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**Research Article**

# PURIFICATION AND CHARACTERIZATION OF POLYSACCHARIDE FROM MICROALGAE BTM 11 AS INHIBITOR OF HEPATITIS C VIRUS RNA HELICASE

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## ABSTRACT

Hepatitis C virus is one of the causative agents for HCV-related liver disease development with high virulence. Antiviral drugs can be discovered through molecular target-based therapy by finding the inhibitors for RNA helicase that play crucial role in viral replication. An inhibitor can be derived from polysaccharides produced by microalgae. In this study, polysaccharide from microalgae BTM11 which had inhibitory activity against RNA helicase have been purified and characterized.On the other hand, the RNA helicase was produced by *E. coli* BL21(DE3)pLyss harboring NS3 RNA helicase HCV gene in pET-21b plasmid. This enzyme then was purified by affinity chromatography and this purified enzyme was used for HCV RNA helicase inhibitory assay. Polysaccharide fractions were separated from the extract of BTM 11 using Sepharose 4B column chromatography. Inhibitor activity was measured using colorimetry ATPase assay based on releasing of phosphate inorganic. The results of SDS-PAGE and Western blot showed that the purified RNA helicase had a molecular weight of 54kDa. The highest inhibition activity of HCV RNA helicase (88 ± 2,4726%) was achieved at fraction 10 of purified polysaccharide. The HPLC result showed that compounds of polysaccharide active fraction were maltopentose ($R_t$ 4.183) and glucose ($R_t$ 5.673). Both of $^1$H-NMR and IR spectra showed hydroxyl and carbonyl groups that present in the polysaccharide structure.

**Key words**: Hepatitis C Virus, RNA Helicase, Microalgae BTM11, chromatography, polysaccharide

## INTRODUCTION

Hepatitis C virus (HCV) is a positive-strand RNA virus of the *Flaviviridae* family. HCV infection, affecting 3% of the world population, leads to chronic hepatitis in up to 85% of the cases, in 10 to 20% of the cases it develops into cirrhosis that requires constant treatment and provokes permanent infirmity, while 1 to 5% of chronically infected patients are diagnosed with hepatocellular carcinoma (Brass *et al.*, 2006). Current treatment consists of a combination of subcutaneously administrated pegylated-interferon-R with the orally dosed nucleoside analogue ribavirin. This combination is generally poorly tolerated, is contraindicated for many patients, and is only effective in controlling the disease in a fraction of the individuals who are eligible for therapy (Pawlotsky, 2006).

Several drugs that target various stages of the replication cycle of HCV are currently in preclinical or clinical development; these include NS3 protease inhibitors, nucleoside and non-nucleoside polymerase inhibitors and cyclophylin binding compounds. So far the NS3 helicase has not been extensively explored as a target for inhibition of HCV replication.

We devised a rational approach for the design of selective inhibitors of the HCV NS3 helicase. It was decided to use this structure as the starting point for the de novo design of novel potential inhibitors. The potential contribution of marine organisms to the discovery of new bioactive molecules is increasingly challenging. Natural products that have been isolated from microalgae have received much attention as a potential source of pharmacologically active agents. Algae include a wide variety of plants that range from

diatoms, which are microscopic, unicellular organisms, to seaweeds extending over 30 m. Unlike land plants, algae do not have true roots, stem, and leaves. They can be cultured in the laboratory with relative ease to provide a consistent source of biologically active secondary metabolites, in comparison with terrestrial plants.

The ability of sulfated polysaccharides from seaweeds to inhibit the replication of enveloped viruses including herpes simplex virus (HSV), human immunodeficiency virus (HIV), human cytomegalovirus, dengue virus and respiratory syncytial virus is well established (Witvrouw and De Clercq, 1997; Schaeffer and Krylov, 2000; Damonte *et al* 2004; Luescher-Mattli, 2003). However, the use of polysaccharides is restricted for various reasons, including a lack of simple methods for isolating them from extracts. In this study, a polysaccharides from microalgae BTM11 have been successfully purified and characterized. We also report that the purified polysaccharides acts as HCV inhibitor through ATPase activity of RNA helicase.

## MATERIALS AND METHODS

### Algae strains and growth conditions

Green microalgae BTM 11 was grown in modified bristol medium sea water (MBM SW). Inoculate (0.2g fresh weight) placed into the medium, grown axenically at 25°C in 20L container containing 15L medium for 50 day, and then harvested at late-log phase.

### Expression and purification of HCV RNA helicase

The expression and purification of HCV helicase was performed as previously described by Utama *et al,* 2000. *E. coli* BL21 (DE3)pLyss that carrying HCV NS3 helicase gene in plasmid was grown in Luria-Bertani (LB) medium supplemented with ampicillin. After the isopropyl-beta-D-thiogalactopyranoside (IPTG) induction at 37°C for 3h, the cells were collected by centrifugation. The cells were suspended in buffer B (10Mm Tris-HCl buffer (pH8.5) 100Mm NaCl, 0,25% tween 20) and disrupted by sonication for 5min on ice. The soluble fraction of the clarified cell lysate was mixed with TALON metal affinity resin (Clontech, Palo Alto, CA, USA). After gentle mixing for 1h at 4°C and wash with buffer B. resin-bound protein was eluted with 2 volumes of buffer B containing 400mM imidazole. Purified HCV NS3 helicase protein was designated NS3HCV wild. The purified enzyme used for screening inhibitor RNA helicase HCV. Protein be analyzed by SDS–PAGE, and visualized by Coomassie blue staining.

