Effect of Ethanol on Physical Chemistry Characterization, Microorganism, and Toxicity of Carrageenan Extracted with the Assistant of Enzyme Viscozyme L

Bui Huy Chich¹,*, Do Van Ninh², Vu Ngoc Boi³, Dang Xuan Cuong⁴,*

¹Department of Science and Technology, Ba Ria-Vung Tau Province, Vung Tau, Vietnam
²Pacific Ocean University, MOET, Nha Trang, Vietnam
³Faculty of Food Technology, Nha Trang University, Nha Trang, Vietnam
⁴Organic Material from Marine Resource, Nhatrang Institute of Technology Application and Research, Vietnam Academy of Science and Technology, Nha Trang, Vietnam

Email address: chichlcntp@gmail.com (B. H. Chich), vaninhcb@yahoo.com (Do V. Ninh), minhboiit@yahoo.com (Vu N. Boi), cuong_mail@ yahoo.com.vn (D. X. Cuong)
*Corresponding author

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Abstract: Carrageenan is a biopolymer found in red algae with high potential in food, functional food, pharmaceutics, and cosmetics. The study focused on the effect of ethanol on physical chemistry characterization and microorganisms of carrageenan that extracted by the enzyme – assisted method and the purification by using ethanol. The results showed the moisture of carrageenan varied from 10.9 to 9.5% DW. After the impact of ethanol, the purification and physical (dispersal in water and rheological) characterization of carrageenan was higher than before the impact of ethanol. For example, dispersal in water, the viscosity of the solution, the solution strength (1.5% of carrageenan and 0.2% of potassium chloride), and carbohydrate content at 20°C corresponded to 1.06, 1.18, 1.07, and 1.11 times, compared to before the impact of ethanol. The content of ethanol-insolubility impurities, total ash, acid-solubility ash, acid-insolubility ash, total protein, sulfate content (SO₄²⁻), and lipid content was 43%, 94.6%, 42.9%, 44.44%, 3.9%, 97.2%, and none-detected in comparison to before the impact of ethanol. The content of lead, arsenic, cadmium, and mercury was 0.01, < 0.01, 0.05, and < 0.01 ppm, respectively. Total aerobic bacterial of carrageenan got the highest value of 2.1 x 10² cells/g. E. coli, coliforms, staphylococcus aureus, salmonella, and bacillus cereus did not occur in carrageenan.

Keywords: Carrageenan, NMR, Rheology, Mineral, Kappaphycus alvarezii, Cam Ranh

1. Introduction

Carrageenans are linear polysaccharides, possess repeating sequences of α-D-galactopyranose and β-D-galactopyranose residues with the 1,3 and 1,4 linkage, named the A residue and B residues, respectively. The difference in the extraction method and the algae species, types of carrageenans can be obtained different, for example, kappa (κ), iota (ι), and lambda (λ) [1-3]. Carrageenans possess good rheological characterizations (forming thermoreversible gels, viscosity) in the salt solutions of small concentration with widely applying into food [4, 5], functional food [5], pharmaceutics [6, 7], and cosmetics [8] in the role of texturing, thickening, suspending, or stabilizing agents [9, 10]. Carrageenan is non-toxic, induces thrombosis, anti-cancer, and anti-inflammatory [11, 12].

Carrageenan content in red algae is up to 40% DW and extracted by using acidic, alkaline, or enzyme depending on the algae species [13]. Almost studies on the carrageenan
extraction used the chemistry method leading to environmental pollution. The enzyme-assisted extraction method was environmental pollution less than the chemistry method. The carrageenan separation out of the cell membrane is effectively better than the chemistry method. Carrageenan is usually purified by the column causing the difference in the application into the food [13-17].

Thus, the study focused on the effect of ethanol on physical chemistry characterization and microorganisms of carrageenan for finding the solution of carrageenan purification easier.

2. Material and Methods

2.1. Material

Kappaphycus alverazii (Doty) cultivated commonly in Nha Trang Bay was harvested, and after cleaning by seawater, they were transferred to the laboratory at the condition under 10°C for further study.

