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THE IMPACT OF ISOPROPYL ALCOHOL AND POTASSIUM CHLORIDE ON THE PRECIPITATION OF ENZYMATICALLY EXTRACTED CARRAGEENAN FROM *Kappaphycus striatum*

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ABSTRACT

Growing evidence points to enzymatic carrageenan manufacturing as an eco-friendly process. This study examines the effects of varying enzyme concentrations (between 0.1% and 0.5%) and altering the precipitation process by combining KCl salt with isopropyl alcohol (IPA) on the qualitative characteristics of carrageenan products. Carrageenan extraction is made easier by cellulase, which breaks down the cellulose component of the seaweed wall. The precipitation process's use of KCl has a major impact on the gel's viscosity, water content, ash content, and sulfate content. The findings show that, compared to IPA alone, the addition of KCl alters the gel strength and sulfate content. Gel strength and viscosity are also highly influenced by different enzyme concentrations, where larger enzyme concentrations result in lower values. The rapid visco analyzer demonstrates improved gel formation and higher final viscosity values (113 cP at 0.1% enzyme usage) with the addition of KCl salt. This study sheds light on optimizing enzymatic carrageenan production for enhanced product quality and efficiency.

Keywords: Carrageenan, Enzymatic, Isopropyl Alcohol, KCl, Precipitation.

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INTRODUCTION

Seaweed hydrocolloids, owing to their unique functional properties, have garnered significant interest in the food industry.[1] Among these hydrocolloids, carrageenan, extracted from red seaweed, particularly *Kappaphycus alvarezii* and *Kappaphycus striatum*, stands out.[2] The current industry preference for carrageenan extraction leans towards alkaline compounds, aiming for extracts with low sulfate content to achieve superior gel strength.[3] However, this method is time-intensive, taking up to a day to complete.[4] Carrageenan, a polysaccharide made up of 3,6-anhydrogalactose and galactose, along with various sulfate esters such as ammonium, calcium, magnesium, potassium, and sodium, undergoes a series of extraction processes including soaking, heating, filtering, gelling, drying, and milling.[5,6] Its versatile properties, including the capacity to thicken, gel, and stabilize, are widely used in the food business., enhancing cheese texture, regulating pudding viscosity, and serving as a filler and stabilizer in meat preparation. The extraction process's quality is influenced by various factors such as seaweed harvest age, post-harvest conditions, storage, distribution, and yield. Although the alkaline extraction method is common, its association with environmental pollution due to alkali residues has spurred the exploration of alternative methods.[7] Researchers have investigated methods like the use of Calcium Hydroxide neutralized with carbon dioxide and enzyme-assisted extraction, aiming for greener and more sustainable approaches.[8] Several studies have explored enzymatic carrageenan extraction, comparing it with the alkaline method.[9] Additionally, the extraction of carrageenan from commercially obtained seaweeds is expected to yield high-purity products, free from protein impurities.[10] Various studies have successfully demonstrated the enzymatic isolation of carrageenans from red seaweed species. For instance, alcalase and papain have been utilized to extract hybrid and lambda carrageenans, respectively, with promising results.[11,12] Furthermore, enzyme-assisted extraction from *Eucheuma cottonii* showed higher yields compared to traditional boiling methods.[13] Notably, studies have shown that carrageenan extracted using pure enzymes from coastal plants' endophytic shoots exhibited higher yields and acceptable properties.[14] Moreover, the interaction between kappa carrageenan and KCl solution has been studied extensively. Kappa carrageenan's gelation is

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enhanced when combined with K^+ ions, attributed to ionic bonding between sulfite chains and potassium ions.[15,16] Several studies have examined the effect of adding KCl solution to kappa carrageenan, demonstrating enhanced gelation due to the presence of K^+ ions.[17] Kappa carrageenan will produce more gelation when mixed with K^+ ions.[18] That sulfite's chain from carrageenan interacts with K^+ by ionic bonding. For instance, two sulfite-anhydrous galactose groups interact simultaneously with the potassium ion.[19] Kappa carrageenan in a sol form at KCl concentrations ranging from 0 to 15 mmol/dm^3.[20] The counterion protects the electrostatic attraction between the coil dimensions and the polymer chain as the concentration of counterions increases in polyelectrolytes. The K^+ ion acts as the counterion in the interaction between kappa carrageenan and KCl solution. Therefore, research has also been done to investigate the impact of mixing kappa carrageenan with KCl solution, which increases gelation when mixed with K^+ ions. When kappa-carrageenan and KCl solution mix, the K^+ ion is the counterion.

