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Short communication

Successful preparation and characterization of biotechnological grade agarose from indigenous *Gelidium amansii* of Taiwan

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ABSTRACT

This paper reports the first successful preparation of biotechnological grade agarose from *Gelidium amansii* found in Taiwan. The scale-efficiency preparation was achieved by shortening EDTA treatment time through dispersing *G. amansii* agar in water in the presence of heat and EDTA, removing agaropectin impurity with a heat-compatible and strong-anion exchange resin, and precipitating agarose with a cost-effective isopropanol method. The yield of agarose from prepared *G. amansii* agar was 11.3%. The acquired agarose has a gel strength of 853 g cm^{-2}, a sulfate content of 0.14%, a pyruvate content of 1.03%, a degree of electroendosmosis of 0.16 and very limited binding affinity to DNA. The excellent properties of agarose from *G. amansii* of Taiwan confirm its potential diverse biotechnological applications. This innovative agarose preparation method with the significantly improved scale-efficiency can be modified for large-scale preparation of agarose for use in biotechnological industry and biochemical research.

1. Introduction

Agar and its sub-fraction, agarose, are two of the most commonly used polysaccharide mixtures obtained from marine red algae (Rhodophyta), which includes the commercially important genera *Gelidium* and *Gracilaria*. Agarose preparations from both genera have important biotechnological applications [1,2] and are widely exploited in the food industry [2,3]. *Gelidium* and *Gracilaria* have played a key role in the field of biotechnology since Koch demonstrated agar was a suitable solid medium for growing microorganisms [1]. For example, agarose gel electrophoresis is currently the predominant method for routine nucleic acid analysis [1,2,4], and has implications in studying nucleic acid–protein interactions [5,6], protein chemistry [7], and viral structure [8]. In addition, agarose-based chromatography is the preferred method in biomolecule separation [1,2]. Moreover, agars and agarose have important applications in a variety of areas including pharmaceutics, cosmetics, tissue engineering, cell-sized liposome preparation and cell encapsulation [2,9,10].

The advantages of using agarose in science and industry derive from its ability to form macroporous matrices with thermoreversible hysteresis and a high gel strength, its non-toxicity after hydrogel formation, and its maintenance of a minimal gel background after rapid staining and destaining [2,4,11]. These essential agarose properties are attributed to the alternating 1,3-linked β-D-galactose and 1,4-linked 3,6-anhydro-α-L-galactose repeating units called agarobiose (Fig. 1; $R_1 = R_2 = R_3 = H$) which polymerize into a long chain [12,13]. The monosaccharide units of natural agarose are often modified with charged groups such as sulfate and pyruvate which transforms agarose into agaropectin (Fig. 1). However, presence of agaropectin is undesirable for the agarose prepared for biotechnological applications [4,14–16]. The negative properties of agaropectin are the consequence of unsaturated chemical bonds in the sulfate and pyruvate substitutions; these bonds bestow high UV absorption in agarose gels and interfere with the detection of nucleic acids after electrophoresis [4,15]. Additionally, these substitutions cause significant biomolecule adsorption on agarose matrices [4,15]. Agarose used in fundamental research and biotechnology is required to have a minimal amount of sulfate and pyruvate modifications.

Preparation of agarose for biotechnological applications has been extensively studied after the seminal work of Araki [12,13].

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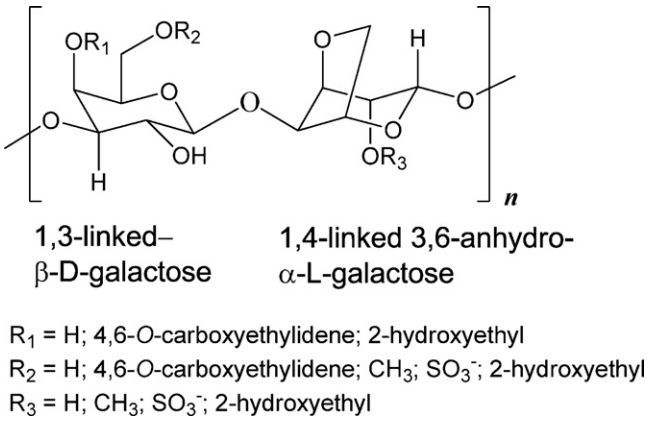

Fig. 1. The generic structure of agarose and agaropectin composed of repeating units of the disaccharide, agarobiose. The structural differences between agarose and agaropectin are indicated by substitutions with various functional groups at R_1, R_2 and R_3.