All chemicals using in the analysis were from Sigma – Aldrich. The distilled water and 96% ethanol was of Vietnam.

2.2. Sample Preparation

2.2.1. Enzyme-assisted Extraction of Carrageenan

*K. alverazii* was macerated in the buffer (pH 5.1) at 42°C for 60 minutes with 1.45% of enzyme according to the solution and algae ratio of 20/1 (v/w). After filtration, the residue was soaked in aqueous at 90°C for 80 minutes with the aqueous to residue ratio of 50/1 (v/w) and collecting the supernatant through the membrane. Carrageenan was continuously precipitated in 80% ethanol and dried by using the method of freeze-drying for the further studies.

2.2.2. Purification of Carrageenan by Using Ethanol

The solution composed of 5% of carrageenan and 25% ethanol was kept at 70°C for 15 minutes for precipitating dissolved protein and impurities. The supernatant was continuously collected by the centrifugation at 10,000 rpm for 15 minutes, and precipitating in 60% ethanol for 40 minutes. After precipitation, the residues were filtered, cleaned twice in 96% ethanol, and dried at 45±2°C by using the freeze drying with the velocity ratio of 2 m/s.

2.3. Quantification Methods

Quantification of moisture was according to the AOAC method [18].

Quantification of solubility in water

The determination of rheology characterization (viscosity and gell strength) was by the machine (Brookfield (American) and CR 500DXS – SunScientific (Japan)), respectively [19].

Quantification of total ash, acid-insoluble ash, and ash soluble in acid was in accordance to the AOACA method (AOAC. 975.12) and Nancy et al. [20].

Quantification of protein content was according to the AOAC method (920.103) based on the nitrogen content with the factor 6.25 [21].

Quantification of sulfate content (SO₄²⁻): One gram of carrageenan was soaked in 50 mL of 0.2 N HCl and boiled for 01 hours. 25 mL of H₂O₂ was then added to the mixture and heated for 05 h. After 05 hours, this solution added to 10 mL of 10% BaCl₂ and boiled for 02 hours. The residues were filtered through an ashless filter (Whatman No. 42) and removed the residual chloride by using the hot distilled water. The filter paper and precipitate were finally burned at 650°C in a furnace and calculating based on equation 5 (JECFA 2007).

Quantification of carbohydrate content was according to the method of Roman (1946) with the standard of glucose, and the absorbance measurement at the wavelength of 490nm [22].

The quantification of lipid content was to base using n-hexane [23].

Quantification of the content of Pb, As, Cd, and Hg was by using inductively coupled plasma mass spectrometry [24].

Quantification of total aerobic bacterial

Quantification of Escherichia coli and Coliforms was according to Method 1604 (2002) [25].

Quantification of Staphylococcus aureus was based on the method of AOAC 975.55 [26].

Quantification of Salmonella was according to Denise et al. [27].

Quantification of Bacillus cereus was according to Irena et al. [28].

2.4. Evaluation of Toxicity

The toxicity assay of single-dose (safety) was on Swiss white mice consisting of four groups and twelve mice per group (ten male and ten female). Group A, B, C, and D drunk the carrageenan solution of 1.5% (w/v), 1.0% (w/v), 0.5% (w/v), and physiological saline, respectively. Clinical manifestations and weight of each rat were observed daily for seven consecutive days. All mice were operated on to see the whole organ in the abdominal and thoracic cavity (Table 1).

The tissue samples will be taken and sent to histopathology at the Department of Pathology and Forensic Medicine, Hue University of Medicine and Pharmacy as finding any abnormalities. Mice numbers were from 101 to 120, 201 to 220, 301 to 320, and 701 to 720, corresponding to group A, B, C, and control (salt solution), respectively (Table 5). Numbered mice were to the first male and late females.

