Fractionation of Agarose and *Gracilaria verrucosa* Agar and Comparison of Their IR Spectra with Different Agar

Agaroz ve *Gracilaria verrucosa* Agarının Fraksiyonlandırılması ve IR Spektrumlarının Değişik Agar ile Karşılaştırılması

Gülşah Balkan¹, Burak Coban¹* and Kasim C. Güven²

¹Zonguldak Karaelmas University, Chemistry Department 67100, Zonguldak, Turkey
²Istanbul University, Institute of Marine Sciences and Management, Vefa, 34470, Istanbul, Turkey

Abstract

Agarose and *Gracilaria verrucosa* agar were fractionated by Sephadex G-25 and SPE columns (butyl, octadecyl, alumina and quaternary amine). IR spectrophotometric and metachromatic methods were used for the identification of the fractions. In contrary to findings of Tsuchiya and Hong, (1965) IR spectrum of agar and agarose were not the same. Agar showed different IR bands than agarose as at 505, 516, 534, 580, 617, 667 cm⁻¹. Agar was not unique compound, after fraction various agars gave different IR spectra. However agar and agarose gave the same metachromatic β band. Hence differentiation of agar and agarose is possible by IR spectra but not metachromatic method.

Keywords: Agarose, *G. verrucosa* agar and agars, fractionation, SPE column, Sephadex, IR Spectra

Introduction

*Gracilaria verrucosa* (Huds.) Papenfuss, a red alga, is abundant in Turkish coastline. It is one of the main sources of raw material for the manufacture of agar.

Agar is a sulphated polysaccharide and contains maximum 9% sulphate group. It is a mixture of polymers as agarose and agarpectin containing 30,000-120,000 Dalton molecular weight. The structure of agar consists of alternating β-1,3 and α-1,4 linked D- and L- galactose units (Araki et al., 1967). Charged residues are sulphated esters and pyruvated ketal groups also present on the polysaccharide chain. The gel properties are highly dependent on the amount and position of sulphated groups as well as the amount of 3,6-anhydrogalactose fraction of the polysaccharide. The repeating sugar unit may be substituted by methoxyl, pyruvate and sulfate groups (Araki, 1966). The red algal polysaccharides are important in practical use due to their well-known gel-forming ability. The gel strength is often being changed considerably by its impurities. Therefore, a simple and effective purification method is required to improve its gel-forming properties.

Agarose consists of alternating units of 3.6 anhydro α – L- galactose and β – D- galactose. Agarose was separated from agar by acetylation method (Araki, 1937a), Sephadex chromatography (Duckworth and Yaphe, 1971), treating with chitosan (Allan et al., 1971), rivanol (Sviridov et al., 1971), acrino (Fuse and Goto, 1971), cetyl-pyridinium chloride (Hjerten, 1962) and precipitation with polyethylene glycol (Russel et al., 1964). Santos and
Doty (1983) obtained agarose from *Gracilaria cylindrica* by precipitation with benzothonium chloride or with *Eucheuma striatum*.

Agarose contain low sulfate value and high 3,6- anhydrogalactose. Low organic sulfate content is an important criterion in determining the quality of agarose since sulfate group is the major contributory factor to the ionic character of agarose (Santos and Doty, 1983). The acceptable value is 0.7 % for sulfate content (Guiseley and Renn, 1970). The most current applications of agarose require value is 0.3 % or less. *Gracilaria cylindrica* agarose have sulfate content of 0.17- 0.42 % (Santos and Doty, 1983). Methoxyl group content of agarose differs due to the origin. Analysis of algal polysaccharides was made by infrared spectrophotometry (Stanley, 1963; Bellion et al., 1981; Rochas et al., 1986; Sur and Güven, 2002).

Agar was extracted from the algae by hot water, dilute acid or alkali media. Agar was widely used in medicine, pharmaceutics, cosmetics and food industry etc.

Agarpectin was obtained by precipitation with ammonium hydroxide (Barteling, 1962), and ammonium sulfate (Egerov et al., 1970).

