the original sites where the sample was added to the blue speckle around the cathode. Then the electro endosmosis was expressed as follows [10]:

$$EEO = \frac{OD}{OA+OD}$$

Application of isolated agarose for HPV DNA identification

With the same procedure as described above, positive HPV samples (PCR products) were separated by an electrophoresis method on a 1% isolated agarose gels (agarose A, agarose B and agarose C) and DNA banding patterns on isolated agarose media were compared with banding patters agarose gel standard (TopVision®).

RESULTS AND DISCUSSION

Agarose yield (%)

Isolation of agarose from 1, 2, 3 % (w/v) agar solution in ethylene glycol gave yield 72. 40, 76.83, and 83.55% as presented in Fig. 1

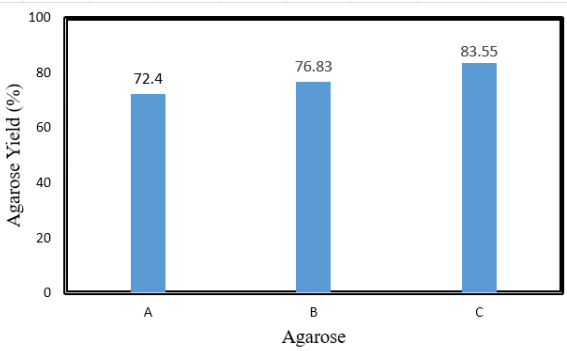

Fig. 1. Agarose yield obtained from different agar concentrations in ethylene glycol (A) Agarose obtained from 1% agar concentration [w/v]; (B) Agarose from 2% [w/v] agar; (C) Agarose from 3% agar. Yield was expressed as a percentage of dry weight

According to the yield results of this experiment, when agar concentration was increased, the agarose yield became higher, this may be due to higher amount of ethylene glycol in the agarose extracted. Furthermore, higher amount of agar makes stickier solution, this makes purification more difficult when isopropanol was added to precipitate the agarose. In this study, agarose being dried at ambient temperature without using oven because elevated temperature may cause Maillard reaction (also known as browning). The Maillard Reaction is a type of non-enzymatic browning which involves the reaction of simple sugars (carbonyl groups) and amino acids (free amino groups) and is responsible for decreasing the quality of agarose.

The above results of yields are different from the concentration reported by Provenchee [2]. The differences were probably caused by variations in agar species, ion exchange, reagents, eluting temperature and filtering method.

Gelling temperature and melting temperature

The measurement of Gelling temperature and melting temperature of agarose A, B and C were shown in Table 1.

Table 1. The gelling temperature and melting temperature of agarose

Sample	Gelling temperature (°C)(1.5% gel)	Melting temperature (°C)(1.5% gel)
TopVision® agarose (standard)	35,50	75.40
Agarose A	36.77	85.57
Agarose B	36.63	86.27
Agarose C	37,96	82.93

From data presented in table 1 can be concluded that Gelling temperature and melting temperature of isolated agarose were close to the agarose standard.

Table 1 shows that the gelling temperature and melting temperature have changed compared with TopVision® agarose. The factors that affect gelling temperature and melting temperature are unknown. Many factors may affect the gelling temperature and melting temperature including the formation mechanism, the type and quantity of substituents, and also the molecular weight. The differences in sulphate content and molecule weight of agarose may cause the change in gelling temperature and melting temperature [5].

Sulphate content

The measurement of total sulphate by calculating with ratio absorbance at wave number 1250/2920 of isolated agarose A,B, and C were 0.13, 0.16, 0.22 % respectively. The measurement of galactose-4-sulphate by calculating the ratio of absorbance at wave number 930/2920 from isolated agarose A,B, and C were 0.28, 0.38, 0.27 % respectively. The measurement of 3,6 anhydrogalactose-2-sulphate by calculating the ratio of absorbance at wave number 805/2920 of isolated agarose A,B, and C were 0.14, 0.12, 0.30 % respectively

It can be concluded that the higher the concentration of agarose in ethylene glycol solution in the extraction process, the higher sulphate content will be found in the isolated agarose.

