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Physico-chemical and gel properties of agar from *Gracilaria tenuistipitata* from the lake of Songkhla, Thailand

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ABSTRACT

Physico-chemical and gelling properties of agar extracted from *Gracilaria tenuistipitata* as affected by alkaline pretreatments using NaOH and KOH at various levels (3–7%, w/v) were investigated. Yield of native agar was 17.1%, whilst those of agars pretreated with NaOH and KOH ranged from 23.6% to 26.1%. Agar with alkaline pretreatment generally showed the better gelling property as evidenced by higher gel strength, gelling, melting temperatures and viscosity with coincidentally increased 3,6-anhydrogalactose (3,6-AG) content than did native agar. Additionally, native agar had a higher sulphate content with lower syneresis ($P < 0.05$) than those with alkaline pretreatment. Regardless of alkaline concentration, NaOH used for pretreatment rendered agar with a higher quality, compared with KOH. Agar (1.5%, w/v) pretreated with 5% NaOH exhibited the highest gel strength (482 g/cm^2) with high yield (25.3%). The decrease in total sulphate content and the increase in 3,6-AG content were observed in agar having 5% NaOH pretreatment as determined by FTIR spectroscopy. Finer and more compact network with smaller pores was visualised in gel from agar with 5% NaOH pretreatment. Therefore, the appropriate pretreatment by using 5% NaOH could increase yield and improve the gelling property of agar from *G. tenuistipitata* harvested from the lake of Songkhla, Thailand.

1. Introduction

Hydrocolloid based gels are gaining importance in food industries due to several features, which can render the unique functional attributes, especially texture (Ross-Murphy, 1994). Agar is frequently used as a thickening and gelling agents in various food formulations, mainly because of cost effectiveness, availability, and the ability to form gel with other liquid foods when cooled (Norton, Jarvis, & Foster, 1999). Agar is a complex mixture of polysaccharides found in the cell matrix of red algae (Rhodophyta), comprising two major components, commonly known as agarose and agaropectin. Agarose is a neutral polysaccharide with a linear structure of repeating units of agarobiose formed by 3 linked β-D-galactose and 4 linked 3,6-anhydro-α-L-galactose (3,6-AG). Agaropectin is an acid polysaccharide containing sulphate groups, pyruvic acid, and D-glucoronic acid conjugated to agarobiose (Araki, 1966). The type, pattern and degree of substitution as well as molecular weight determine the gelling properties of agar (Villanueva, Sousa, Gonçalves, Nilsson, & Hilliou, 2010). Additionally, numerous studies have been conducted on extraction and characterisation of agar from different cultivations and geographical area (Kumar & Fotedar, 2009; Yenigül, 1993). Properties of the agar differ, depending on species (Praiboon, Chirapart, Akakabe, Bhumibhamon, & Kajiwara, 2006), season variation (Kumar & Fotedar, 2009), environment factors (Bird, 1988) and extraction process (Yousefi, Islami, & Filizadeh, 2013).

Gracilaria is one of the genera, belonging to Rhodophyta family (Freile-Pelegrın & Murano, 2005). It is available in tropical regions and has high potential for culturing. However, agar produced from *Gracilaria* spp. renders gel with a low quality. This is associated with high sulphate content. Sulphate acts as kinks in agar helix formation, thereby hindering gel network formation. Generally, alkaline pretreatments are well demonstrated for desulphation, by which L-galactose-6 sulphate is converted to 3,6-AG (Rees, 1972). Alkaline pretreatment variables, such as alkaline type and concentration or heating time and temperature affected the yield and quality of the agar (Arvizu-Higuera, Rodríguez-Montesinos, Murillo-Álvarez, Muñoz-Ochoa, & Hernández-Carmona, 2008). Yousefi et al. (2013) reported that soaking of *Gracilaria corticata* with 5% NaOH at a ratio of 1:200 for 1 h at 40 °C, followed by extraction at 80 °C for 2.5 h

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yielded the agar with prime quality. Freile-Pelegrín and Robledo (1997) found that pretreatment of *Gracilaria cornea* with 3% or 5% NaOH rendered the agar with the highest gel strength and low sulphate content.

Gracilaria tenuistipitata (G. tenuistipitata) is currently dominant in Pattani and Songkhla provinces, Thailand. It has high growth rate and is tolerant to a wide range of environments. It is normally found in shallow mudflat area. In general, it is sold as fresh or dried form for direct consumption and export. Due to its abundance and for better exploitation, *G. tenuistipitata* has become an economically important raw material for agar production. Montaño, Villanueva, and Romero (1999) reported that agar from *G. tenuistipitata* harvested from the Philippines had a higher gel strength than did the agar from *Gracilaria arcutata*. However, a little information regarding the extraction and characterisation of agar from this species has been reported. Therefore, the objective of this study was to investigate the influence of alkaline pretreatments using NaOH and KOH at different concentrations on yield and physicochemical and gelling properties of agar from *G. tenuistipitata*.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. Resorcinol was purchase from Sigma Chemical Co. (St. Louis, MO, USA). Potassium sulphate and barium chloride were procured from Ajax Finechem, Pty. Ltd. (Taren Point, NSW, Australia). A commercial agar powder was obtained from HiMedia laboratories Pvt. Ltd (Mumbai, India).

2.2. Collection and preparation of G. tenuistipitata

G. tenuistipitata were gathered from the lake of Songkhla, Koh Yo (7°09′30.0″ N, 100°32′08.7″ E), Songkhla province, Thailand during March and April, 2014. Harvested algae were transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 1 h. Algae were washed with tap water to remove the impurities. The clean algae were sun-dried, cut into small pieces (1.5 cm in length) and kept in plastic bag at 4 °C until use.

2.3. Alkaline pretreatment and agar extraction

Alkaline pretreatment was performed as per the method of Freile-Pelegrín and Robledo (1997) with a slight modification. Prepared algae (30 g) were soaked in 1500 mL of NaOH or KOH solutions at different concentrations (3%, 5% and 7%, w/v) for 24 h at room temperature (27–30 °C). The mixtures were subsequently heated at 90 °C in a temperature controlled water bath (Memmert, Schwabach, Germany) for 3 h with a continuous stirring using an overhead stirrer (W20.n, IKA®-Werke GmbH & CO.KG, Stanfen, Germany). After alkaline pretreatment, the algae were washed with running tap water to remove the alkali for 2 h. Thereafter, the pretreated samples were neutralised in 0.025% H_2SO_4 with an algae/solution ratio of 1:50 for 1 h. The neutralised samples were washed with running water until neutral pH was obtained (pH 6.8–7.0).