EXPERIMENTAL

Material and Methods

Commercial cellulase enzymes were purchased from Novozyme and dried seaweed *Kappaphycus striatum* obtained from the East Nusa Tenggara region were utilized. KCl, IPA, and distilled water were used in the carrageenan production process. The carrageenan extraction process involved the utilization of various tools, including a shaker water bath, Erlenmeyer flask, analytical balance, basin, gelling basin, powder machine, centrifuge, pH paper, timer, and hot plate. Additionally, the quality analysis phase employed a range of tools such as porcelain cups, desiccators, Erlenmeyer flasks, beaker glass, hotplates, stirrers, funnels, spatulas, ovens, furnace ovens, filter paper, thermometers, curd tension apparatus, casts, Rapid Visco Analyzers, and TA-XT Texture Analyzers.

Seaweed Pre-treatment

Contaminants such as sand and salt are removed from dried *Kappaphycus striatum* seaweed through a washing process, followed by sun-drying. After that, the seaweed is bleached by soaking it for two hours in a 1% H_2O_2 solution. After bleaching, the seaweed is again dried until its water content reaches 12% by the AOAC[21] standard, rendering it suitable for extraction. This bleaching and drying cycle is repeated until the seaweed achieves a neutral pH level.

Extraction of Carrageenan

There were several stages involved in removing carrageenan from dried *Kappaphycus striatum*. First, 1000 milliliters (1000 mL) of Novozyme cellulase enzyme solution with varying concentrations (0.1–0.5%) was added to 40 g of dried seaweed. The mixture was heated in a shaking water bath for an hour at 60°C. The remaining seaweed was then boiled for two hours at 90°C after being mixed into a pulp. After the resultant solution was separated, the supernatant was obtained by centrifuging it at 3500 rpm for 15 minutes. This was done in a 1:1 ratio with distilled water. The diluted extract was mixed with 6.25% KCl solution (10% concentration) to encourage the production of gels. The resulting mixture was then put into isopropyl alcohol and left for about 15 minutes. Consequently, a carrageenan gel clump developed. This gel was subsequently dried in an oven for eighteen hours at 50°C. Finally, the dried carrageenan sheets were ground into a fine powder using a ball mill, yielding the desired carrageenan extract ready for further analysis and application in various industries. The use of different concentrations of Novozyme cellulase enzyme solution allowed for the evaluation of its impact on carrageenan extraction efficiency, ensuring reliable and reproducible results for future research and applications.

Carrageenan's Characteristics Analysis

Several characteristics were assessed, such as gel strength, yield, water content, sulfate content, ash content, and viscosity. The FMC Corp. (1977) method was used to measure the yield, gel strength, viscosity, sulfate concentration, and ash content.[22] However, the AOAC (1995) protocol was followed in the analysis of the water content.

RESULTS AND DISCUSSION

Yield, Water Content, Ash Content, and Sulfate Content

The carrageenan yield obtained through different extraction methods, specifically using KCl precipitation and enzymatic treatment, exhibits significant variations. The disparity in yield is logically attributable to

the salt content present in carrageenan precipitated with KCl, which leads to an increase in the overall yield. These distinctions are summarized in Table-1.