Agarose in the from of aqueous gels has been widely used in electrophoresis, chromatography, culture media, immunological analysis and gel cloning as well.

IR spectrum has a role for differentiacion of algal polysaccharides. The IR absorption bands of sulfate groups of algal polysaccharide are shown at 1240-1250 cm\(^{-1}\) generally for ester sulfate and 805 cm\(^{-1}\) attributed to sulfate on C\(_2\) of 3.6 anhydro galactose (Anderson et al., 1968). The band at 705 cm\(^{-1}\) is probably due to sulfate on C\(_4\) galactose (Rohas et al., 1986).

The weak absorption peak at 850 cm\(^{-1}\) indicate the presence of a low content of 4 sulfate in the 1.3- linked galactose units (Zablackis and Santos, 1986).

The peaks at 1960 and 1180 cm\(^{-1}\) (Cross, 1964) were attributed to sulfate ester linked.

Absorbances at 2960, 2845, 1640 and 895- 900 cm\(^{-1}\) were also observed in IR spectra of agar. Absorbance at 2960 cm\(^{-1}\) is associated with CH\(_2\), absorbance at 2845 cm\(^{-1}\) due to O- CH\(_3\) occurs as a shoulder and the band at 2920 cm\(^{-1}\) in spectra of highly methylated agar (Ji et al., 1985).

The peaks at 2830 and 2815 cm\(^{-1}\) were attributed to O\(_{-}\)CH\(_3\) group (Araki et al., 1967) and the peak at 1780 was 6 mono methyl group of agar (Christiaen and Bodard, 1983). The band at 1640 cm\(^{-1}\) was attributed to water (Zundel, 1969). The band at 930 cm\(^{-1}\) (Stanley, 1963) and also at 1070 cm\(^{-1}\) were usually attributed to 3.6 anhydro- galactose (Christiaen and Bodard, 1983).

A sharper band at 930-940 cm\(^{-1}\) indicated O ether bond of 3.6 anhydro – D- galactose. The band at 897 cm\(^{-1}\) was attributed to 1.3 linked β- D galactose pyranosyl units (Barker et al., 1956).

The metachromatic method was used for identification of algal polysaccharides. Metachromasy is a case of the \(\lambda_{\text{max}}\) (α-band) of the dye changes and another \(\lambda_{\text{max}}\) appears (β-band) which can be observed visually and by using spectrophotometer. Metachromasy was used in histological staining of tissues first by Ehrlich (1887). Lison (1935) showed that agar gave metachromatic reaction with cressyl blue. Metachromatic phenomenon of algal polysaccharides was studied by various workers (Michaelis, 1947; Shubert and Levin, 1953; Stone et al., 1963; Suzuki et al., 1969; Graham, 1971; Stone, 1972; Gangolli et al., 1973). Identification of the algal polysaccharides such as agar, carragenan and alginate has been studied in detail by Güven and Güvener, (1985a,b). Agar gave one metachromatic band with acridine orange, toluidine blue, two bands with Azur A and finally three bands with methylene blue (Güven and Güvener, 1985a). The method can be also used for qualitative and quantitative identification of agar.
fractions.

The method used for fractionation of agar in this work are as follows: Sephadex is a modified
dextran which forms cross-linked three-dimensional network of polysaccharide chains. It is
suitable for gel filtration chromatography which is usually used for separating biological
macromolecules according to their molecular weights. The substances are eluted from a
Sephadex bed in the order of decreasing molecular size (Annon. 1966).

Solid phase extraction (SPE) technique based on fractionation of the sample has been used since
1970, for analysis.

This paper reports the fractionation of agar and agarose and comparison of IR spectra and also
different agar and their metachromatic properties.

**Material and Method**

Agarose (Sigma),
Pure agar (Merck),
Commercial agar,
Difco agar,
Pasteur agar,
Aqua agar,
Gracilaria verrucosa agar obtained in our laboratory.