Agar extraction was carried out from native algae (without pretreatment) and those pretreated with different alkaline conditions. The samples (30 g) were mixed with 1500 mL of distilled water at 95 °C. Extraction was performed for 1.5 h with continuous stirring. At the designated time, all extracts were prefiltered through a cheesecloth and filtered under the pressure using a Buchner funnel with a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). All filtrates were transferred into plastic container to obtain a thickness of 2 cm. Filtrates were allowed to gel at room temperature, frozen for 24 h and thawed at room temperature for approximately 4 h. Thereafter, the thawed gels were oven-dried at 55 °C until a constant weight was obtained. The dried agars were subsequently ground using a National Model MX-T2GN blender (Taipei, Taiwan). The yields of agars were calculated based on the dry weight of the starting algae. The agar powders were subjected to analyses.

2.4. Determination of chemical compositions

2.4.1. Sulphate content

Sulphate content of agar samples was measured turbidimetrically using $BaCl_2$-gelatin method after hydrolysis in 0.5 M HCl as described by Dodgson and Price (1962) with a slight modification. Agar powder (40 mg) was hydrolysed in 6 mL of 0.5 M HCl at 105–110 °C for 12 h. After cooling, the solutions were mixed thoroughly and filtered with a Whatman No. 1 filter paper. A portion (0.2 mL) was transferred to a 10-mL tube containing 3.8 mL of 3% trichloroacetic acid and 1 mL of $BaCl_2$-gelatin reagent. The solution was mixed vigorously and incubated at room temperature for 20 min. The absorbance at 360 nm was measured using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Blank was prepared in a same manner, except the distilled water was used instead of agar sample. Commercial agar was used as a reference. A standard curve was prepared using K_2SO_4 at concentration ranging from 0.1 to 1 mg/mL (~0.0533–0.533 mg SO_4^{2-}/mL). The sulphate content was calculated and expressed as the percentage (dry weight basis).

2.4.2. 3,6-AG content

3,6-AG content was determined colourimetrically using the resorcinol-acetal method as described by Yaphe and Arsenault (1965). Agar solution (0.02 mg/mL, 2 mL) was transferred into 20 × 150 mm screw cap tube containing 10 mL of cold resorcinol-acetal reagent. The mixtures were mixed thoroughly and allowed to cool in ice bath for 3 min. The tubes were placed in 20 °C water bath for 4 min and then heated at 80 °C for 10 min in the dark. After cooling, the absorbance at 555 nm was read within 15 min. Commercial agar was used as a reference. A standard curve was prepared using D-fructose at concentration ranging from 0.018 to 0.09 mM. The 3,6-AG content was calculated and expressed as the percentage (dry weight basis).

2.5. Determination of gel properties

Agar gels were prepared by the method of Kumar and Fotedar (2009). Agars were dissolved in boiling distilled water to obtain a final concentration of 1.5% (w/v). The solution was stirred using a hot plate stirrer (IKA Labortechnik stirrer, Selangor, Malaysia) until agar was solubilised completely. The agar solutions were transferred to a cylindrical mold with 3 cm diameter and 2.5 cm height. The solutions were incubated at a refrigerated temperature (4 °C) for 12 h. The gels were equilibrated at room temperature (25–30 °C) for 2 h before analysis. A commercial agar (HiMedia, Mumbai, India) was used as a reference.

2.5.1. Gel strength

The gel strength was determined at 25–30 °C using a texture analyser (Stable Micro System, Surrey, UK) with a load cell of 5 kg, cross-head speed of 1 mm/s, equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger as per the method of Lee, Namasivayam, and Ho (2014). The maximum force (gram), taken when the plunger had penetrated 5 mm into the agar gels, was recorded. Gel strength was calculated and expressed as g/cm^2.

2.5.2. Texture profile analysis

Texture profile analysis (TPA) was performed using a TA-XT2 texture analyser (Stable Micro Systems, Godalming, Surrey, UK) with a load cell of 25 kg. Cylindrical aluminium probe (50 mm diameter) was used. The samples were placed on the instrument's base. The tests were run with two compression cycles. TPA textural parameters were measured at room temperature with the following testing conditions: crosshead speed of 5 mm/s, 30% compression of the original sample height, surface sensing force of 99 g and threshold of 30.0 g. Time interval between the first and second compression was 10 s. Hardness, cohesiveness, springiness, chewiness and resilience were calculated from the force–time-curves generated for each sample.

2.6. Determination of physical properties

2.6.1. Colour

The colour of agar powders was measured by a colourimeter (ColourFlex, Hunter Lab Reston, VA, USA) and reported in the CIE system. L^*, a^*, b^*, ΔE^*, and ΔC^* representing lightness, redness/greenness, yellowness/blueness, total difference of colour, and the difference in chroma, respectively, were reported. ΔE^* and ΔC^* were calculated as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^*, and Δb^* are the differentials between colour parameters of the samples and those of the white standard ($L^* = 92.82$, $a^* = -1.22$, $b^* = 0.49$)

$$\Delta C^* = C^*\text{sample} - C^*\text{standard}$$

where $C^* = \sqrt{(a^*)^2 + (b^*)^2}$

2.6.2. Gelling and melting temperatures

Gelling and melting temperatures of the different agar samples were measured following the method of Hilliou, Larontonda, Sereno, and Gonçalves (2006) using a controlled stress rheometer (RheoStress RS 75, HAAKE, Karlsruhe, Germany). The measuring geometry used was a 6 cm parallel plate and the gap was set at 1.0 mm. The agar solution (1.5%, w/v) was prepared in the same manner as described previously. The solution (3 mL) was loaded on the peltier plate (pre-heated at 80 °C for 5 min). The excess sample was removed and its periphery was coated with paraffin oil to minimise the evaporation. The measurement was performed at a scan rate of 2 °C/min, frequency of 1 Hz, oscillating applied stress of 5 Pa during cooling from 80 to 10 °C and heating from 10 to 120 °C. The gelling and melting temperatures were calculated, where tan δ became 1 or δ was 45°, during cooling and heating, respectively.

2.6.3. Viscosity

Rheological viscosity of different agars was determined using a controlled stress rheometer. Three millilitres of agar solution (1.5% w/v) were loaded on the plate (pre-heated at 80 °C). The viscosity measurements were conducted at 80 °C using a constant shear rate of 50 1/s. The viscosity at shearing time of 30 min was recorded.

2.6.4. Syneresis

The syneresis of agar gel was determined as described by Banerjee and Bhattacharya (2011). Hot dispersions (1.5% w/v; 30 mL) of different agars were poured into 50 mL graduated centrifuge tubes and their masses (m_1) were recorded. The samples were allowed to cool at room temperature for gel formation. Gels were matured at 4 °C for 12 h. Before measurement, gels were equilibrated at room temperature for 2 h. Then, these samples were centrifuged at 2150 × *g* at 25 °C for 10 min using a refrigerated centrifuge (Beckman Coulter, Palo Alto, CA, USA). After centrifugation, gels along with the tubes were weighed (m_2) again after discarding the separated water. The percentage of syneresis was calculated as follows:

$$\textit{Syneresis}(\%) = \frac{(m_1 - m_2)}{m_1} \times 100$$

2.7. Characterisation of selected agars

2.7.1. Fourier transform infrared (FTIR) spectroscopy

FTIR measurements of the selected agars were carried out on a KBr pellets using a Bruker Model EQUINOX 55 FTIR (Bruker, Ettlingen, Germany) spectrometer equipped with a deuterated L-alanine triglycine sulphate (DLATGS) detector. The spectra were obtained at the wavenumber range of 4000–500 cm^{-1}. Automatic signals were collected in 16 scans at a resolution of 4 cm^{-1}. Analysis of spectral data was carried out using the OPUS 3.0 data collection software program (Bruker, Ettlingen, Germany).