Various methods have been developed to prepare high-grade agarose from either high quality agars or low-grade agarose. Preparation has included fractional precipitation methods [14,15,17–19] such as isopropanol (IPA) precipitation, adsorption methods [20,21] such as aluminum hydroxide treatment, or chromatography methods [14] such as DEAE ion exchange chromatography. Prepared agarose is generally evaluated using parameters which include gel strength, the temperatures at which it solidifies and melts, sulfate content, pyruvate content, and electrophoretic properties of the gel [1,15]. Typically, biotechnological grade agarose has a gel strength of at least 1000 g cm^{-2}, a gelation temperature of 36 °C, a melting temperature of 85 °C, a sulfate content of 0–0.15% (w/w), a pyruvate content of 0–0.1% (w/w) and a degree of electroendosmosis (EEO) at 1.0% (w/w) agarose concentration of 0.04 or less [15].

As a potential source for high quality agarose, *Gelidium amansii* is very abundant in northeastern Taiwan and has long been a staple in traditional Taiwanese diets. *G. amansii* agar is easily prepared from the alga by heating, filtering and cooling without chemical treatments [4], which is in sharp contrast to that of *Gracilaria* agar preparation required more complicated procedures [22]. Various *G. amansii* applications underscore the importance of the algal extracts for the Taiwanese food industry and suggest a plausible role in the pharmaceutical industry [23]. However, to date, there has been no attempt to use Taiwanese *G. amansii* to prepare high quality agarose for biotechnological use.

In this study, high quality agarose was prepared from indigenous Taiwanese algae. The unique and scale-efficient method for agarose preparation adopts three critical procedures: heat dispersion of *G. amansii* for efficient EDTA chelation while significantly reducing treatment time from 4 d to 1 d, effective separation of agarose from impurities by a heat-compatible anion exchange resin, and a cost-effective IPA precipitation. Its low absorption under UV illumination, good electrophoretic properties, and lack of DNA adsorption in the agarose gel are indicative of the excellent chemical and physical properties of agarose prepared using indigenous Taiwanese *G. amansii*. Our successful method to prepare biotechnological grade agarose from *G. amansii* native to Taiwan with the scale-efficiency reveals significant potential in biotechnology applications. Finally, the innovative preparation method can be modified to accommodate industrial-scale production.

2. Materials and methods

The materials and detailed experimental methods are described in Supplemental data.

3. Results

3.1. Temperature and concentration optimizations of EDTA treatments for agarose preparation

Two essential determinants of agarose suitability for biotechnological applications are the concentration of sulfate and pyruvate [15]. Kirkpatrick et al. based on their analysis methods to conclude that agarose with a sulfate content of 0–0.15% (w/w) and a pyruvate content of 0–0.1% (w/w) is required to qualify as biotechnological grade [15]. Treating agar or low-grade agarose with EDTA effectively reduces the concentration of both of sulfate and pyruvate in the samples when used in combination with subsequent separation methods such as adsorption [21] and IPA precipitation [15]. The original method of Barteling used 20 mM EDTA in the first agar wash for 2 d and 10 mM EDTA in the second agar wash for another 2 d at room temperature [21]. We reasoned that higher concentrations of EDTA in the presence of heat would result in more effective EDTA chelation of divalent cations from the agaropectin and facilitate preparation of biotechnological grade agarose.

Increasing both the temperature of the agar solution and the EDTA concentration to 30 mM during incubation significantly reduced the content of sulfate and pyruvate in the prepared agarose (Fig. 2). First, 45 °C was selected as the incubation temperature of the agar solution during EDTA treatment due to a concern of energy consumption by the prolonged incubation. In addition, the EDTA concentrations were changed from 20 mM to 40 mM for the first wash and 10 to 20 mM for the second wash. The method of Barteling requires 4 d to complete two EDTA washes [21], a process too time-consuming for industrial processing requirements. Therefore, the time of each EDTA wash was shortened to only 12 h to allow the overall EDTA treatment steps completed in 1 d. Results indicated that the optimal conditions for agarose preparation were 30 mM EDTA for the first wash, 15 mM EDTA for the second wash, and an incubation time of 12 h for each wash at 45 °C (Fig. 2). Since 45 °C might not be the optimal temperature for the EDTA treatment, the 30 mM/15 mM EDTA wash steps were conducted at several different incubation temperatures to determine the optimal temperature to further reduce sulfate and pyruvate contents. Incubation of the agar solution at 50 °C during the 30 mM/15 mM EDTA treatment step provided the lowest agarose sulfate and pyruvate content (Fig. 2). It is concluded that the 30 mM/15 mM EDTA treatment with an incubation time of 12 h for each wash at 50 °C is the optimal EDTA treatment method to prepare *G. amansii* agarose.

