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Research Article

ISOLATION OF SULPHATE POLYGALACTAN FROM *Eucheuma alvarezii* Doty AND ITS ANTICOAGULANT ACTIVITY

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ABSTRACT

A sulphate polygalactan carrageenan was isolated from *Euchema alvarezii* Doty with yield 54.36%. The sulphate polygalactan was fractionated with KCl (aq) 2.5% result of a sulphate polygalactan of soluble fraction with the yield 60.34% and a sulphate polygalactanof insoluble fraction with the yield 34.53%. Based on the properties and supported by IR spectrum, the sulphate polygalactan of soluble fraction is κ-carrageenan, whilethe sulphate polygalactan of insoluble fraction is ι-carrageenan. Anticoagulant activitiy of the carrageenans were based on their prolongation effects on Activated Partial Thromboplastine Time (APTT) and Prothrombine Time (PT) of sodium citrate. On the assays, the carrageenans exhibited promising anticoagulant activities.

Key words: *Eucheumaalvarezii* Doty, κ-carrageenan, ι-carrageenan, anticoagulant activity, APTT and PT

INTRODUCTION

Many species of seaweed (marine micro algae) are used as food and they have also found use in traditional medicine because of their perceived health benefits.Seaweeds are rich sources of sulphate polysaccharides, including some that have become valuable additivesin the food industry because of their rheological properties as gelling and thickening agents. Sulphate polysaccaharides in seaweeds are agar, carrageenan, alginate, and laminaran. In addition, sulphate polysaccharides are recognized to possess a number of biological activities including anticoagulant, antiviral, and immuno-inflammatory activities that might find relevance in nutraceutical/functional food, cosmetic/cosmeceutical, and pharmaceutical applications (Jiao *et al*., 2011). Anticoagulant and anti-thrombotic activities are among the most widely studied properties of marine algae (Farias *et al*., 2000, Pereira *et al*., 2002, Mourao *et al*., 2004, Fonseca *et al*., 2008, and Rodrigues *et al*., 2011). Result of these researches as a rule, molecular size and sulphate content are among the most important prerequisites for these polymers to have anticoagulant activity. There are few reports of anticoagulant activity for sulphate polysaccharides isolated from green algae. Matsubara *et al*. (2000) isolated a highly sulphate galacto-arbinoglucan from the green algae *Codium pugniformis* with anticoagulant activity. A sulphate polygalactan with anti-coagulant activity was also extracted from *Codium cylindricum* (Matsubara *et al.,* 2001), *Caulerpa racemosa* with anticoagulant and antiviral (Ghos *et al.,* 2004), genus of *Monastroma* as anticoagulant (Mao *et al*., 2008 and Zhang *et al*., 2008). The sulphate polygalactan is a carrageenan. Based on these result, a carrageenan from green algae and marine algae has potential as anticoagulant activity. In this study, sulphate polygalactan (as a carrageenan) was isolated from the marine red algae *Eucheuma alvarezii* Doty, and some characteristics and potential anticoagulant activity of the sulphate polysaccharides were investigated. Common structure of carrageenan (*as a sulphate polygalactan*) is shown in Figure 1. Doty is alternative of anticoagulant resource that can be developed. Objective of this research is to isolate a sulphate polygalactan compound from *Euchema alvarezii* Doty employing APTT and PT assays.

METHODOLOGY

Materials

The marine red algae Doty was collected from Waworada Bay (once of Indonesian island) West Nusa Tenggara – Indonesia. The materials was cleaned from epiphytes, washed

with distilled water, dried in the sun light, and stored at cold-room until use. The specimen determineated by Laboratory of Herbarium Bogoriense–Bogor–Indonesia. The chemicals that used were sodium hydroxide, sodium chloride, barium chloride, potassium chloride, hydrochloric acid, ethanol, sulphuric acid, hydrogen peroxide, calcium chloride, phenol (it all from Merck), APTT and PT reagents, sodium citrate, blood as human plasma, heparin, and filter paper Whatman No.42.

Instrumentations

The instruments in this research were blender, centrifuge, rotary vaccum evaporator, glassware, magnetic stirrer, hotplate with stirrer, pH meter, furnace, water-bath, analytical balance, Spectronic-20, Shimadzu FT-IR Spectrophotometer type 1600, and Shimadzu Coagulometer Ca-50.