### Table 1. Criteria for evaluating clinical manifestations in safe laboratory rats.

| Symptom                                             | Evaluation (% | Appearance (date) |
|-----------------------------------------------------|---------------|-------------------|
| Struggling / stimulating / tiptoeing                 | No / Yes      |                   |
| Shaggyness, poor reflexes with the outside           | No / Yes      |                   |
| Ruffled feathers                                    | No / Yes      |                   |
| Shortness of breath                                 | No / Yes      |                   |
| Exudation (watery eyes, runny nose, saliva)          | No / Yes      |                   |
| Shivering/sweating                                  | No / Yes      |                   |
| Distention                                           | No / Yes      |                   |
| Vomiting                                             | No / Yes      |                   |
| Diarrhea                                             | No / Yes      |                   |
| Paralysis or increase/decrease in muscle tone        | No / Yes      |                   |
2.5. Determination of Carrageenan Purification

The determination of carrageenan purification was by using the NMR spectrum, and carrageenan content before and after purification in 96% ethanol. 1H-NMR (500 MHz, CDCl₃) and 13C-NMR (125 MHz, CDCl₃) spectrum were determined on the machine Bruker Avance-500 MHz with internal standards of TMS.

2.6. Data Analysis

All experiments were in triplication (n=3) and removing unnormal value by the method of Duncan. Statistic analysis was by using the software of MS Excel 2010.

3. Results and Discussion

3.1. Physical Characterization of Carrageenan

The results showed ethanol affected the physical chemistry of carrageenan that extracted with the assistance of enzyme Viscozyme L and the purification by using ethanol. For example, before the impact of ethanol, viscosity of solution (1.5% of carrageenan) at 75°C and the solution strength (1.5% of carrageenan and 0.2% of potassium chloride) at 20°C the content of the moisture corresponded to 80.5±2.01 (cPs) and 615±22.76 (g/cm²), respectively. After the impact of ethanol, the viscosity and the strength of the carrageenan solution were 95.3±2.76 (cPs) and 657±15.11 (g/cm²) (Table 2), respectively. Therefore, ethanol caused the improvement of the physical characterization (viscosity and strength of the solution) of carrageenan.

| Order | Analysis target | Unit | The results |
|-------|----------------|------|-------------|
|       |                |      | Before purification | After purification |
| 1     | Moisture       | % DW | 10.9±0.23 | 9.5±0.27 |
| 2     | Dispersal in water | % DW | 92.5±1.76 | 98.2±2.46 |
| 3     | Ethanol-insolubility impurities | % DW | 1.74±0.04 | 0.74±0.02 |
| 4     | Viscosity of solution (1.5% of carrageenan) at 75°C | cPs | 80.5±2.01 | 95.3±2.76 |
| 5     | The solution strength (1.5% of carrageenan and 0.2% of potassium chloride) at 20°C | g/cm² | 615±22.76 | 657±15.11 |
| 6     | Total ash content | % DW | 20.3±0.37 | 19.2±0.52 |
| 7     | Acid-solubility ash | % DW | 0.7±0.02 | 0.3±0.01 |
| 8     | Acid-insolubility ash | % | 0.9±0.02 | 0.4±0.01 |
| 9     | Total protein content | % | 5.1±0.17 | 2.0±0 |
| 10    | Sulfat content (SO₄²⁻) | % | 17.8±0.52 | 17.3±0.4 |
| 11    | Carbohydrate content | % | 45.5±1.64 | 50.6±1.42 |
| 12    | Lipid content | % | 0.4±0.02 | - |
| 13    | Lead content (Pb) | mg/kg | 0.023 | 0.01 |
| 14    | Arsenic content (As) | mg/kg | 0.038 | <0.01 |
| 15    | Cadmium content (Cd) | mg/kg | 0.105 | 0.05 |
| 16    | Mercury content (Hg) | mg/kg | 0.026 | <0.01 |

3.2. Microorganisms on Carrageenan

The microorganisms causing the human diseases did not occur in carrageenan, except for total aerobic bacterial. For example, before and after the impact of ethanol, total aerobic bacterial of carrageenan corresponded to 2.1×10³ and 10² Cells/g, respectively. According to the standard of FAO on carrageenan [29], total aerobic bacterial was not excess 5000 CFU/g (Table 3). Therefore, carrageenan in the current study got the standard of FAO.