3.2. Biotechnological grade agarose preparation with excellent electrophoretic properties

We further substituted the $Al(OH)_3$ adsorption method with ion exchange column chromatography to obtain agarose with lower sulfate and pyruvate content to meet the demands of biotechnological applications. A prerequisite to effectively removing more of the extensively modified agaropectin from the agarose preparation during ion exchange column chromatography is to completely dissolve agarose, generally required to maintain the solution temperature over 80 °C, in an aqueous solution containing ion exchange resin. The study was thus motivated to exploit commercially available, heat-stable, and strong anion exchange resins. The properties of the hydrophilic SuperQ-650M anion exchange resin are ideal for preparing agarose suitable for biotechnological applications (Table 1). However, the sulfate and pyruvate content in the prepared agarose were still not low enough to meet biotechnological grade requirements [0.99% sulfate content (w/w); 1.40% pyruvate content (w/w)].

The preparation of biotechnological grade agarose was achieved by further purifying agarose using an improved IPA precipitation

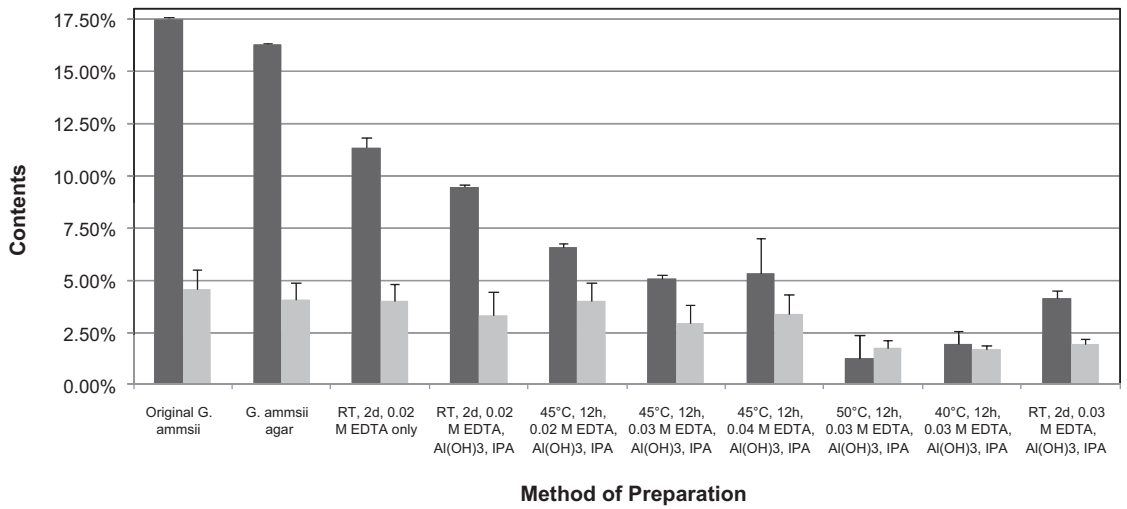

Fig. 2. Sulfate and pyruvate content in *G. amansii*, *G. amansii* agar and *G. amansii* agarose prepared by various methods. The content of sulfate (black bar) and pyruvate (gray bar) in samples were determined by the methods detailed in Section 2. Data are expressed as means ± standard deviations of triplicate determination.

Table 1
Chemical and physical properties of biotechnological grade agarose prepared from Taiwanese *G. amansii* compared to commercially available agarose. Data are expressed as means ± standard deviations of triplicate determination.