Procedure

Isolation of sulphate polygalactan

The dried powder of red algae sea weed *Eucheuma alvarezii* Doty (15g) was suspended in 1000mL of water and the pH was adjusted to 8.0 with aqueous solution of sodium hydroxide 2%. The mixtures were stirred with magnetic stirrer for 3h at 80°C, centrifuged at 3500rpm until 30min, in addition it were tested by methylene blue to show sulphate polygalactan, it was indicated by purple fiber. The centrate was added with aqueous solution of sodium chloride 10%, evaporated (with rotavapor) up to its volume to a third the initial volume. Concentrated centrate poured into ethanol 96% (1:2.5v/v) and obtained a precipitate. The precipitate as sulphate polygalactan (carra-genan), it was then filtered and dried to obtain crude sulphate polygalactan (crude carrageenan).

In additon, the sulphate polygalactan (or crude carrageenan) was dissolved in distilled water. The solution was added drop-wise of H_2O_2 30% while stirred until was resulted colourless solution. The solution was precipitatedby ethanol 96% and then filtered and dried by calcium chloride anhydrous. By the process given "*pure*" sulphate polygalactan (pure carrageenan).

Fractionation of sulphate polygalactan

Sulphate polygalactan was fractionated by Stancioffand Stanley method. Pure sulphate polygalactan (carrageenan) was dissolved in solution of potassium chloride 2.5% (1:100m/v), stirred for 1 hour, and allowed to stand at room temperature until 20h. The mixture was re-stirred (for 1 h) and then centrifuged (3500rpm, 30min), and then was decanted. Residue was added with 50mL of solution potassium chloride 2.5%, was stirred, and then centrifuged at 350 0rpm for 30min; it is resulted precipitate and centrate. The centrate was used to determine sulphate polygalactan of soluble fraction in KCl and the precipitate was used to determine insoluble fraction in KCl solution.

The centrate was evaporated up to its volume to a third of the initial volume. Concentrated centrate poured into ethanol 96% (1:2.5v/v) and obtained a precipitate. The precipitate was then filtered and dried to obtain sulphate polygalactan (carrageenan) of soluble fraction in KCl 2.5%. The part of sulphate polygalactan that not dissolved in KCl 2.5% was diluted in KCl 2.5%, centrifuged at 3500rpm for 30min, and decanted. The residue was diluted in 800mL water and heated at 85°C for 1 hour. The result was filtered to obtain residue and centrate. The centrate was added 20mL of NaCl 10%, stirred and concentrated by rotary vacuum evaporator. Concentrated centrate was precipitated with ethanol 96% (1:2.5v/v) and obtained a precipitate. The precipitate was dried and called as a sulphate polygalactan (carrageenan) of insoluble fraction in KCl 2.5% solution.

Characterization and identification of sulphatepolygalactan.

Total sulphate content

The total sulphate content was determined by gravimetric method. "*Pure*" sulphate polygalactan (1g) was refluxed in 50 mL HCl 0.2M for 1hour. Its mixture was reacted with 25mL of H_2O_2 10%, allowed for 5hours and obtained clear solution. The solution was boiled, and reacted with $BaCl_2$ (aq) 10% to obtain precipitate (as $BaSO_4$), and then it was separated and calculated to determine total sulphate content.

Content of total galactose

The total galactose content was determined by phenol-sulphuric acid analysis (Dubois method) using D-galactose as standard. Sulphate polygalactan (1mg) was dissolved in 10mL of water. One mL of this solution is taken, and added 1 drop of phenol 80% and 5mL of concentrated H_2SO_4. The solution was shaken in a water-bath at room temperature. In addition, made of the blank solution and standard solution, and then its absorbance were measured by Spectronic-20 at 485nm.

Solubility test

Sulphate polygalactan was dissolved in water at room temperature until a saturated solution is obtained. 10 mL of the solution was taken and added with ethanol 96%, precipitate was obtained and calculated. Solubility ($g.mL^{-1}$) in water of sulphate polygalactan is weight of precipitate (in gram) per volume of water (in mL).

Total ash content

Into crucible has been glowed was poured 0.2g of sulphate polygalactan and then it continued glowing to ash. The amount of ash that obtained as total ash content.