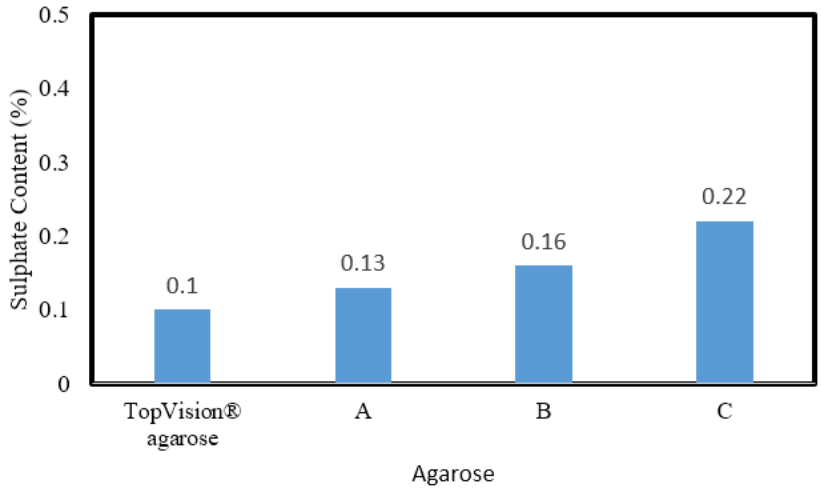

Fig. 2. The sulphate content of agarose samples, (A) Agarose obtained from 1% [w/v] agar; (B) Agarose from 2% [w/v] agar; (C) Agarose from 3% [w/v] agar

Sulphate content was obtained from characteristic infrared absorbance bands and determined by base line method using Nicolet™ IS™ 10 FT-IR Spectrometer. The IR technique is rapid, non-destructive and requires only a few mg of sample. There is an inverse relationship between gelation and sulphate content of agar and agarose [6]. In this experiment, the amount of sulphate contained in different agarose samples were projected (Fig. 2).

According to the results, sulphate content of TopVision® agarose, agarose A, agarose B, agarose C were 0.1%, 0.13%,0,16%, 0,22%, respectively. Sulphate content of isolated agarose A and B as well as a commercially available TopVision® agarose, and sulphate content of agarose C was higher than that of isolated agarose A and B. This may

be due to incomplete separation Agarosa from Agaropectin. It need to be considered that all sulphate in agar is probably not in the form of half esters, double ester sulphates are also conceivable.

The absorbance at 1250 has been used as a quantitative method for determining the total sulphate content in the agarose. Bands at 1250 are due to sulphate, The band may be used in comparison with the absorbance of C-H band at 2920 as an index relating to total sulphate band [3] because C-H represents a good reference for total sugar content because the number of C-H groups remains constant irrespectively of changes in the ratio of galactose to 3,6- anhydrogalactose and sulfate content of the galactans [3].

Gel strength

One of the most important factors contributing to the success of agarose as an anti-convection medium is its ability to exhibit high gel strength at low concentrations. The gel strength of the agarose extracted in present study was shown in Fig. 3.

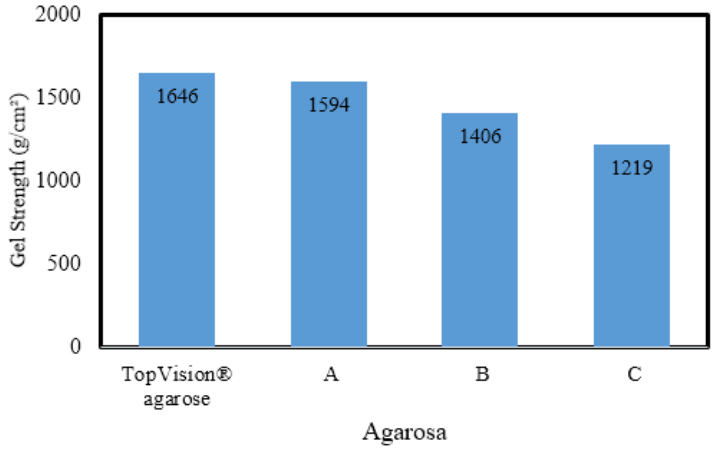

Fig. 3.Agarose gel strength (g/cm²) obtained from different agar concentrations in ethylene glycol. (A) Agarose obtained from 1% agar concentration [w/v]; (B) Agarose obtained from 2% [w/v] agar; (C) Agarose obtained from 3% [w/v] agar. Gel strength was measured with 1.5% agarose gel

According to the results, all agarose samples (at 1.5% agarose concentration) exhibited good gel strengths (g/cm²) and were in the range required for commercial agarose (>800 g/cm²) or other agarocolloids [8]. Gel strength of TopVision® agarose, agarose A, agarose B, agarose C were 1646 g/cm², 1594 g/cm², 1406 g/cm², 1219 g/cm², respectively.

The gel strengths (1.5% gel) agarose A, agarose B, agarose C and TopVision® agarose were determined and compared with their sulphate content. The gel strengths were enhanced as the sulphate content decreases. The sulphate content is one of the main factors affecting the gel strength of agarose since the agarose contains just a small amount of embranchment structures and other substituents. In the application as stationary phase of gel electrophoresis method, gel strength of agarose is very important; therefore, conditions involved in the agarose preparation must be then carefully controlled.

The low gel strength attributed with the agarose C may be caused by insufficient transformation and conformation of the substitute groups in the agar polymer.