Agarose samples	Sulfate content (%, w/w)	Pyruvate content (%, w/w)	Gel strength ($g\,cm^{-2}$)	Gelling temperature (°C)	Melting temperature (°C)	Degree of EEO
SuperQ-650 M (from this study)	0.14 ± 0.01	1.03 ± 0.07	853 ± 11	34 ± 0	75.8 ± 0.3	0.16 ± 0.01
SeaKem LE (Lonza)	0.35 ± 0.01	1.07 ± 0.10	945 ± 16	36 ± 1.5[a,b]	>90[a,b]	0.22 ± 0.01
BIO-41027 (Bioline)	5.36 ± 0.28	0.44 ± 0.04	154 ± 10	37–39[a]	88–90[a]	0.18 ± 0.01

[a] Data provided by the manufacturers (http://lonza.com/group/en.html and http://bioline.gene-quantification.info/).
[b] Measured in 1.5% agarose gels.

method. Kirkpatrick et al. previously demonstrated that the addition of IPA to agarose solutions (2:1 volume ratio of IPA to agarose solution) could selectively precipitate agarose [15]. With the goal of developing a more cost-effective IPA precipitation procedure by decreasing volume of IPA without sacrificing the efficiency of agarose precipitation, the volume of IPA was systematically reduced to determine the smallest volume of IPA required to effectively precipitate agarose (Fig. S1). The results indicated that, by using a 1.5:1 volume ratio of IPA to agarose solution, the agarose yields were comparable to that of a 2:1 ratio. Thus, a volume ratio of IPA to agarose solution of 1.5 was determined to be the optimal condition for the IPA precipitation in preparing biotechnological grade agarose. The prepared agarose had sulfate and pyruvate contents of 0.14% and 1.03%, respectively (Table 1 and Fig. S1). These levels were lower or comparable to two commercial biotechnological grade agarose products (Lonza SeaKem LE and Bioline BIO-41027). The low sulfate and pyruvate contents of the SuperQ-650M-prepared agarose also contributed a good EEO value of 0.16, which is better than two commonly used, commercial biotechnological grade agarose products.

The excellent biotechnological properties of the SuperQ-650M-prepared agarose were demonstrated when DNA samples were analyzed simultaneously by electrophoresis in gels prepared from either the SuperQ-650M or the Lonza SeaKem LE agarose (Fig. 3). The SuperQ-650M-prepared agarose gel provided better DNA resolution even through with a slightly higher background signal when compared to that of the gel prepared using Lonza SeaKem agarose (signal intensity, quantified by ImageQuant software, of 9×10^5 in SeaKem gel and of 1.1×10^6 in SuperQ-650M-prepared gel). Moreover, net DNA band signals after electrophoresis in the SuperQ-650M-prepared agarose gel had higher intensity counts and, thus, better DNA detection sensitivity than that of the SeaKem agarose (net signal intensity of 202 bp = 6.6×10^6 in the SeaKem gel and 7.3×10^6 in the SuperQ-650M-prepared gel for 202 bp DNA). Similarly, evidence of better electrophoretic properties of the SuperQ-650M-prepared agarose gel was provided when compared with electrophoresis results of the Bioline agarose gel (results not shown). The SuperQ-650M-prepared agarose gel also showed no detectable DNA binding ability (Fig. S2). The outstanding electrophoretic results obtained when using gels prepared using the SuperQ-650M agarose are consistent with the relatively low sulfate and pyruvate content, and a low degree of EEO of the agarose, which further supports the potential of the *G. amansii* agarose for diverse biotechnological applications.

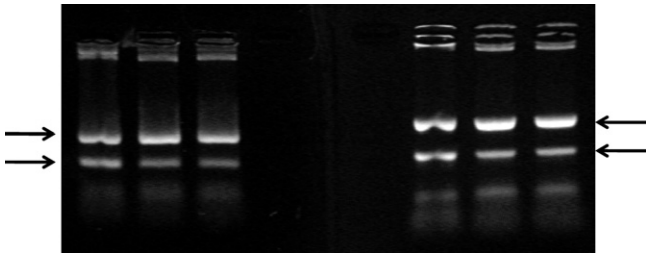

Fig. 3. Comparison of excellent gel electrophoresis properties of SuperQ-650M-prepared agarose and SeaKem LE agarose. DNA used for gel electrophoresis contained mostly 100-bp and 202-bp DNA fragments amplified from the pGEM-T vector as described in Section 2. A DNA sample (32 ng of 202 bp and 36 ng of 100 bp) was loaded into each well of 3% gels prepared from the SeaKem LE (left) or from the SuperQ-M650-prepared (right) agarose in TBE, and electrophoresed in both gels simultaneously in the same standard electrophoresis unit. Triplicate DNA samples were applied to each agarose gel to ensure result reproducibility. The top arrow indicates the location of the 202-bp DNA marker; the bottom arrow represents migration of the 100-bp DNA marker after electrophoresis.