### Preparation of crude polysaccharides

Five grams of microalgae BTM 11 powder were mixed with 100mL of distilled water. The cells were disrupted by sonicator. The resulting sample was incubated in a water bath at 100°C and then centrifuged at 4500rpm for 10min. Trichloroacetic acid (TCA) was added to the samples, which were centrifuged at 7000rpm for 15min and the pellet was removed. The supernatant was dialyzed against de-ionized water for 24h. The crude polysaccharides were precipitated with absolute ethanol and then lyophilized for 48h (Shi *et al*., 2007).

### Purification of polysaccharide

The purification of polysaccharide inhibitor as described Shi *et al*., 2007. Column chromatography was performed with Sepharose 4B. One milliliter of the extracts was subjected to chromatography. The column was eluted with de-ionized water at the flow rate of 1mL/min. The isolated fractions were measured by the phenol–sulphuric acid method.

### Colorimetric ATPase assay

Screening of RNA helicase inhibitor has performed through screening of ATPase inhibitor due to RNA helicase possesses ATPase activity. Extract of microalgae (5µL) was added to reaction mixture (50µL) containing 10mM MOPS buffer (pH 6.5), 2mM ATP, 1mM MgCl2, appropriate RNA helicase, and incubated in a 96-well plate at room temperature for 40min. The reaction was stopped by adding 100 µl of dye solution (water : 0.081% malachite green : 5.7% ammonium molybdate in 6N HCl : 2.3% polyvinyl alcohol = 2 : 2 : 1 : 1). After the addition of 25µL of 30% sodium citrate, $OD_{620/405}$ measured. Mixture reaction without supernatant was used as control. Percentage of

inhibitor calculated as ($OD_{620/405}$ sample with supernatant / $OD_{620/405}$ sample without supernatant) x 100%. The data were presented as means ± standard deviations of three determinations.

**Analysis of polysaccharide**

The polysaccharide was determined by the phenol–sulfuric acid reaction, using glucose as standard (Cuesta *et al*., 2003).

**HPLC analysis**

Samples were determined by HPLC on Aminex® HPX-87H 300 x 7.8mm columns and eluted with 0.008 N of sulfate acid solution at a flow rate of 1mL $min^{-1}$ at 35°C. Elution was monitored by a refractive index detector (Zhang *et al.,* 2004)

**FTIR analysis**

Infrared spectra of the purified polysaccharide was recorded with a Nicolet 5DXB FT-IR spectrometer in the range 4000-600$cm^{-1}$, using the KBr disk method (Wang *et al.,* 2004).

**NMR analysis**

$^{1}H$ NMR spectra of the purified polysaccharide in $D_2O$ was recorded at 22°C on a Delta NMR spectrometer (JEOL, USA). Chemical shifts were measured in part per million (ppm) with signal of $D_2O$ (4.672ppm) as reference.

## RESULTS AND DISCUSSION

**Expression and purification of HCV RNA helicase**

The HCV NS3 helicase gene was expressed in *E. coli* BL21 (DE3) pLysS for the helicase production and ATPase activity assay. Large-scale cultures were raised under IPTG induction, and the cells were processed to get the soluble. The soluble fractions in each case was subjected to $Co^{2+}$-chelating affinity chromatography. The bound proteins were eluted in 400mM of imidazole, and the SDS-PAGE and Western blot analysis indicated that the purified HCV NS3 helicase proteins were 54 kDa (Figure 1). These results are in accordance with those of Utama *et al.* (2000) that HCV NS3 helicase protein had 54 kDa of molecular weight. The NS3 helicase affects two steps in the life cycle of HCV. First, RNA replication step of virion in which this protein is required to unwind the double-stranded RNA intermediate during RNA-dependent replication. Second, NS3 helicase assists in virus assembly and can also act as scaffold for interaction with viral cofactors (Fatima *et al.,* 2014). In addition, this protein binds to DNA and RNA with an equilibrium dissociation constant in the low nM range, a binding site size of 7-8 nucleotides, and little or no reported cooperativity (Raney *et al*., 2010).

**Culture of microalgae BTM 11**

Microalgae BTM 11 was isolated from Batam Sea, Indonesia. Growth of microalgae BTM 11 was identified with the appearance of cell-formed filamen until green color reflected. Microalgae BTM 11 was grown in MBM SW medium for various time of incubation (Figure 2). Along cultivation periods (1 to 50 days), the cells-free extract from sample culture was analyzed for inhibitory activity against ATPase to find out the optimum harvest time. As the result, microalgae BTM 11 started to produce the inhibitor chemical compound of the ATPase activity of HCV RNA helicase after 7 days of incubation, and remained constant until 50 days of incubation (data not shown). Cells were harvested in late-log phase to prevent secretion of polysaccharides into medium. According to de Jesus Raposo *et al.* (2013), several marine microalgae able to release polysaccharides into the surrounding medium during stationary phase.