| Order | Microorganism | Unit | The results |
|-------|---------------|------|-------------|
|       |               |      | Before purification | Before purification |
| 1     | Total aerobic bacterial | Cells/g | 2.1.10³ | 10⁴ |
| 2     | Escherichia coli | Cells/g | None detected | None detected |
| 3     | Coliforms | Cells/g | None detected | None detected |
| 4     | Staphylococcus aureus | Cells/g | None detected | None detected |
| 5     | Salmonella | Cells/25g | None detected | None detected |
| 6     | Bacillus cereus | Cells/g | None detected | None detected |

3.3. Chemical Composition and Characterization

The results showed ethanol affected the chemical composition and characterization of carrageenan that extracted with the assistance of enzyme Viscozyme L and the purification by using ethanol. For example, before the impact of ethanol, the content of the moisture, the ethanol-insolubility impurities, total ash, acid-solubility ash, acid-insolubility ash, total protein, sulfate (SO₄²⁻), carbohydrate, and lipid of carrageenan corresponded to 10.9±0.23, 1.74±0.04, 20.3±0.37, 0.7±0.02, 0.9±0.02, 5.1±0.17, 17.8±0.52, 45.5±1.64, 0.4±0.02% DW, respectively, and was 1.14, 2.35, 1.05, 2.33, 2.25, 2.55, 1.03, and 0.9 times, compared to after the impact of ethanol, respectively (Table 2). Lipid did not exist in carrageenan after
the impact of ethanol, the viscosity of solution (1.5% of carrageenan) at 75°C, and the solution strength (1.5% of carrageenan and 0.2% of potassium chloride) at 20°C of carrageenan after the impact of ethanol were higher than before the impact of ethanol. Heavy metal content (lead, arsenic, cadmium, and mercury) of carrageenan after the impact of ethanol was lower than before the impact of ethanol. Cadmium content got the highest value, compared to other metal content for both carrageenan kinds. The maximum value of the content of lead, arsenic, and mercury was ≤ 0.01 ppm.

Figure 1. The 1H-NMR spectrum of carrageenan before the impact of ethanol.

Figure 2. The 13C-NMR spectrum of carrageenan before the impact of ethanol.
The anomeric proton signals (1H in the β-D-Gal residue of carrageenans) exhibited in the range of 4.49 to 4.54 ppm (Figure 1) and 4.5 to 4.57 ppm (Figure 3) in the 1H NMR spectrum. The signals of 1H in α-D-AnGal residue, αD-AnGal residue, the methyl proton in 6-O-methyl Gal, and methyl hydrogen of carrageenan did not occur in both of 1H spectrum. Methylene and methine hydrogens of the carrageenan exhibited in the range of 3.76 to 4.5 (Figure 1) and 3.5 to 4.81 ppm (Figure 3). The signal range at 102.5, 91.7, 91.7, and 95.8 ppm exhibited anomeric carbon resonance pairs attributed to the pyruvated α-, methylated α- and ι carrageenans, respectively (Figure 2). The anomeric carbon
resonance pairs belonging to pyruvate and carrageenan occurred in the signal range at 102, 95.5 (Figure 4), 60.4 & 60.6 (Figure 2), and 61.3 (Figure 4) of the carbon resonance were belonging to the methylated C-6 of 3-linked galactose. 13C NMR resonances at 101.0 ppm indicated the acetel group of the pyruvate unit. C-4 and C-5 of the 3-linked pyruvate galactose unit exhibited in the signals at 67.8 and 67.9 ppm. The signal at a range of 6 to 7 ppm was the characterization for protein impurities that existed in carrageenan. This signal was consistent with the results of the analysis of physical and chemical indicators of pre-purified carrageenan samples and showing that the protein existed in the initial carrageenan sample. The peak at 6 ÷ 7ppm did not occur in figure 4. Some peaks at the range of 4 ppm in figure 2 were more than figure 4, was the characterization of protein and lipid. The information was suitable for the analysis results of physical chemistry of carrageenan before and after the purification by ethanol. Thus, ethanol was useful to the purification of carrageenan.