Table-1: Carrageenan Yield (%) by IPA Precipitation with or without KCl

Enzym(%)	IPA w/o KCl(%)	IPA w/ KCl(%)
0.1	10.73	29.41
0.2	17.84	39.48
0.3	11.76	41.23
0.4	15.79	32.85
0.5	16.49	35.96

Moreover, our investigation into the impact of varying the amount of cellulase enzymes used in the extraction process reveals irregular yield values. Notably, the treatment utilizing a 0.2% enzyme concentration consistently demonstrates a notably high yield, regardless of the extraction method employed. The values for water content and ash content obtained from various enzyme concentrations did not have a high significance, where the water content obtained ranged from 12.52% to 18.20% and the ash content ranged from 10.6% to 22.65%. Tables-2 and 3 below provide the water and ash content values.

Table-2: Carrageenan Water Content (%) by IPA Precipitation with or without KCl

Enzyme(%)	IPA w/o KCl(%)	IPA w/ KCl(%)
0.1	13.85	18.16
0.2	13.53	18.20
0.3	12.45	15.63
0.4	13.25	16.47
0.5	12.52	13.91

By WHO standards, high-quality carrageenan should not contain more than 12% water to avoid microbial and fungal growth. Nevertheless, prior research has shown that the quantity of carrageenan produced enzymatically varies from 7% to 10%. This discrepancy suggests that the use of IPA during the enzymatic process has an impact on the water content of the carrageenan produced.

Table-3: Carrageenan Ash Content (%) by IPA Precipitation with or without KCl

Enzym(%)	IPA w/o KCl(%)	IPA w/ KCl(%)
0.1	10.60	19.83
0.2	12.98	21.22
0.3	13.34	22.65
0.4	13.65	22.13
0.5	13.78	20.36

The results presented in Tables-2 and 3 demonstrate that the carrageenan samples' ash and water content were significantly impacted by the addition of KCl. Specifically, KCl increased the water content of the carrageenan gel matrix, which in turn helped to maintain the matrix's stability by preventing erosion or decay.[23] The increase in acid-insoluble ash content observed in samples with added KCl is likely due to the ionization of KCl into K^+ and Cl^-, with potassium being one of the compounds that contribute to ash formation. Consequently, the ash content rose as a result of the KCl addition to the carrageenan solution, which caused an accumulation of insoluble potassium.

Table-4: Carrageenan Sulfate Content (%) by IPA Precipitation with or without KCl

Enzym(%)	IPA w/o KCl(%)	IPA w/ KCl(%)
0.1	16.43	11.02
0.2	16.77	15.33
0.3	16.50	15.89
0.4	15.62	15.74
0.5	16.88	15.08

Due to its effect on the gel strength and viscosity of the carrageenan solution, the sulfate level is a crucial component that determines the quality of seaweed products.[24] The carrageenan sulfate content in this study is shown in Table-4, where it ranges from 11.02% to 16.88%. According to FAO[25], this value is lower than the standard value of carrageenan for its application as a food ingredient, which is between 15% and 40% sulfate content. Furthermore, this value is still lower than the sulfate content of carrageenan produced using

alkaline compounds. This lower sulfate content may be due to the use of raw materials with a low sulfate content. The adding the extraction process, the sulfate bond is separated from the polysaccharide bond. Sulfate reduction is a necessary reaction that may be required to improve gelation properties. High sulfate levels affect carrageenan solution characteristics such as gel strength and viscosity.[4] Additionally, the precipitation process indicates that samples with KCl tend to reduce sulfate levels. This reduction occurs because K^+ ions can convert sulfate groups into galactose groups[26], resulting in a decrease in sulfate content.

Rapid Visco Analyzer

The study utilized RVA to measure viscosity and distinguish the gelation characteristics of different treatments, such as enzyme pretreatment and precipitation. By examining multiple profiles, the study identified the properties of the carrageenan produced, which can be used for future applications. The viscosity of the carrageenan solution was assessed utilizing the Rapid Visco Analyzer (RVA 4500, Perten Instruments) to characterize its viscosity profile. 28 g of distilled water and 0.28 g of the hydrocolloid sample were mixed to provide a 1% w/w aqueous solution for the testing process. Before initiating the test, the gum was thoroughly dissolved in water using a non-stick stirrer. The viscosity value was measured during a 70-minute rotation at 160 rpm as the temperature dropped steadily from 80°C to 20°C. The viscosity profiles were found to be influenced by both the duration of alkalization and the quantity of enzyme applied during pretreatment, demonstrating a correlation with temperature and time. Subsequently, a descriptive analysis was conducted on the viscosity data.[27,28] Figures-1 and 2 display the carrageenan profile, and Tables-3 and 4 illustrate the changes in viscosity over time.