Content of insoluble ash in water

The ash was obtained as total ash content and water were boiled for 5mins and then filtered. The precipitate obtained was washed with hot water and glowed, then cooled, its result as insoluble ash in water.

Content of insoluble ash in acid

The ash was obtained as total ash content and 25mL hydrochloric acid 0.1M were boiled for 5 minutes and then filtered. The precipitate obtained was washed with hot water and glowed, then cooled, its result as insoluble ash in acid.

Identification of sulphate polygalactan

A "*pure*" sulphate polygalactanor carrageenan (*see above*) was identified its functional groups by infrared spectro-hotometric by KBr pellet.

Anticoagulant assays by APTT and PT

All clotting assay for study of anticoagulant activity were carried out using normal human plasma similarly with carried out by Clinical Pathology Laboratory Baptis Hospital Batu – East Java. The assay was carried out to evaluate the prolongation effect on Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT) of sodium citrat.

Assay for APTT and PT test carried-out by providing 11 test-tubes containing citrate 3.8%. In each test-tube was poured 200µL solution of sulphate polygalactan (total), sulphate polygalactan of soluble fraction, and sulphate polygalactan of unsoluble fraction with each concentration are 0.3, 0.15, and 0.05%. One of the 11 test-tubes was poured 200µL heparinsolution (5.000unit/mL), and one test-tubes is blank. On the 11 test-tubes have been contained the mixture was poured 1.8 mL normal human blood. In addition, test-tubes were carried-out of APTT test with procedure accordingly SOP (standard operating procedure) of Clinical Pathology Laboratory in Baptis Hospital Batu – East Java. Note: instrument that used for APTT and PTT testsis Coagulometer Ca-50. The record of cloting time was measured on second.

RESULTS AND DISCUSSION

Yield of sulphate polygalactan from seaweed

In the present study, yield of isolation sulphate polygalactan from *Eucheuma alvarezii* Doty was carried out by extraction is 58.90% as "crude sulphate polygalactan"and 54.36% as "pure sulphate polygalactan". The sulphate polygalactan was fractionated by solution of KCl (*aq*) 2.5%. The result of fractionation is 60.34% as sulphate polygalactan of soluble fraction and 34.53% as sulphate poly-galactan of in soluble fraction in solution KCl 2.5%.

The properties of sulphate polygalactan

The characteristic of many sulphate polygalactan (Table I) from red seaweed of *Eucheuma alvarezii* Doty is accordwith standard quality from USP XXI/NF XVI 1985.

Table I. Characteristic of sulphate polygalacatan from *Euchema alvarezii Doty*

No	Properties	Fraction of sulphate polygalactan in KCl (*aq*) 2.5%		
		Total	**Soluble**	**Insoluble**
1	Total sulphate (%)	17	1	Total sulphate (%)
2	Total galactose (%)	64.90	2	Total galactose (%)
3	Toal ash (%)	35	3	Toal ash (%)
4	Insoluble ash in acid (%)	17	4	Insoluble ash in acid (%)
5	Insoluble ash in water	1.5	5	Insoluble ash in water
7	Solubility in water (g/mL)	0.0025	7	Solubility in water (g/mL)

Table II. The prolongation effect of sulphate polygalactan on the clotting time (PTT and APTT) of sodium citrat (n=3) .

Sample	Concentration (%)	**PTT** (Second)		**APTT** (Second)	
		Mean	**SD**	**Mean**	**SD**
Blank*		11.3		36.5	
Control	0.00**	12.1	0.07	36.3	0.43
Heparin	5000***	****	-	****	-
Sulphate	0.30	19.7	0.14	****	-
polygalactan (total)	0.10	13.7	0.23	152.9	0.78
	0.05	12.6	0.19	99.1	0.75
Sulphate	0.30	13.2	0.29	96.1	0.49
polygalactan	0.10	13.5	0.25	61.5	1.68
soluble fraction in KCl(*aq*) 2.5%	0.05	12.6	0.27	49.4	0.60
Sulphate	0.30	14.5	0.27	****	-
polygalactan	0.10	14.5	0.27	****	-
insoluble fraction in KCl(*aq*) 2.5%	0.05	14.2	0.27	****	-

*Note: * = without sample, ** = sodium citrat only, *** = unit/mL, **** = undetected*
Test-tubes number 2-12 were added sodium citrate 3.8%

According to the standard quality, current research goals are focused on identifying new properties from "old" compounds from low-cost natural sources for different industrial applications. Based on the characteristic, standard quality USP XXI/NF XVI 1985, and infra-red spectrum of these compounds, sulphate ploygalactan shown a carrageenan, and it was sugested that the polygalactan sulphate of soluble fraction is ι-carragenan, while the sulphate polygalactan of insoluble fraction is κ-carrageenan. Structure of the carrageenan (κ- and ι-carrageenan) is shown in figure 1 and IR spectra is shown in figure 2a and 2b.