3.3. Physical properties of the biotechnological graded agarose

The physical parameters for the SuperQ-650M-prepared agarose were compared to Lonza SeaKem LE and Bioline BIO-41027 agarose (Table 1). While the gel strength was slightly lower than that of SeaKem agarose, the SuperQ-650M-prepared agarose had more desirable lower gelling and melting temperatures, critical properties to exploit agarose in nucleic acid gel electrophoresis applications. The appropriate physical properties of the SuperQ-650M-prepared agarose meet the requirements for applications in biotechnology.

4. Discussion

Successful preparation of biotechnological grade agarose from indigenous *G. amansii* of Taiwan with scale-generated efficiency was attained by decreasing EDTA treatment time, incorporating a novel anion exchange resin to purify agarose from impurities, and decreasing the required volume of IPA to effectively precipitate agarose. Washing *G. amansii* agar in the presence of higher EDTA concentrations and elevated temperatures not only contributed decreases of the degree of EEO and the levels of sulfate and pyruvate in the prepared agarose but also significantly reduced the EDTA washing time from 4 d to 1 d which eliminates 3 d from previously reported EDTA methods [15,21].

Ion exchange column chromatography was indispensable in this study to obtain agarose suitable for biotechnological application. Essential properties of the SuperQ-650M resin allowed for a more effective separation of agaropectin from solutions and resulted in agarose with lower sulfate and pyruvate content and good electrophoretic properties (Table 1 and Fig. 3). However, special precaution must be taken during the agarose-SuperQ-650M resin incubation to prevent damage during mixing. Unattended mixing while suspending the SuperQ-650M resin in the agarose solution can rupture the resin beads. Resin debris can pass through the filter disk, contaminate the agarose, and deleteriously affect the electrophoretic properties of the prepared agarose gel. As stated in Materials and methods in Supplementary data, we carefully stirred agarose mixtures by hand for 30 min to ensure the anion exchange resin remained suspended in solution while avoiding breakage of the SuperQ-650M resin. We never observed debris of the SuperQ-650M resin in agarose preparation if the same vigilant measures were taken.

The development of a method to reduce the required IPA volume for effective agarose precipitation is also an important finding of the current study. By using less IPA during the preparation of agarose, industry operation costs can be reduced. In addition, the improved IPA precipitation method can be a more environmentally friendly process if IPA is accidentally released during industrial agarose preparation.

The SuperQ-650M-prepared agarose demonstrated physical properties comparable or better than two commercially available agarose products (Table 1). Specifically, the SuperQ-650M-prepared agarose has lower gelling and melting temperatures, and comparable gel strength than those of SeaKem LE gel. Interestingly, the gel strength and the sulfate and pyruvate content of both the Lonza and Bioline agarose, which were measured in the current study, differed from those reported by the manufacturers. Discrepancies in values between reported physical and chemical parameters and those presented in this study may be the result of differing measurement methods. No standard protocols for agarose characterization have been accepted by academia or industry. Current results, therefore, provide no basis for disputing the accuracy of agarose specifications documented by the manufacturers. Nevertheless, the SuperQ-650M-prepared agarose unequivocally offers ideal properties for biotechnological applications. Research is proceeding to improve and refine the procedures to enable industrial production of agarose from indigenous *G. amansii* of Taiwan as a way to achieve its full economic potential.

Author contributions

T.-P. W. designed, analyzed data and wrote manuscript; L.-L. C. performed research and analyzed data; S.-N. C. provided expertise, performed research and analyzed data; E.-C. W. provided expertise; L.-C. H. provided useful reagents; Y.-H. C. provided useful discussions and analytic tools; Y.-M. W. provided useful discussions and analyzed data.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2011.12.015.

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