*Gracilaria verrucosa* (Huds.) Papenfuss was collected, in Izmir Bay, Turkey in September
2001. The sample was cleaned from foreign materials, washed with distilled water, dried and
powdered. Agar was extracted with water from algae at 110°C in autoclave for 30 min. It was
filtered from cheese cloth and the filtrate was put in the freezer (Freezing drying technique or
the extract was precipitated by adding of ethyl alcohol (95%) or isopropyl alcohol).

**Fractionation of agar**

1. **Sephadex G25 column**

The volume of the column was determined by using 0.5% dextrane-blue solution.

100 mg of crude agar was fractionated by Sephadex G-25 (AB Pharmacia, Uppsala) in a
1x50cm glass column; distilled water used as mobile phase. The sample was dissolved in 4mL
water and applied to the column. The flow rate was adjusted to 6 drops/min. Each 5mL of
fractions was collected and controlled with 0.5% Azur A solution for metachromatic reaction.
Each fraction was lyophilized and its IR and UV spectra were taken.

2. **SPE column** (J.T.Baker)

The columns used and solvent system are;

2.1. Butyl column, elution solvent:
2.1.1. 1mL distilled water, 1N NaOH and 2mL distilled water
2.1.2. 1mL distilled water, 1N NaOH and 2mL distilled water and 1mL 96% alcohol
2.1.3. Acetonitrile: distilled water (1:1), acetonitrile:distilled water (1:2) and 1mL 96%
    alcohol
2.1.4. Acetonitrile:distilled water (1:1), acetonitrile:distilled water (1:2) and isopropyl
    alcohol
2.1.5. Isopropyl alcohol / %96 ethanol / acetonitrile – distilled water (1:1) / distilled water, 0.1 N NaOH, distilled

2.2. Alumina column, elution solvent:
1mL distilled water / 1N NaOH, 2mL distilled water.

2.3. Amino column, elution solvent:
2 ml acetonitrile distilled water (1:1), 2 ml 0.1 N HCl.

2.4. Quarternier column, elution solvent:
Distilled water / 0.1 NaOH/distilled water / acetonitrile- distilled water (1:1), 2 ml 0.1 N HCl

2.5. Octadecyl column, elution solvent:
Distilled water.

UV spectrum was taken after addition of 1 drop 0.5% Azur A (Gurr) solution. The analysis was made by UV-Visible spectrophotometer (Shimadzu-UV 1601).

IR spectra were taken on agar fractions in KBr tablet by FTIR spectrophotometer (Shimadzu-PC8601).

Results and Discussion

IR spectrum of agarose, *Gracilaria verrucosa* agar and commercial agar are shown in Fig 1-3.

The differences on the IR bands between agarose with various agar and *G. verrucosa* agar are: at 505, 516, 534, 580, 617, 650, 667 cm\(^{-1}\) observed in IR spectra of agar but was not found on the spectra of agarose.

The absorbance of sulfate groups at 705, 805 and 1070 cm\(^{-1}\) were not observed on IR spectra of agarose and also of sulfate groups at 1240-1250, 705 and 850 cm\(^{-1}\) are not found in agarose due to it low contains of sulfate groups.

The band at 2920 cm\(^{-1}\) indicated high methylated group not found in agarose.

This study on IR spectra of various agars gave not the same absorption band.

The absorption bands of *G. verrucosa* agar were not observed in various agars as:

Pure agar: 443, 457, 584, 607, 634, 642, 665, 705, 750, 790, 848, 875, 935, 1066, 1126, 1236, 1325, 1488, 1496, 1506, 1569, 1635, 1647, 1716 and 1739 cm\(^{-1}\);

Commercial agar: 534, 578, 771, 869, 931, 968, 1072, 1157, 1373, 1643, 2115, 2343, 2513 and 2898 cm\(^{-1}\);

Pasteur agar: 424, 580, 617, 650, 869, 891, 1045, 1157, 1218, 1251, 1525, 1544, 1643, 2043, 2898 cm\(^{-1}\);