Table-5: Carrageenan Viscosity (cP) on Cellulase Concentration by IPA and KCl Precipitation

Temp	Enzym (%)				
°C	0.1	0.2	0.3	0.4	0.5
80	20	23	18	35	19
70	20	23	16	32	15
60	23	25	17	34	16
50	24	28	18	36	17
40	28	33	19	39	19
30	32	37	23	43	21
21	113	123	63	179	52

In Fig.-1, the relationship between carrageenan concentration and cellulase concentration for precipitation using a combination of IPA and KCl is depicted. The measurements taken by Yu *et al*. are consistent with the trend seen in this picture[29], wherein the viscosity of the solution initially remains low and subsequently increases as the temperature decreases. In contrast, Fig.-2 displays a distinct profile that does not exhibit a viscosity spike, making it difficult to determine the gel point in the carrageenan extract sample subjected to IPA precipitation without KCl addition.

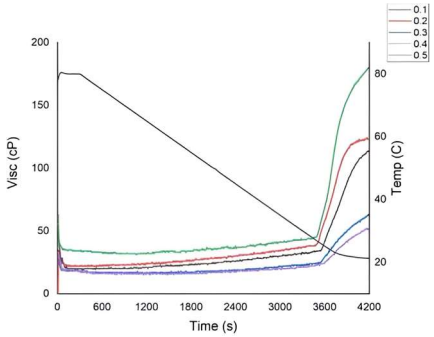

Fig.-1: RVA profile of Carrageenan Extract at Various Cellulase Concentrations with IPA and KCl Precipitation

As a result, the information derived from these figures shows how the addition of KCl affects the precipitation of carrageenan and the subsequent gel formation.

The inclusion of cellulase enzymes appears to have an impact on the rapid visco analyzer readings, where, in general, higher concentrations of cellulase result in higher viscosity values. Specifically, the highest

viscosity value of 179 cP was attained when using an enzyme concentration of 0.4%. These results align with the findings reported by Laksono *et al*.[2], which also demonstrated an increase in cellulase concentration leading to higher viscosity values in extracted carrageenan. In contrast, the process without KCl precipitation showed comparatively lower viscosity values for all carrageenan samples, as opposed to the addition of KCl precipitation. Nonetheless, a common trend emerged in both processes, where higher enzyme concentrations resulted in higher achieved viscosities.

Table-6: Carrageenan Viscosity (cP) on Cellulase Concentration only by IPA Precipitation

Temp	Enzym (%)				
°C	0.1	0.2	0.3	0.4	0.5
80	18	23	16	24	27
70	17	21	14	23	25
60	17	22	16	26	29
50	19	25	18	28	32
40	22	29	20	33	37
30	26	34	24	38	43
21	30	39	27	45	50

However, it is noteworthy that a peculiar phenomenon was observed in the KCl precipitation process when employing a 0.5% enzyme concentration, wherein the viscosity value exhibited a drastic drop. This observation raises intriguing questions concerning the potential interaction between isopropyl alcohol (IPA) and KCl during the carrageenan precipitation and recovery stages. To fully comprehend the fundamental mechanics underlying this phenomenon, more research is necessary.