2.7.2. Microstructure analysis

The microstructures of the selected agar gels were visualised using a scanning electron microscopy (SEM). The agar gels having a thickness of 2–3 mm were dehydrated in ethanol with a serial concentration of 25, 50, 70, 80, 90 and 100% (v/v). Samples were critical point dried using CO_2 as transition fluid. Dried samples were mounted on a bronze stub and sputter-coated with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 15 kV.

2.8. Statistical analysis

Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel & Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Yields of agar prepared without and with different alkaline pretreatments

The yields of agar from *G. tenuistipitata* pretreated without and with NaOH or KOH solutions at different concentrations (3–7% w/v) are shown in Table 1. The native agar (without alkaline pretreatment) had a yield of 17.1%. When alkaline pretreatment was applied, higher yields were obtained with the range of 23.6–26.1%. Nevertheless, no difference in yield was found between agars prepared from algae pretreated with different conditions used in the present study ($P > 0.05$). Fidelis et al. (2014) reported that sulphated polysaccharide from *Gracilaria birdae* extracted using water (alone) had a lower yield, compared to those extracted from the combination method of alkaline solution, proteolysis and sonication. Generally, agar is accumulated in the cell walls of agarophyte algae and is embedded in a structure of fibers of crystallised cellulose, constituting its polysaccharide reserve (Armisen, Galatas, Phillips, & Williams, 2009). Extraction was carried out at high temperature, indicating that high energy was required to disrupt bondings stabilising the extracelluar structure. Alkaline pretreatment prior to extraction might destabilise the cross-links occurring in cell wall of *G. tenuistipitata*. This resulted in higher amount of

Table 1
Yield of *G. tenuistipitata* agar powders without and with different alkaline pretreatments.

Agars	Yield (%)
Native	17.1 ± 1.6[b,*]
Pretreated with 3% NaOH	26.1 ± 1.5[a]
Pretreated with 5% NaOH	25.3 ± 1.3[a]
Pretreated with 7% NaOH	24.6 ± 0.9[a]
Pretreated with 3% KOH	24.2 ± 1.2[a]
Pretreated with 5% KOH	25.2 ± 5.0[a]
Pretreated with 7% KOH	23.6 ± 1.6[a]

Mean ± S.D (n = 3).
*Different lowercase superscripts within the same column indicate the significant differences (P < 0.05).

agar released from a swollen or disrupted structure during extraction. Since *G. tenuistipitata* is cultivated in tropical region, its structure might be more complex and resistant to a high temperature environment. Yang, Dong, and Kim (2012) reported that the main axes of *G. tenuistipitata* were dense and irregular. Its medulla consists of 4–5 cells that increase markedly in size towards the centre and reaches up to 130–200 μm in diameter, with strong thick walls of 10–25 μm in diameter. The cortex consists of 1–2 layers of rounded cells with 14–22 μm in diameter. Additionally, the presence of high cellulose content in cell wall of algal cell might interfere the extraction of galactans from this species. As a result, harsher extraction conditions were required to obtain the higher yield. However, no difference in yield between agars from algae pretreated with NaOH and KOH at all levels used (P > 0.05). Ganesan, Rao, and Jha (2004) reported that agar extracted from *Gracilaria edulis* pretreated with 0.5% acetic acid for 1 h followed by neutralisation with 1% KOH for another 1 h had the maximum yield (69.2%). Praiboon et al. (2006) found that agars extracted from *Gracilaria fisheri* and *G. edulis* pretreated with 5% NaOH showed a higher yield (34.3–39.6%), compared to the native agars (10.9–13.3%). On the other hand, alkaline treatment at high temperatures (80–100 °C) for *Gracilaria* prior to extraction caused the decrease in yield of agar due to the degradation of polysaccharide and agar loss associated with diffusion during pretreatment process (Armisen et al., 2009; Freile-Pelegrın & Murano, 2005; Freile-Pelegrín & Robledo, 1997; Marinho-Soriano, 2001). On the other hand, Montaño et al. (1999) found a tremendous decrease of agar yield extracted from *G. tenuistipitata* harvested from the Philippines upon alkali modification (~33% for native agar and ~16% for alkali-treated agars). Although the same species was used, they might have the varying morphology features when grown at different regions. Villanueva et al. (2010) noted that the agars extracted from non-treated *Gracilaria vermiculophylla* (native agars) had the low recovery upon partial dehydration with ethanol (after the freeze-thawing step), leading to lower yields. It was suggested that different processes used for agar recovery affected the yield. Thus, alkaline pretreatment either by NaOH or KOH, in the concentration range of 3–7% w/v could increase the yield of resulting agar.

3.2. Chemical compositions of agars prepared without and with different alkaline pretreatments

Different agars contained varying sulphate and 3,6-AG contents (Fig. 1A and B). Native agar showed higher sulphate content (4.3%) than those with alkaline pretreatment (1.6–2.6%) (P < 0.05). Rebello, Ohno, Ukeda, and Sawamura (1997) reported that sulphate content of non-alkaline pretreated *G. tenuistipitata* from China was 2.3%, whilst Montaño et al. (1999) noted that the native agar from the Philippines had sulphate content of 3.9%. The result revealed that the agar from *Gracilaria* spp. from different geographical

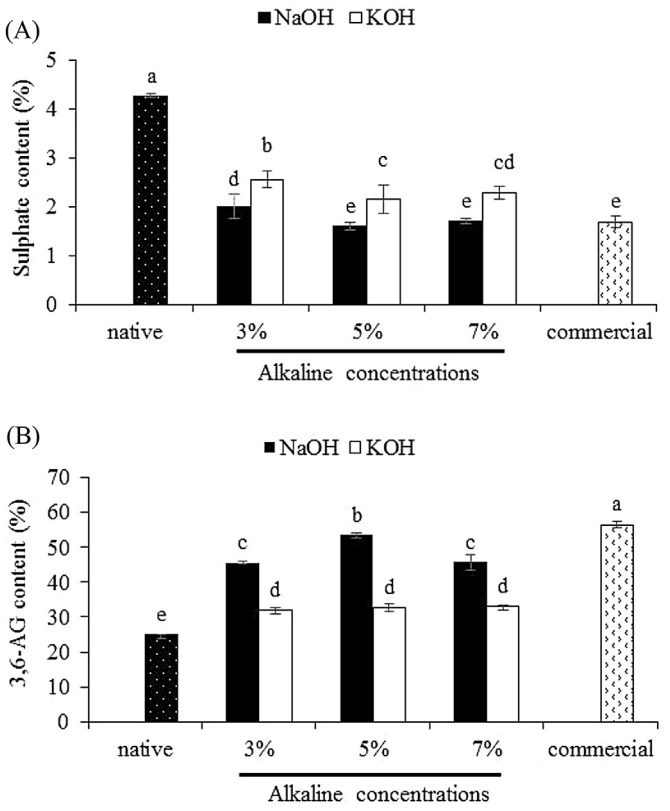

Fig. 1. Sulphate (A) and 3,6-AG (B) contents of agar from *G. tenuistipitata* without and with different alkaline pretreatments. Commercial agar was used as a reference. Different lowercase letters on the bar denote significant difference (P < 0.05). Bar represent the standard deviations (n = 3).