Difco agar: 424, 650, 690, 713, 771, 869, 891, 931, 989, 1045, 1072, 1218, 1251, 1525, 1544, 1643, 2933 cm\(^{-1}\);

Aqua agar: 424, 435, 501, 617, 650, 869, 891, 1045, 1072, 1157, 1218, 1251, 1525, 1544, 1647, 2358, 2933, 3419 cm\(^{-1}\);

According to these findings agars are not completely similar products. The composition of agars varies according to the algae used and also extraction techniques.
The absorbance of the IR bands of agars provide information on the presence of 3.6 anhydro galactose (930 cm\(^{-1}\)), sulfate 1370 cm\(^{-1}\) galactose –4- sulfate (845 cm\(^{-1}\)), galactose –2- sulfate 830 cm\(^{-1}\), galactose –6- sulfate 820 cm\(^{-1}\) and 3.6 anhydro galactose –2- sulfate 805 cm\(^{-1}\).

Absorbance at 805 cm\(^{-1}\) is attributed to sulfate on C\(_2\) of 3.6 anhydro galactose (Anderson et al., 1986). It was not observed in IR spectra of agarose and agar.

The absorbance at 930 cm\(^{-1}\) and 1070 cm\(^{-1}\) is attributed to 3.6 anhydro- galactose (Stanley, 1963). These were found in IR spectra of agarose and agar.

The absorbance at 1060, 1180, 1070 cm\(^{-1}\) and 1370 cm\(^{-1}\) (Cross, 1964), 1250 cm\(^{-1}\) (Akahane and Izumi, 1976) are attributed to the O-CH\(_3\) group (Araki et al., 1967). The bands 1060 and 1180 cm\(^{-1}\) were not observed in IR spectra of agarose.

The absorption bands of sulfate groups as 1240- 1250, 705 and 850 cm\(^{-1}\) are not found in agarose while it contains lower sulfate groups.

The band at 2920 cm\(^{-1}\) indicated highly methylated group was not found in agarose.

Santos and Doty (1983) are investigated of gel strenghts of agarose and found some differences.

Tsuchiya and Hong. (1965) have studied IR spectra of agar, agarose and agarpectin from *Gelidium amansii* and *Gracilaria sp.* and found that the IR spectra of all tested compounds are similar. In contrary to this findings we found that IR spectra of agar and agarose are not similar.

The different band are observed on the IR spectra of agar as 505, 516, 534, 580, 617 and 667 cm\(^{-1}\).

The metachromatic properties of agarose and agar fraction were also studied. When agar precipitated by addition of 96% ethyl alcohol on crude agar solution their UV spectrum of gave no difference at \(\alpha\) and \(\beta\) bands.

\(\lambda\) max of the metachromatic \(\beta\) band of agar and fractions and \(\alpha\) band of dye are listed below.

<table>
<thead>
<tr>
<th>Agar obtained</th>
<th>(\beta) band</th>
<th>(\alpha) band</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>565</td>
<td>634</td>
</tr>
<tr>
<td>Precipitated by ethanol</td>
<td>565</td>
<td>639</td>
</tr>
<tr>
<td>by isopropyl alcohol</td>
<td>567</td>
<td>634</td>
</tr>
<tr>
<td>Agar fractioned by SPE (butyl)</td>
<td>546</td>
<td>634</td>
</tr>
<tr>
<td>SPE octadecyl</td>
<td>551</td>
<td>615</td>
</tr>
<tr>
<td>SPE Alumine</td>
<td>547</td>
<td>634</td>
</tr>
</tbody>
</table>

As can be seen in the table, the metachromatic bands of agar and fractions and agarose are the same. Hence differenciation of agar and agarose is possible by IR spectra but not metachromatic method.
Table 1. The IR bands of various agars, its fractions and agarose