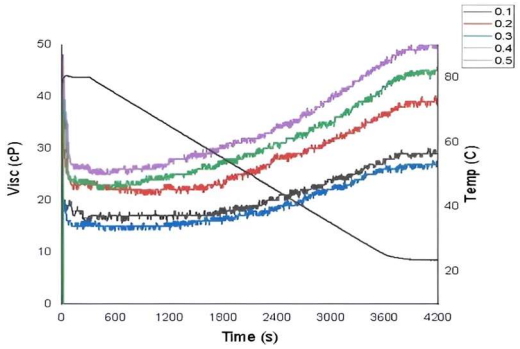

Fig.-2: RVA Profile of Carrageenan Extract at Various Concentrations of Cellulase with IPA Precipitation without the Addition of KCl

Several RVA patterns for carrageenan have been identified by Schierbaum.[28] These patterns allow differentiation between kappa, iota, and hybrid types of carrageenan, as demonstrated in Fig.-3. Analysis of the RVA pattern can help identify the type of carrageenan present in unknown samples. While iota carrageenan is detected by RVA analysis of carrageenan extracted without KCl addition (Fig.-2), the retrieved carrageenan is actually of the kappa type (Fig.-3). This finding is contrary to the assumption that the sample was extracted from *Eucheuma spinosum*, which is the source of iota carrageenan, as it is obtained from *Kappaphycus striatum*. Mendoza *et al*. reported that *Kappaphycus striatum* contains a significant amount of iota carrageenan, although it is dominated by kappa carrageenan, with iota carrageenan content ranging from 4-10% mol.[30] The addition of KCl during precipitation can increase the dominance of kappa carrageenan over iota carrageenan in carrageenan extracted from *Kappaphycus striatum*. However, further investigation is required to validate this hypothesis.

An intriguing finding emerged from the carrageenan extract profile that was precipitated using alcohol alone. As mentioned previously, the profiles of these carrageenan extracts did not exhibit an increase in viscosity. This behavior is likely due to the weak intermolecular bonds between carrageenan molecules. This differs from carrageenan which is supplemented with KCl, where K^+ ions promote strong bonding between carrageenan molecules, as depicted in Fig.-4, resulting in the formation of a more viscous solution upon dissolution.

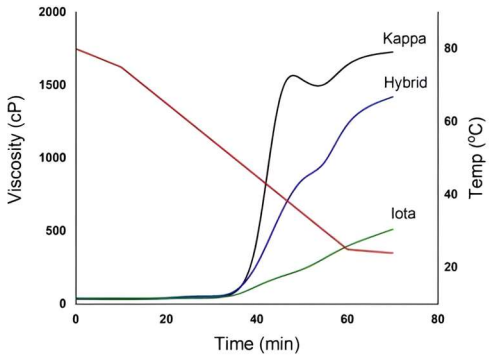

Fig.-3: RVA Profile for Several Types of Carrageenan [28]

Furthermore, the presence of K^+ ions can facilitate the conversion of sulfate groups to galactose groups, leading to an increase in viscosity. The precipitation process using IPA is intended to enhance the quality of carrageenan production, as the resulting carrageenan is expected to be purer. Thus, the use of salt (in this case KCl) significantly impacts the viscosity of enzymatically-extracted carrageenan extracts.

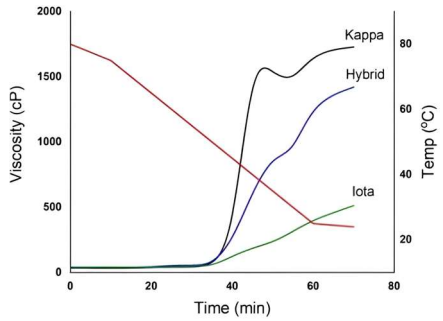

Fig.-4: Carrageenan Gelation Mechanism[2]

Gel Strength

The determination of carrageenan's characteristics is critical in assessing its uses, and gel strength is among the essential features used to achieve this end. The strength of carrageenan's gel is proportional to its sulfate content. A solution was made by dissolving 3 grams of the substance in 197 ml of distilled water and adding 0.6 grams of KCl to measure the sample's gel strength. After homogenizing the mixture, it was heated for 15 minutes at 80°C. The resulting samples were allowed to cool at 20°C for 16-17 hours, followed by an additional hour at room temperature before measuring the gel strength. The TA-XT Texture Analyzer tool was used to perform the measurements in Compression mode. The sample was placed at the center of the probe, which was a P/0.5R cylindrical probe equipped with a 5 kg load cell. There were three speeds used: 1.0 mm/s for the pre-test, 10 mm/s for the test, and 15 mm for the post-test. The probe was injected into the gel to a depth of 15 mm when a trigger force of 5 g was reached, and the maximum force measurement was noted as the sample's "Gel Strength."Table-7 presents the study's conclusions about the gel strength values.