origins had different sulphate contents. In general, agar from *Gracilaria* spp. showed high sulphate content, which was associated with weak gels (Armisen, 1995). Sulphate unit at C-6 in polysaccharide chains causes the kinks in the helical structure responsible for gel formation (Rees, 1972). With alkaline pretreatment, the lower sulphate content was observed in agar from algae pretreated with NaOH, compared with those with KOH pretreatment at all concentrations used (P < 0.05). NaOH might hydrolyse sulphate ester to a higher extent during pretreatment process, in comparison with KOH. Generally, sulphate content decreased with increasing alkaline concentrations up to 5% (P < 0.05), irrespective of alkaline type. Nevertheless, no significant difference in sulphate content was observed between agars with pretreatment using alkali at 5% and 7% (P > 0.05). It was noted that the agar with 5% or 7% NaOH pretreatment had similar sulphate content to the commercial agar (P > 0.05). The result suggested that the employment of alkaline treatment prior to agar extraction was able to cleave sulphate ester at C-6 of the L-galactose with simultaneous formation of 3,6-AG, thereby improving the gelling properties (Murano, 1995).

The 3,6-AG contents of different agars are depicted in Fig. 1B. The highest 3,6-AG content was observed in the commercial agar (56.5%) (P < 0.05). All agars extracted from *G. tenuistipitata* had lower 3,6-AG contents than commercial counterpart (P < 0.05). The lowest 3,6-AG content was found in native agar (24.9%). A similar 3,6-AG content (25.3%) of native agar from this species harvested from China was reported (Rebello et al., 1997). For agar with NaOH pretreatment, the 3,6-AG content increased with increasing alkaline concentration up to 5% (P < 0.05). Montaño et al. (1999) found that pretreatment of *G. tenuistipitata* with 10% NaOH prior to extraction rendered agar with a higher 3,6-AG content (42.1%),

compared with native agar (34.0%). However, a decrease in 3,6-AG content was observed when 7% NaOH was used for pretreatment ($P < 0.05$). 3,6-AG content was related with gelling property of agar (Murano, 1995). Pretreatment with NaOH at high concentration plausibly caused a chain breakage rather than the formation of 3,6-AG. The result was in agreement with Rebello et al. (1997) who found a drastic decrease in 3,6-AG of Chilean agar when 7% NaOH was used for pretreatment. When KOH was used for pretreatment, similar 3,6-AG contents (32.1–33.1%) were observed for all agars, regardless of the used concentrations ($P > 0.05$). Although sulphate content was decreased after KOH pretreatment (Fig. 1A), a similar 3,6-AG content was noticeable, irrespective of KOH concentration. It was suggested that KOH more likely played a role in chain hydrolysis rather than the induction of 3,6-AG formation. However, 3,6-AG contents of agar with KOH pretreatment were lower than those from algae pretreated with NaOH ($P < 0.05$), at all alkaline concentrations used. This was possibly because KOH contained significantly more water due to its higher hygroscopic nature. As a consequence, it might be less effective in converting sulphated α-galactopyranose residues into their cyclised forms. In general, the increase in 3,6-AG content was in accordance with the decrease in sulphate content.

3.3. Properties of agar gels

3.3.1. Gel strength

Gel strength of agars extracted from *G. tenuistipitata* pretreated without and with alkaline solutions at varying concentrations (3–7%) is shown in Fig. 2. Gel strength is one of the most important quality indices of agars. When comparing the gel strength of all agar samples from *G. tenuistipitata* and commercial agar, the formers had the lower gel strength ($P < 0.05$). The lowest gel strength (95 g/cm^2) was found in the native agar ($P < 0.05$) and it was related well with the higher sulphate content (Fig. 1A). Basically, gelation of agar occurs through the aggregation of helical conformation of agar polymers via hydrogen bonds. The presence of charged groups (sulphate group) more likely interfered the intermolecular hydrogen bonding for double helices formation (Lahaye & Rochas, 1991). Gel strength increased by 77.5–80.4% (419–482 g/cm^2) and 76.7–78.8% (406–446 g/cm^2) when NaOH and KOH with the concentrations range of 3–7% were used for pretreatment, respectively. Closure of the ring to form the 3,6-AG and cleavage of the C-6 sulphate group by alkaline hydrolysis contributed to the chain straightening and regularity in the polymer. This resulted in the increase capability of forming double helices, which directly strengthen the gel network (Rees, 1972). Amongst all samples, agar from algae pretreated with 5% NaOH showed the highest gel strength (482 g/cm^2) ($P < 0.05$). When KOH was employed for pretreatment, gel strength of resulting agar increased, compared with native agar (without pretreatment). Nevertheless, the KOH concentrations used did not cause the difference in gel strength of agars obtained ($P > 0.05$). Montaño et al. (1999) found that gel strength of native agar and 10% NaOH pretreated agar extracted from the same species was 304 and 606 g/cm^2, respectively. Different environments where algae grow and various processes used might determine the properties of agar. Freile-Pelegrín and Robledo (1997) found that gel strength of agar from *Gracilaria cornea* pretreated with NaOH increased to 1758 g/cm^2 when NaOH at concentrations of 5% was used. Therefore, alkaline pretreatment was required for increasing gel strength of agar and the use of 5% NaOH for pretreatment resulted in the highest gel strength ($P < 0.05$).

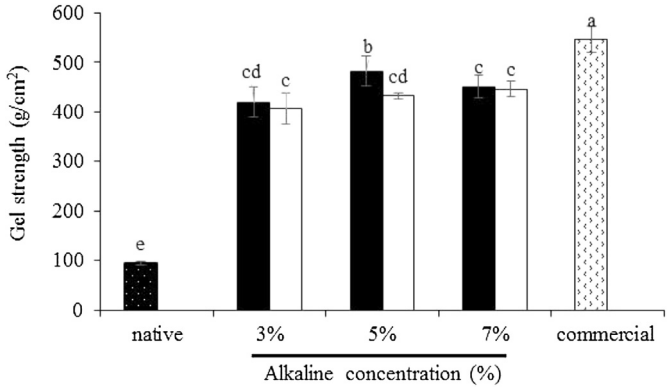

Fig. 2. Gel strength of agar from *G. tenuistipitata* without and with different alkaline pretreatments. Commercial agar was used as a reference. Different lowercase letters on the bar denote significant difference ($P < 0.05$). Bar represent the standard deviations ($n = 3$).