Agarose gel formation characteristics caused by the three H atoms in the 3,6-anhidro-L-galactose, which forces the molecules to form a helical structure. Helix-helix interaction of these structure, will lead to the formation of a gel. This helical structure would not have happened if all galactose in an agarose present in the form of galactose-6-sulfate. Sulphate are charged ions and require a large space in the molecule. Thus in the presence of sulphate, ionic force occurred between charged molecules, so that the distance between the molecules become further and the gel strength become lower.

Detection of HPV DNA by gel electrophoresis method

Described by Pastrana [9]. A total of 10 samples of cervical scrapes and paraffin-embedded tissue biopsies submitted for HPV assessment were collected. They were first analyzed by PCR using GP5+/6+ primer set with 150 bp length amplicon described by Sotlar et al [7]. After being electrophorized, all samples showed near identical band size as the expected HPV PCR product (10 samples were HPV positive). The same samples (positive samples) were analyzed by gel electrophoresis using Polymerase Chain Reaction (PCR) with consensus primers can potentially

detect most mucosal HPV types. There are several consensus primers isolated agarose A, B, and C and the banding patterns were compared with commercially available agarose TopVision®.

Electro-endosmosis (relative mobility (—mr))

Electro-endosmosis (EEO) is one of the quality standards of agarose. The EEO of isolated agarose A, B, C, and TopVision® agarose are shown in Table 2.

Table 2. Electro-endosmosis (relative mobility (—mr))

Agarose	Electroendosmosis (EEO)
TopVision® agarose	0,16
A	0,18
B	0,29
C	0,36

The EEO of TopVision® agarose, isolated agarose A, B, and C were 0.16, 0.18, 0.29, 0.36, respectively. The EEO of agarose A is 0.18 which meets the quality standard (0.16–0.18) of EEO Sigma agarose for electrophoresis. EEO is one of the most important quality standards of agaroses, which reflects the amount of negative charges carried by the agarose gel [11].

Application of isolated agarose for HPV DNA identification

Data obtained from the electrophoresis method presented descriptively in the form of a gel photograph (Figure 4).

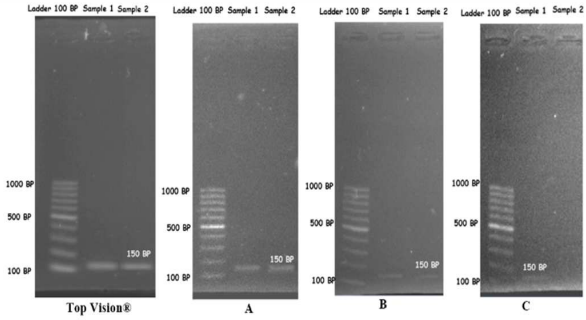

Fig. 4. Comparison of the results of gel electrophoresis. 100 kb DNA ladder marker (from 1000 bp to 100 kb) and positive samples were fractionated in 1% agarose gels prepared from commercial agarose (TopVision®) and agarose A, B, and C in this study

According to the experiment, all separation process showed positive results with the banding pattern parallel to the DNA ladder at 150 bp. The results of DNA reactions on isolated agaroses were almost similar to the banding pattern of TopVision® agarose and was verified with precise banding pattern without any smearing or high background fluorescence. DNA bands on agarose B and C are slightly less clear than the TopVision® agarose and agarose A, This may be influenced by the colour visual appearance of agarose gel B and C which have a rather yellowish colour in comparison to agarose A and agarose TopVision®.

The banding patterns of the DNA ladder of isolated agaroses were verified to be free from contaminants that will cause inhibition or slowdown of the DNA fraction migration [6]. This was an evidence of the purity and suitability of the isolated agarose for practical purposes in DNA separation.

It can be concluded that isolated agarose obtained from these experiments can be used for separating DNA fraction by electrophoresis method.

CONCLUSION

Agarose can be separated from 1,2,3 % (w/v) of agar solution in ethylene glycol with yield of 72,40, 76.83, 83.55%. The physical properties of isolated agaroses were determined and compared with commercially available TopVision® agarose (electrophoresis grade). Gelling point and melting point of isolated agarose were comparable with agarose standard. The sulphate content, electro-endosmosis (EEO) and gel strength (1.5%) of agarose A, B, and C were 0.13%, 0.18, and1594 g/cm^2; 0.16%, 0.29, 1406 g/cm^2; 0.22 %, 0.36, 1219 g/cm^2, respectively. Agarose A, agarose B, and agarose C can be used as medium for separation and identification of HPV DNA.

It was found that sulphate content of isolated agaroses were slightly higher than agarose standard however it has no bad influence to HPV DNA separation and EEO value.

Electrophoresis was conducted on gel of agarose A that showed clear separate extinction-banding pattern of DNA ladder and the positive results at 150 bp (as also observed from commercial TopVision® agarose) and indicated the suitability of the agarose for the separation of HPV DNA fractions.

Acknowledgments
This study has been supported by The research competency grant Programme from Minister of Research-Technology and High Education, Republic of Indonesia.

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