Table-7: Carrageenan Gel Strength (gr/cm^2) by IPA Precipitation with or without KCl

Enzym(%)	IPA w/o KCl(%)	IPA w/ KCl(%)
0.1	413.69	319.15
0.2	674.85	365.92
0.3	205.93	235.15
0.4	633.57	285.96
0.5	500.13	160.99

The study's findings show that the carrageenan produced by enzyme treatment had gel strength values ranging from 160.99 to 674.85 gr/cm^2. These findings indicate that the viscosity value attained does meet the minimum quality standard of carrageenan set by the FAO for its application as a food ingredient, which is 500 gr/cm^2. Gel strength exhibited a tendency to decline with the addition of enzymes during the extraction process, which may be attributed to the residual enzymes interfering with and remaining in the carrageenan extract. The enzymes can cleave long chains into smaller fragments, resulting in carrageenans

with low gel strength.[14] Furthermore, impurities in the extracted carrageenan or other cellular impurities may cause the gel strength to be lower than that of carrageenan extracted using an alkaline solution. The strength of the carrageenan gel is further impacted by the extraction process's lack of an alkaline solution since the hydrocolloid produced using water has a larger sulfate content than that of the alkaline approach.[31] Based on the findings, the sample precipitated with IPA without the addition of KCl exhibits gel strength that adheres to the standards established by the World Health Organization (WHO). This is because, during the analysis of the gel strength using a Texture analyzer, KCl was added once again in line with the method devised by FMC Corp. Food Marine Colloids (1977), leading to excessive salt content in the gel. Lopez-Caballero found that this high salt content could cause a decline in gel firmness, which encompasses breaking strength and hardness.[32]

CONCLUSION

The precipitation process is a crucial step in obtaining high-quality carrageenan, often achieved through the addition of salts such as KCl, NaCl, or $CaCl_2$. Additionally, alcohols, including ethanol, methanol, and IPA, can also serve as recovery materials for carrageenan extracts. In this study, an interaction was observed between the enzyme and the precipitant material during the extraction process, with clear impacts on viscosity and gel strength. These results, however, contradict previous findings by Laksono *et al*. The impact of cellulase enzyme concentration on water, ash, and sulfate content analysis was found to be statistically insignificant. However, significant effects were observed on the results of RVA and gel strength analysis, particularly with the addition of KCl. The use of KCl as a precipitant was found to reduce gel strength values due to the softer and more easily crushed gel produced by this method. The difference in properties between carrageenan extracted using IPA alone and IPA with KCl can be attributed to the nature of the precipitation process. The addition of KCl to IPA can increase the solubility of carrageenan in the extract, potentially leading to higher yield and viscosity. However, the presence of Additionally, KCl may cause the carrageenan's ash level to rise, which could reduce the gel strength. However, because KCl is not present in carrageenan extracted with IPA alone, it may have a lower ash level and higher purity, which would improve the gel strength and decrease the viscosity, sulfate content, and water content. Further research is necessary to investigate the enzymatic extraction of carrageenan from seaweed samples collected from different regions since variations in seaweed content may lead to non-standard outcomes with cellulase. The current study focused on *Kappaphycus striatum* seaweed from the East Nusa Tenggara region, which resulted in a carrageenan extract with low sulfate content. This low sulfate content may be attributed to the seaweed's inherent low sulfate content, leading to lower sulfate levels in the extracted carrageenan. Thus, a comprehensive investigation of different seaweed varieties from diverse regions is essential to identify the factors contributing to differences in carrageenan extracts. Furthermore, additional research is needed to determine whether carrageenan extracted from *Kappaphycus striatum* using the precipitation process with IPA without KCl can yield carrageenan that is more dominant in iota carrageenan than kappa carrageenan.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID IDs, given below:

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