3.3.2. Textural properties

Textural properties of agar from *G. tenuistipitata* pretreated without and with various alkaline solutions (NaOH or KOH) at different levels are presented in Table 3. In comparison with the native gel, those pretreated with alkaline solutions using both NaOH and KOH, showed the increase in hardness, representing the force required to compress sample to attain a given deformation (Intarasirisawat, Benjakul, Wu, & Visessanguan, 2014) ($P < 0.05$). At the same concentration, no difference in hardness was observed between agar extracted from algae pretreated with NaOH and KOH ($P > 0.05$). Similar hardness was observed in gel of agar prepared from algae with 5% NaOH pretreatment to that of commercial agar ($P > 0.05$). The result was in accordance with the high gel strength (Fig. 2).

For gumminess (the energy required to breakdown a semi-solid food ready for swallowing) and chewiness (the required energy to chew the sample to the point required for swallowing it) (Intarasirisawat et al., 2014), the agar extracted from algae pretreated with 5% NaOH showed the higher values than commercial agar ($P < 0.05$). It was also noted that the decrease in gumminess and chewiness were found when the pretreatment with 7% NaOH or KOH was implemented ($P < 0.05$). The lowest gumminess and chewiness were observed for native agar gel ($P < 0.05$). Sousa and Gonçalves (2015) reported that gel of agar with alkaline pretreatment was firmer than native gel as evidenced by the higher gel strength and failure stress.

The springiness presents how well a product physically springs back after it has been deformed during the first compression (Intarasirisawat et al., 2014). There was no difference between native agar and those with alkaline pretreatments, except for that pretreated with 7% KOH, which showed the lowest value ($P < 0.05$). In general, all agars with alkaline pretreatment had the similar springiness to commercial agar ($P > 0.05$).

For cohesiveness (capability in breaking down the internal gel structure) (Intarasirisawat et al., 2014), all agars with alkaline pretreatment showed the higher value than commercial agar ($P < 0.05$), except that prepared from algae pretreated with 7% NaOH, which had similar value to commercial agar ($P > 0.05$).

The resilience, (the energy accumulated that allows the sample to recover its original shape after deformation) (Intarasirisawat et al., 2014), of all agars was similar ($P > 0.05$), except agar with pretreatment using 7% KOH, which had the lower resilience than others and commercial agar. It was found that native agar showed the similar resilience to that with 5% NaOH pretreatment ($P > 0.05$).

The result suggested that textural characteristic of agar gel varied with alkaline pretreatments. Both type and concentration of

Table 2
Physical properties of *G. tenuistipitata* agar without and with different alkaline pretreatments.

Agars	Colour					Gelling temperature (°C)	Melting temperature (°C)	Viscosity (cP)	Syneresis (%)
	L*	a*	b*	ΔE*	ΔC*				
Commercial	83.93 ± 0.15^{a,*}	1.68 ± 0.00^{b}	13.38 ± 0.12^{b}	15.93 ± 0.13^{d}	12.17 ± 0.12^{b}	33.2 ± 0.6^{b}	102.8 ± 0.3^{a}	25.33 ± 1.22^{a}	1.15 ± 0.28^{bc}
Native	58.02 ± 1.10^{e}	2.30 ± 0.24^{a}	19.25 ± 0.44^{a}	39.69 ± 1.18^{a}	18.08 ± 0.46^{a}	30.4 ± 0.3^{c}	85.6 ± 0.2^{c}	8.12 ± 0.13^{e}	0.75 ± 0.02^{d}
Pretreated with 3% NaOH	77.33 ± 1.07^{c}	0.71 ± 0.05^{d}	9.18 ± 0.15^{d}	17.88 ± 0.98^{c}	7.90 ± 0.15^{d}	34.2 ± 0.6^{ab}	102.9 ± 0.9^{a}	15.57 ± 1.50^{c}	1.18 ± 0.07^{b}
Pretreated with 5% NaOH	81.93 ± 0.74^{b}	0.06 ± 0.07^{f}	9.16 ± 0.44^{d}	13.98 ± 0.84^{e}	7.84 ± 0.44^{d}	35.5 ± 0.6^{a}	103.3 ± 1.3^{a}	24.76 ± 0.25^{a}	1.07 ± 0.18^{c}
Pretreated with 7% NaOH	84.80 ± 0.41^{a}	−0.43 ± 0.02^{g}	8.06 ± 0.23^{f}	11.06 ± 0.39^{f}	6.76 ± 0.23^{e}	36.0 ± 1.1^{a}	103.5 ± 0.0^{a}	18.78 ± 1.05^{b}	1.05 ± 0.20^{c}
Pretreated with 3% KOH	73.24 ± 1.02^{d}	0.43 ± 0.04^{e}	10.10 ± 0.37^{c}	21.88 ± 1.09^{b}	8.80 ± 0.37^{c}	34.9 ± 0.2^{ab}	97.2 ± 0.4^{b}	10.20 ± 2.14^{d}	1.54 ± 0.07^{a}
Pretreated with 5% KOH	77.34 ± 1.77^{c}	1.08 ± 0.19^{c}	9.66 ± 1.00^{cd}	18.14 ± 1.13^{c}	8.34 ± 0.90^{cd}	34.9 ± 0.9^{ab}	103.3 ± 1.5^{a}	14.63 ± 0.17^{c}	1.33 ± 0.28^{ab}
Pretreated with 7% KOH	81.82 ± 0.06^{b}	0.23 ± 0.06^{f}	8.02 ± 0.25^{f}	13.42 ± 0.11^{e}	6.70 ± 0.25^{e}	34.7 ± 1.3^{ab}	103.1 ± 0.6^{a}	15.31 ± 2.33^{c}	1.37 ± 0.21^{ab}

Mean ± S.D (n = 3).
*Different lowercase superscripts within the same column indicate the significant differences (P < 0.05).

alkali used determined textural properties of resulting agars. Overall, agar extracted from algae pretreated with 5% NaOH had the hardness, springiness and resilience equivalent to commercial agar, but showed the higher cohesiveness, gumminess and chewiness. Also, it possessed higher hardness, gumminess and chewiness than native agar (P < 0.05).