3.4. Toxicity of Carrageenan After Purification

3.4.1. Clinical Manifestations in Mice

After seven days of testing, mice were given carrageenan at different concentrations without any clinical symptoms compared to the control samples (Table 4).

### Table 4. Clinical manifestations of mice drinking Carrageenan and control.

| Symptoms                          | Group A | Group B | Group C | Control group |
|-----------------------------------|---------|---------|---------|---------------|
| Struggling / stimulating / tiptoeing | None    | None    | None    | None          |
| SluggishNESS, poor reflexes with the outside | None    | None    | None    | None          |
| Ruffled feathers                  | None    | None    | None    | None          |
| Shortness of breath               | None    | None    | None    | None          |
| Exudates (watery eyes, runny nose, saliva)| None    | None    | None    | None          |
| Shivering/sweating               | None    | None    | None    | None          |
| Distention                       | None    | None    | None    | None          |
| Vomiting                         | None    | None    | None    | None          |
| Diarrhea                         | None    | None    | None    | None          |
| Paralysis or increase/decrease in muscle tone | None    | None    | None    | None          |

Note: None: none-detection.

#### 3.4.2. Mice Weight

### Table 5. Mice weight drinking Carrageenan and control after seven days.

| Mice code | Weight (g) per day | Mice code | Weight (g) per day |
|-----------|--------------------|-----------|--------------------|
| D0        | 21.1               | D0        | 21.0               |
| D1        | 21.0               | D1        | 21.2               |
| D2        | 21.4               | D2        | 21.4               |
| D3        | 21.8               | D3        | 21.6               |
| D4        | 21.9               | D4        | 21.7               |
| D5        | 21.7               | D5        | 21.7               |
| D6        | 21.8               | D6        | 21.9               |
| D7        | 21.9               | D7        | 21.5               |

| Mice code | Weight (g) per day | Mice code | Weight (g) per day |
|-----------|--------------------|-----------|--------------------|
| D0        | 21.1               | D0        | 21.0               |
| D1        | 21.0               | D1        | 21.2               |
| D2        | 21.4               | D2        | 21.4               |
| D3        | 21.8               | D3        | 21.6               |
| D4        | 21.9               | D4        | 21.7               |
| D5        | 21.7               | D5        | 21.7               |
| D6        | 21.8               | D6        | 21.9               |
| D7        | 21.9               | D7        | 21.5               |

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Carrageenan oral mice: mice weight increased from 9.7 to 10.5 g/rat (increased by 50-55%, respectively), compared to the initial weight. The increase in mice weight was not different insignificance (p>0.05) between other groups. Therefore, purified carrageenan by ethanol was non-toxicity.

3.4.3. Pathology
Abnormalities in the organs belong to the abdominal and thoracic of the rat were not found after surgery (Figure 5). The lymph nodes, tumors, bleeding signs, abnormal fluid retention in the abdominal and chest cavities did not appear.

**Figure 5.** Mice drunk physiological saline (a) and carrageenan (b).

With the results of clinical observation, weight monitoring, and anatomy, it said that carrageenan was non-toxic and safe.

4. Conclusion
After the impact of ethanol, the purification and physical (dispersal in water and rheological) characterization of carrageenan was higher than before the impact of ethanol. For example, dispersal in water, the viscosity of the solution, the solution strength (1.5% of carrageenan and 0.2% of potassium chloride), and carbohydrate content at 20°C corresponded to 1.06, 1.18, 1.07, and 1.11 times, compared to before the impact of ethanol. The content of ethanol-insolubility impurities, total ash, acid-solubility ash, acid-insolubility ash, total protein, sulphat content (SO₄²⁻), and lipid content was 43%, 94.6%, 42.9%, 44.44%, 3.9%, 97.2%, and none-detected in comparison to before the impact of ethanol. The content of lead, arsenic, cadmium, and mercury was 0.01, < 0.01, 0.05, and < 0.01 ppm, respectively. Total aerobic bacterial of carrageenan got the highest value of 2.1 x 10^8 cells/g. E. coli, coliforms, staphylococcus aureus, salmonella, and bacillus cereus did not occur in carrageenan. Purified carrageenan by using ethanol was non-toxic.

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