Anticoagulant activity of sulphate polygalactan

The research is need to discover the new anticoagulant compounds polygalactan sulphated. Sulphate polygalactan has been obtained last year ago. However, sulphate polygalactan area potentially attractive sourcesof macromolecules that investigated as anticoagulant because its structure was similar with heparin. Anticoagulant activity of the sulphate polygalactan (a carrageenan) were assessed using human plasma from healthy donors based on APTT and PT test. Assays of the samples using different concentration, i.e 0.30, 0.10, and 0.05%. Result of APTT and PT assays shown in table II.

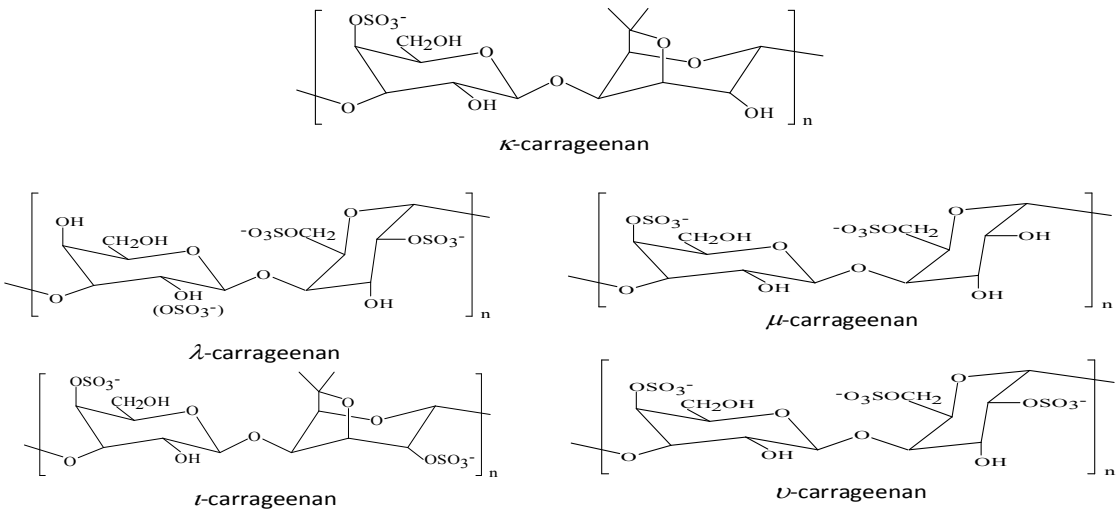

Figure 1 Structure of carrageenans

Based on this fact, we determined the anticoagulant activity of these sulphated polysaccharides. The anticoagulant activity mechanism of carrageenan can be shown via thrombin inhibition. The anticoagulant properties of the carrageenan were compared with citrate solution. The results obtained in an APTT assay showed that carrageenan had very high anticoagulant activity when compared to citrate (Table II) on concentration test. However, anticoagulant activity of this compound is lower than heparin (Matsubara *et al*., 2001). According to Boisson-Vidal *et al.* (200), polysaccharides in which the native sulphated pattern was intact that were more potentially than polymers of equivalent molecular weight and more potentially than the overall degree of sulphatation in which this pattern had been disrupted by partial desulphatation. The main basis of the anticoagulant activity of carrageenan appears to be an anti thrombic properties (Shanmugam & Mody, 2000). APTT is a non-specific test of the intrinsic system. Taken in conjunction with a normal prothrombin time, it is the most useful screening test for detecting deficiencies of factors VIII, IX, XI, and XII. The APTT will also be prolonged in any deficiency involving the common pathways (deficiencies of factors V, X, II and, to a lesser extent, fibrinogen)and in the presence of inhibitors. The presence of some therapeutic inhibitor coagulation such as heparin will also prolong APTT. It is important to exclude the possibility that such treatments have been employed in the initial investigation of prolonged APTTs. PT test reflects the overall efficiency of the extrinsic system. It is sensitive to change in factors V, VII, and X, and less so to factor II (prothrombin). It is also unsuitable for detecting minor changes in fibrinogen level, but may be abnormal if the fibrinogen level is very low or if an inhibitor is present. The sensitivity of the test is influenced by the reagent and technique used, and it is important to establish a reference range locally. The pathway measured by the prothrombin time. The PT reagent, often termed thromboplastin, contains tissue factor and phospholipids. Thus, the APTT test sugested that anticoagulant sulphate polygalactan isolated from *E. spinosum* acted on intrinsic and/or common pathways (Sutrisno *et al*., 2010). Whereas the PT test indicated that its compound did not act the extrinsic pathways on the coagulation sascade (Rodrigues *et al*., 2011).