3.4. Physical properties of agars prepared without and with different alkaline pretreatments

3.4.1. Colour

Differences in colour were observed amongst agars from *G. tenuistipitata* pretreated without and with alkaline (NaOH and KOH) solutions at various concentrations (Table 2). Commercial agar exhibited a slight creamy-yellowish in colour. Native agar (without pretreatment) had brown yellowish colour as evidenced by the lowest L*-value and highest a* and b*-values (P < 0.05). This coincided with highest ΔE* and ΔC*-values of native agars (P < 0.05). Pigments, e.g. chlorophyll, carotenoid, phycoerythrobilin or others in algae, leached out during extraction most likely contributed to the dark colours of native agars (Li, Yu, Jin, Zhang, & Liu, 2008). During the alkaline pretreatment, the algal cells were destructed and pigments localised in cell were prone to photolysis. Li et al. (2008) noted that phycoerythrobilin was removed from the phycoerythrin during the alkaline pretreatment of *Gracilaria lemaneiformis*. As a result, those pigments could be released from cell with ease during alkaline pretreatment. With NaOH pretreatment, lower b*, ΔE* and ΔC*-values of resulting agar were obtained, compared to those pretreated with KOH. The decreases in b*, ΔE* and ΔC*-values were more pronounced, when NaOH and KOH at a concentration of 7% was used for pretreatment. This result reconfirmed the effectiveness of alkali on destruction or removal of pigments in algae, in which the colour of agar could be more improved. Additionally, L*-value increased as the concentration of alkaline solution increased (P < 0.05). Thus, alkaline solution at high concentration was able to remove most pigments from algal cell.

3.4.2. Gelling and melting temperatures

Gelling and melting temperatures of agars without and with different alkaline pretreatments are presented in Table 2. Commercial agar had gelling and melting temperatures of 33.2 and 102.8 °C, respectively. The lower gelling and melting temperatures of native agar were found at 30.4 °C and 85.6 °C, respectively. Native agars from *Gracilaria blodgettii* and *Gracilaria crassissima* collected from the Yucatán Peninsula (Freile-Pelegrın & Murano, 2005), *G. tenuistipitata* from the Philippines (Montaño et al., 1999) and *Gracilaria corticata* from Kenya (Oyieke, 1993) also showed the different gelling and melting temperatures, which were in the range of 42–45 °C and 86–88 °C, respectively. The variation in both gelling and melting temperatures depended on the regions, where the algae were harvested. Additionally, the different processes used for extraction had the influence on both gelling and melting temperatures. For agar with alkaline pretreatment, those with NaOH pretreatment became gel at temperature range of 34.2–36.0 °C, whilst those with KOH pretreatment formed gel at 34.7–34.9 °C. The corresponding gels molt at 102.9–103.5 °C and 97.2–103.3 °C, respectively. It was found that agar had higher gelling and melting points when alkaline pretreatment was performed before agar extraction. Higher melting temperature was ascribed the higher energy required to break down the network, indicating that gels of agar with alkaline pretreatment were more stable than native agar. Villanueva et al. (2010) reported that gelling and melting temperatures of *Gracilaria vermiculophylla* agar were lowest (21.6–26.4 °C and 62.7–70.0 °C, respectively), however pretreatment using NaOH concentration up to 4% resulted in the increases in both gelling and melting temperatures (31.0–35.8 °C and 73.6–80.4 °C, respectively). Generally, gelling temperature has been shown to be negatively related with sulphate content and is also affected by the methoxyl content and its location (Andriamanantoanina, Chambat,

Table 3
Textural properties of *G. tenuistipitata* agar without and with different alkaline pretreatments.

Agars	Hardness (N)	Springiness (cm)	Cohesiveness	Gumminess (N)	Chewiness (N × cm)	Resilience
Commercial	0.37 ± 0.01^{a,*}	0.81 ± 0.01^{bc}	0.46 ± 0.08^{c}	0.17 ± 0.03^{bc}	0.14 ± 0.02^{bcd}	0.45 ± 0.01^{bc}
Native	0.06 ± 0.00^{e}	0.92 ± 0.01^{a}	0.75 ± 0.00^{a}	0.04 ± 0.00^{e}	0.04 ± 0.00^{e}	0.52 ± 0.00^{a}
Pretreated with 3% NaOH	0.31 ± 0.00^{bc}	0.84 ± 0.01^{abc}	0.65 ± 0.03^{ab}	0.20 ± 0.01^{ab}	0.17 ± 0.01^{ab}	0.45 ± 0.01^{bc}
Pretreated with 5% NaOH	0.34 ± 0.01^{ab}	0.87 ± 0.02^{ab}	0.65 ± 0.02^{ab}	0.22 ± 0.01^{a}	0.19 ± 0.01^{a}	0.49 ± 0.10^{ab}
Pretreated with 7% NaOH	0.27 ± 0.01^{cd}	0.83 ± 0.01^{abc}	0.47 ± 0.05^{c}	0.12 ± 0.01^{d}	0.11 ± 0.02^{d}	0.39 ± 0.02^{cd}
Pretreated with 3% KOH	0.26 ± 0.00^{cd}	0.87 ± 0.02^{ab}	0.69 ± 0.00^{ab}	0.18 ± 0.00^{bc}	0.16 ± 0.00^{abc}	0.47 ± 0.00^{ab}
Pretreated with 5% KOH	0.28 ± 0.01^{bcd}	0.85 ± 0.02^{abc}	0.64 ± 0.04^{b}	0.18 ± 0.00^{bc}	0.17 ± 0.00^{ab}	0.45 ± 0.01^{bc}
Pretreated with 7% KOH	0.24 ± 0.09^{d}	0.77 ± 0.14^{c}	0.68 ± 0.13^{ab}	0.16 ± 0.04^{c}	0.12 ± 0.05^{cd}	0.36 ± 0.02^{d}

Mean ± S.D (n = 3).
*Different lowercase superscripts within the same column indicate the significant differences (P < 0.05).

& Rinaudo, 2007). Melting point is correlated with pyruvic acid content of agar (Young, Duckworth, & Yaphe, 1971). Additionally, the variations in gelling or melting temperature were known to be related with molecular weight distribution of agar, which strongly determined the gelling properties (Freile-Pelegrın & Murano, 2005). No differences in both gelling and melting temperatures were observed amongst all samples with alkaline pretreatment, except that extracted from algae with 5% KOH pretreatment, which had the lower melting temperature. It was noted that agar with 5% and 7% NaOH pretreatment had higher gelling temperature than that of commercial agar ($P < 0.05$). Thus, alkaline pretreatment condition also played a role in gelling and melting temperatures of resulting agar.