<table>
<thead>
<tr>
<th>AGAR</th>
<th>cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>pure agar</td>
<td>418 - 443, 516 - 566, 695 - 700, 705 - 710, 848 - 875, 935 - 1005, 1126 - 1224, 1290 - 1320, 1417 - 1438, 1500 - 1521, 1624 - 1629, 1643 - 1650, 1716 - 1739</td>
</tr>
<tr>
<td>Table 1. Continued</td>
<td></td>
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<td>-------------------</td>
<td></td>
</tr>
<tr>
<td>Frozen agar fit. Fr. XIII</td>
<td>430</td>
</tr>
<tr>
<td>Frozen agar fit. Fr. XIV</td>
<td>440</td>
</tr>
<tr>
<td>Frozen agar fit. +Alcohol Fr.XII</td>
<td>450</td>
</tr>
<tr>
<td>Frozen agar fit. +IPA- aqueous part</td>
<td>420</td>
</tr>
<tr>
<td>Frozen agar fit. +EA-aqueous part</td>
<td>420</td>
</tr>
<tr>
<td>Frozen agar fit. +EA-precipitate</td>
<td>420</td>
</tr>
<tr>
<td>Frozen agar fit. +IPA-precipitate</td>
<td>420</td>
</tr>
<tr>
<td>Autoclaved agar +IPA-aqueous part</td>
<td>420</td>
</tr>
<tr>
<td>Autoclaved agar +EA-aqueous part</td>
<td>420</td>
</tr>
<tr>
<td>Autoclaved agar +IPA-precipitate</td>
<td>420</td>
</tr>
<tr>
<td>Autoclaved agar +EA-precipitate</td>
<td>420</td>
</tr>
<tr>
<td>Sephadex G-25 (5-10) Fr.1</td>
<td>410</td>
</tr>
<tr>
<td>Sephadex G-25 (11-15) Fr.2</td>
<td>410</td>
</tr>
<tr>
<td>Sephadex G-25 (16-20) Fr.3</td>
<td>470</td>
</tr>
<tr>
<td>Sephadex G-25 (21-25) Fr.4</td>
<td>470</td>
</tr>
<tr>
<td>Sephadex G-25 (26-30) Fr.5</td>
<td>410</td>
</tr>
<tr>
<td>Sephadex G-25 (31-35) Fr.6</td>
<td>410</td>
</tr>
<tr>
<td>Sephadex G-25 (36-40) Fr.7</td>
<td>410</td>
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<tr>
<td>Table 1. Continued</td>
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<tr>
<td>Sephadex G-25</td>
<td>470</td>
</tr>
<tr>
<td>(31-35) Fr.6</td>
<td></td>
</tr>
<tr>
<td>SPE-Al₂O₃ (d/water +NaOH+d/water)</td>
<td>420</td>
</tr>
<tr>
<td></td>
<td>470</td>
</tr>
<tr>
<td>SPE-C₄ (d/water +NaOH+d/water)</td>
<td>420</td>
</tr>
<tr>
<td></td>
<td>470</td>
</tr>
<tr>
<td>SPE-C₄ (d/water +NaOH+d/water+alcohol)</td>
<td>470</td>
</tr>
<tr>
<td>SPE-C₄ (acetonitrile/dist.water+EA)</td>
<td>420</td>
</tr>
<tr>
<td>SPE-C₄ (acetonitrile/dist.water+IPA)</td>
<td>420</td>
</tr>
<tr>
<td>SPE-C₄ (acetonitrile/dist.water+IPA+precip.)</td>
<td>420</td>
</tr>
</tbody>
</table>

100
Fig. 2. IR spectra of agarose

Bu çalışmada ayrıca agarın tek bir madde olmadığı ve fraksiyonlanması sonucunda farklı IR spektrumlarına sahip maddelerden oluştuğu tespit edilmiştir.

Diğer tarafından agarın ve agarozun ve agar fraksiyonlarının UV’de metakromatik ile spektrumları alındı burada ise bir farklılık saptanmadı.

Sonuçta agar ve agarozun IR spektrunları arasındaki fark ile ayrılabileceği ve fakat metakromatik yol ile ayrılamayacağı saptandı.

**References**


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