CONCLUSION

The sulphate polygalactan isolated from *Euchema alvarezii* Dotyis a carrageenan with yield 54.36%. The sulphate polygalactan as a carrageenan. Fractionation of total sulphate

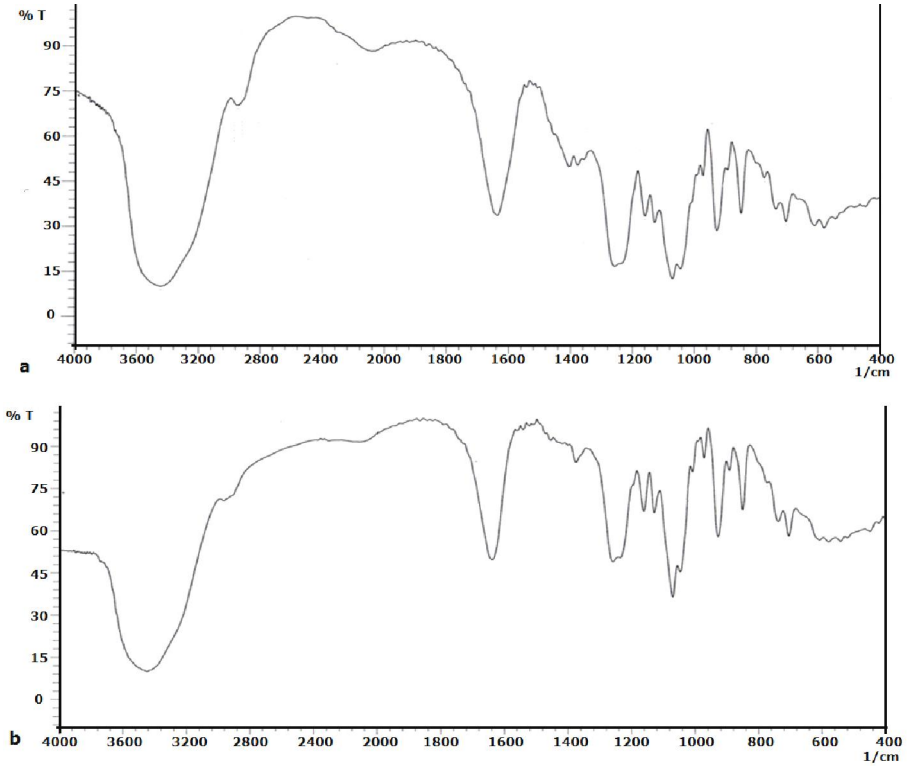

Figure 2. IR spectrum of carrageenan of soluble fraction (a) and insoluble fraction (b) in KCl (aq) 2.5%

polygalactan by KCl (*aq*) 2.5% were resulted a sulphate polygalactan of soluble fraction (yield 60.34%) and a sulphate polygalactan of insoluble fraction in KCl (*aq*) 2.5% (yield 34.53%). Based onthe properties and supported by IR spectra, the sulphate polygalactan of soluble KCl solution is ι-carragenanand the sulphate polygalactan of insoluble fraction is κ-carrageenan. Anticoagulant activitiy of the carrageenan was based on their prolongation effects on Activated Partial Thromboplastine Time (APTT) and Prothrombine Time (PT) of sodium citrate. On the test concentration, both of carrageenans (ι-carragenan and κ-carrageenan) exhibited anticoagulant activities by prolonging the APTT and PT of sodium citrate.

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