3.4.3. Viscosity

The variation in viscosity of agars with different alkaline pretreatments was observed (Table 2). The viscosity of agar solution reflects the molecular weight or the chain length of molecules (Murano, 1995). All agars with alkaline pretreatment had higher viscosity (10.20–24.76 cP) than native agar (8.12 cP). However, their viscosity was lower than that of commercial agar (25.33 cP). Nevertheless, the agar with 5% NaOH pretreatment showed similar viscosity to that of commercial agar ($P > 0.05$). The contamination of non-gelling components in native agar might contribute to the lower viscosity (Praiboon et al., 2006). Moreover, the presence of sulphate group in its structure possibly resulted in the electrostatic repulsion between polysaccharide chains. As a consequence, the entanglement of polysaccharide chain might be lowered. After alkaline pretreatment, the chains were modified, in which 3,6-AG content increased. As a result, the degree of chain entanglement might be enhanced. The result was correlated well with higher gelling and melting temperatures of agar with alkaline pretreatment. Generally, viscosity of agar from algae pretreated with NaOH was higher than those pretreated with KOH at all concentrations used ($P < 0.05$). Polysaccharide chain might be more degraded by KOH. For NaOH pretreatment, agar extracted from algae with high alkaline concentration (7% NaOH) had the decreased viscosity. Praiboon et al. (2006) reported that the decreases in viscosity of agar using 5% NaOH pretreatment were caused by alkaline hydrolysis of polysaccharide chain. High viscosity associated with higher melting temperatures was reported to be related with high molecular weight of polymer (Selby & Wynne, 1973; Whyte & Englar, 1981). Nevertheless, similar viscosity between agar with 5% and 7% KOH pretreatments was observed ($P > 0.05$). Thus, alkaline pretreatment affected viscosity of agar solutions.

3.4.4. Syneresis

Syneresis of different gels prepared from agar without and with alkaline pretreatments is shown in Table 2. Syneresis is defined as the water loss of agar gels during ageing, indicating instability of gel network. This is attributed to the contraction of the polymer network by a slow further aggregation of helices (Stanley, 2006). The lowest syneresis was noticeable in native agar gel ($P < 0.05$). The lower syneresis was obtained in gels of agar extracted from algae with NaOH pretreatment than KOH pretreated counterpart. Lower syneresis might be related with strong gel network, which could imbibe more water. During cooling, agar polymer forms three-fold left-handed helices, stabilised by the presence of water molecules bound inside the double helical cavity (Labropoulos, Niesz, Danforth, & Kevrekidis, 2002). Exterior hydroxyl groups allow aggregation of up to 10,000 of these helices to form micro domains of spherical microgels (Boral, Saxena, & Bohidar, 2008). Owing to the less sulphate remained in agar with alkaline pretreatment, the bundles of helix were more compact by self-aggregation, leading to the liberation of water from gel matrix (Labropoulos et al., 2002). As a consequence, the syneresis was

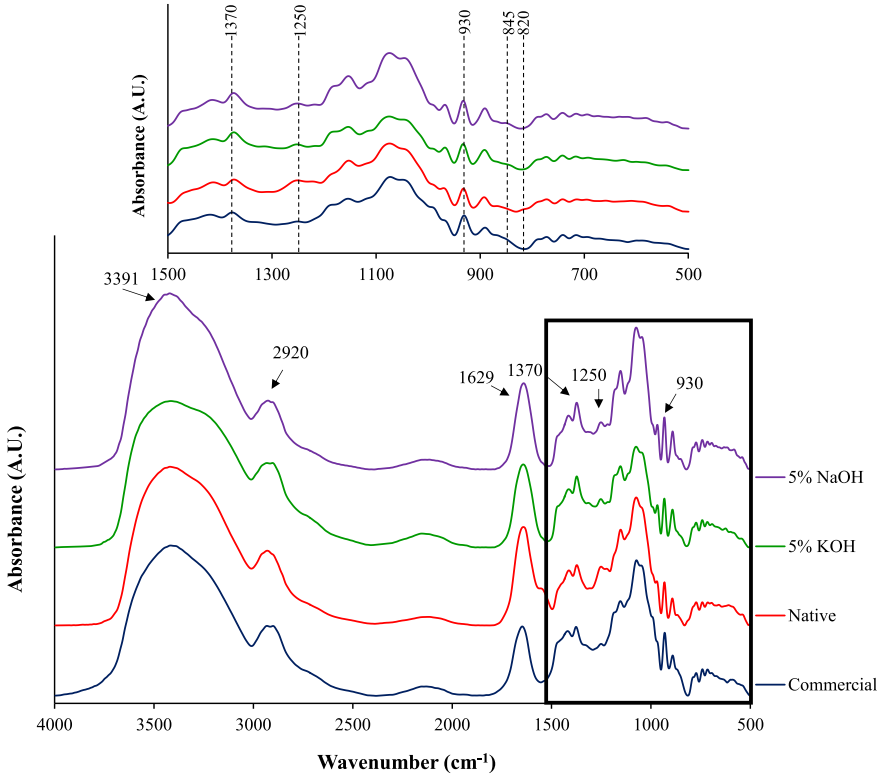

Fig. 3. FTIR spectra of native agar and agar with 5% NaOH or KOH pretreatments from *G. tenuistipitata*. Commercial agar was used as a reference. Bands assigned to total sulphate (1250 cm^{-1}), 3,6-AG (930 cm^{-1}), sulphate at 6-position in galactose units (820 cm^{-1}, weak signal) and total sugar content (C–H; 2920 cm^{-1}) are marked accordingly.

more pronounced, compared with native gels. Similar result was observed by Montaño et al. (1999) who found that syneresis of native agars from *Gracilaria arcuate* and *G. tenuistipitata* was lower than those of agar with alkaline pretreatment. With increasing alkaline concentration, there was no difference in syneresis index, irrespective of alkaline types. Although agar with KOH pretreatment contained a higher sulphate content than did NaOH counterpart (Fig. 1A), more water liberated in the resulting gel was observed as evidenced by the higher syneresis. KOH pretreatment resulted in the decreased viscosity (Table 2). This might contribute to poorer gel matrix with low water holding capacity.

3.5. Characteristics of selected agars

3.5.1. FTIR-spectra

FTIR spectra in the 4000–500 cm^{-1} region of native agar from *G. tenuistipitata* and those with 5% NaOH or KOH pretreatments are shown in Fig. 3. All agars showed the typical spectra of agar-like galactans (Sousa et al., 2012; Villanueva et al., 2010). The most important bands were found at 1370, 1250, 1072, 931, 845 and 820 cm^{-1}, typically recognised as agarocolloid (Melo, Feitosa, Freitas, & de Paula, 2002). Based on spectra, the dominant band was observed at 3391 cm^{-1} associated with OH groups. The absorbance at 2932 cm^{-1} was attributed to CH_2 groups of agar. The band at 1629 cm^{-1}, assigned to amide I vibrations, suggests the presence of proteins (Guerrero, Garrido, Leceta, & de la Caba, 2013). During extraction of agar at high temperature, the destruction of algal cell occurred. As a result, the soluble proteins were more likely co-extracted into the agar. The presence of high amount of protein in the agar of this study might be caused by the extraction process used, in which less pure product was obtained. Moreover, it was possibly because of protein rich raw material used for agar extraction. Benjama and Masniyom (2012) reported that protein content of *G. tenuistipitata* was 21.6%, whilst *Gracilaria fisheri* had protein content of 11.6% (dry weight basis). The highest amplitude for this band was found in the native agar. Band at 1370 cm^{-1} and 1250 cm^{-1} are corresponded to ester sulphate groups, whilst the band with wavenumbers of 1072 cm^{-1} is equivalent to the skeleton of galactans (Melo et al., 2002). In addition, the region around 931 cm^{-1} was attributed to the C–O–C group of 3,6-anhydro-α-L-galactopyranose (3,6-AG) and the absorption at 845 and 820 cm^{-1} indicated the presence of sulphate groups on the C-4 and C-6 of galactose, respectively (Sousa et al., 2012). As expected, native agar had the largest band at 1250 cm^{-1} and also showed a minor shoulder at 820 cm^{-1}. The result was in accordance with the highest sulfate content determined turbidimetrically for this agar (Fig. 1A). However, the decrease in amplitude at wavenumber of 1250 cm^{-1} was found and no band around 820 cm^{-1} was detected after alkaline pretreatments (both KOH and NaOH). It was suggested that the elimination of unstable sulphate group at C-6 was achieved upon alkaline pretreatment. Additionally, the minor peak at 845 cm^{-1}, indicating an axial sulphate group at C-4, was present in all samples. The presence of sulphate group after alkaline pretreatment indicated the availability of alkaline-stable sulphate groups on sugar residues in agar structure (Sousa et al., 2012). Furthermore, alkaline pretreatment resulted in an increase of the band intensity around 930 cm^{-1}, especially when 5% NaOH was used for pretreatment. This was postulated that alkaline

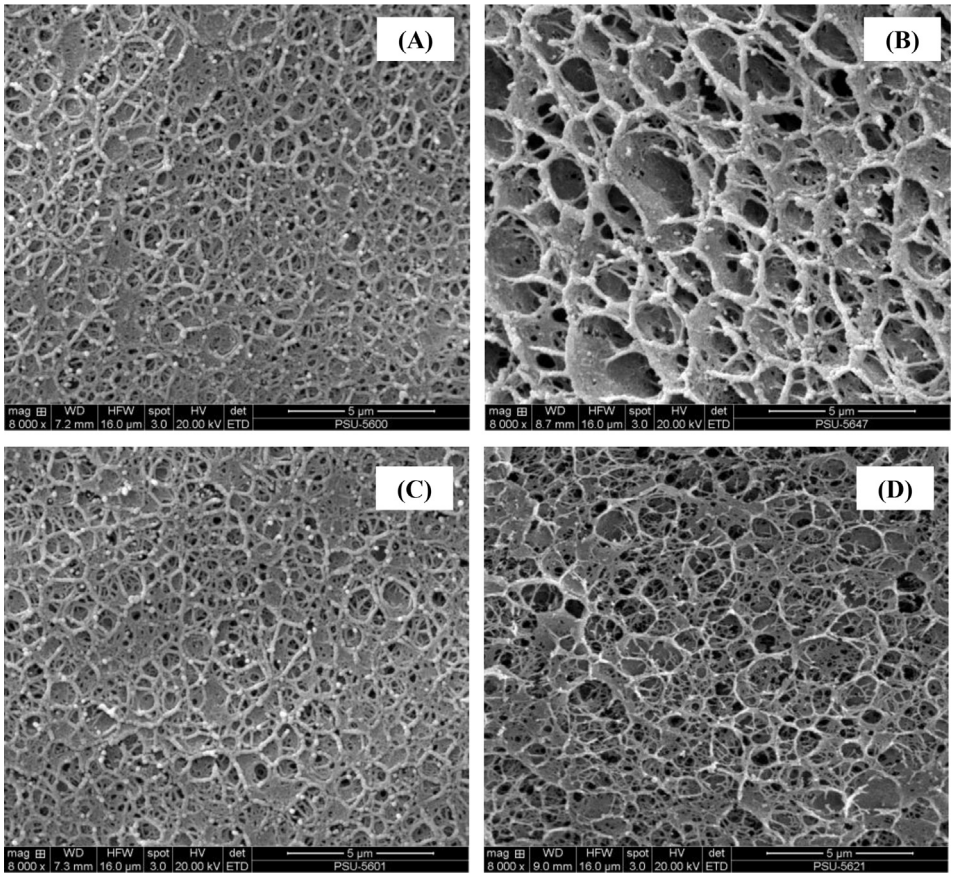

Fig. 4. Microstructures of gels of different agars from *G. tenuistipitata*. (A) commercial agar; (B) native agar; (C) agar with 5% NaOH pretreatment; (D) agar with 5% KOH pretreatment. Magnification: 8000×. Agar solutions (1.5%, w/v) were used for gel preparation.

pretreatment could convert sulphate substitution to 3,6-AG amongst 4-linked residues. The residues of 6-sulphate-α-L-galactose are known to be precursors of 3,6-anhydro-α-L-galactose (Talarico et al., 2004). The result was in agreement with the higher 3,6-AG content as measured colourimetrically in the agar with 5% NaOH pretreatment (Fig. 1B). Villanueva et al. (2010) reported the higher proportion of A_{935}/A_{1250} of alkaline treated agar than that of native agar, indicating the increased relative concentration of 3,6-AG to total sulphate. In the present study, the peak ratios of A_{930}/A_{1250} for commercial agar, native agar, agar with 5% KOH and NaOH were 0.94, 0.80, 0.85 and 0.95, respectively. Thus, the structure and functional group of agar from *G. tenuistipitata* were affected by alkaline pretreatment.

3.5.2. Microstructure of agar gel

Microstructures of the gel from commercial agar and native agar from *G. tenuistipitata* agars with 5% NaOH or 5% KOH pretreatments are illustrated in Fig. 4. All agar gels showed a network of interconnection with fairly uniform strands. The gel from commercial agar showed a finest network with very small voids (Fig. 4A), whilst that from native *Gracilaria* agar had coarsest network with the largest voids (Fig. 4B). When comparing gel structure between native agar and that with alkaline pretreatment, the latter became more order and denser with a finer strand (Fig. 4C and D). The finer structure was observed for gel of agar with 5% NaOH pretreatment, compared with that of gel having 5% KOH pretreatment. Alkaline pretreatment resulted in desulphation, in which more interconnection of polysaccharide chain could be developed. The result was correlated well with higher gel strength observed in gel of agar with alkaline pretreatment, compared with native agar (Fig. 2). Sousa et al. (2013) also noted that a lower density of the network was observed in native agar from *Gracilaria vermiculophylla*, reflecting fewer junction zones between helices. Gel strength of agar is governed by molecular weight capability to form 3-dimensional lattices between water and gel helices (Bird, Hanisak, & Ryther, 1981). Differences in structure of agar gel were mediated by their physico-chemical properties.

4. Conclusion

Agar was successfully extracted from *G. tenuistipitata* with appropriate alkaline pretreatment. Alkaline pretreatment using NaOH, especially at a concentration of 5%, increased yield of agar and improved gelling property, viscosity. NaOH pretreatment showed the higher efficacy in improvement of agar properties than KOH as indicated by higher increase in 3,6-AG and lower sulphate content of resulting agar. Therefore, the pretreatment of *G. tenuistipitata* with 5% NaOH prior to agar extraction was recommended for production of agar.

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