**Anti-HCV activity of purified poly-saccharide**

The crude polysaccharides of microalgae BTM11 was purified by gel chromatography on Sepharose 4B, showing one active fraction (fraction 10) which inhibit about 87.7±2.47% activity of HCV RNA helicase (Figure 3). Purification quality of polysaccharide will be evaluated by HPLC analysis. This result reveal that HCV helicase inhibition can be directly observed from ATPase activity. Hidrolysis of ATP is proposed to release closed conformation. The amount of phosphate (Pi) release in ATP hidrolysis is related to unwinding rate of RNA duplex (Raney *et al*., 2010).

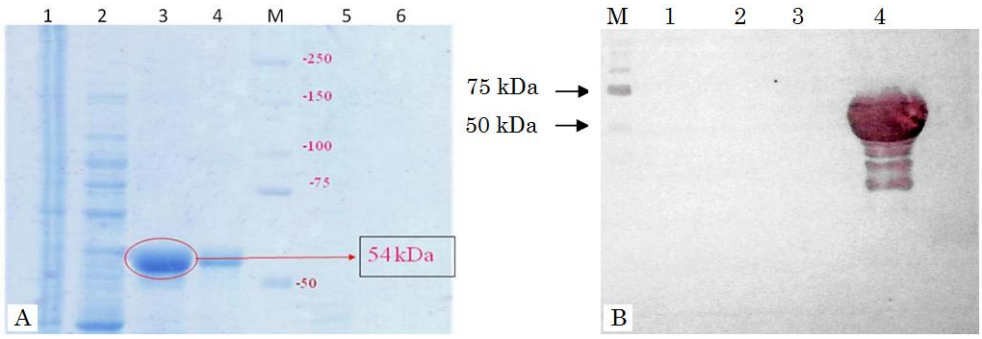


Figure 1.(A). SDS-PAGE analysis of purified HCV NS3 proteins. The gel was stained with Coomassie blue. (1) extract-free cell; (2) inner volume; (3) and (4) elution; (M) protein marker. (B) Western blot analysis of puried HCV NS3 proteins. (1) Inner volume; (2): washing 1; (3) washing 2; and (4) elusion; M: protein marker)

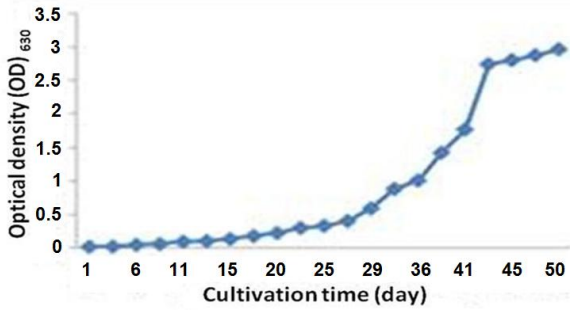


Figure 2. Growth curve of microalgae BTM 11 corresponds to OD and incubation time.

Drug targeting the unwinding activity could act via one or more of the following mechanism: (a) inhibiting ATPase activity by interfering with ATP binding and therefore limiting the energy available for the unwinding, (b) inhibiting ATP hydrolisis or release of ADP by blocking opening or closing of domains, (c) inhibiting RNA substrate binding, (d) inhibiting unwinding by sterically blocking helicase translocation, or (e) inhibiting coupling of ATPase and RNA binding capability of NS3 (Borowski *et al.* 2002, Salam *et al.* 2012). As a macromolecule, this study suggests that polysaccharide may mask one or both of RNA and ATP binding domain. This will impact on reduction of viral replication due to lack of energy from ATP hydrolyzed.

**Characterization of the purified polysaccharide**

In order to confirm the purity of polysaccharide, HPLC was shown that fraction 10 have two peaks with retention time (Rt) 4.183 and 5.673, respectively (Figure 4). This result also compared with saccharides standard (maltopentose and glucose) showed that the monosaccharides are components of the polysaccharide reside in the sample. Furthermore, the results also indicated that maltopentose ($R_t$ 4.183) was the predominant monosaccharides of fraction 10 with low levels of glucose ($R_t$ 5.673). Fraction 10 contains total sugar of 3.066mg/mL that indicate the proportion of polysaccharide in the sample.

The IR spectra of the fraction 10 are shown in figure 5. Strong-broad peak at 3340-3439cm$^{-1}$ range are assigned to the hydroxyl group vibration, while the rest of peak at 1635 and 1029cm$^{-1}$ are assigned to the carbonyl group. These functional groups are compound of primary structure of polysaccharide. The $^{1}$H NMR spectra of the fraction 10 are shown in figure 6. Majority proton signals at 1.41, 1.55, 2.66 and 3.26ppm are typical of polysaccharide due to presence of sugar residues. However, one strong signal at 3.71ppm has been assigned to halogen bond (R-X) which is polysaccharide may associate with this atom by covalent or non-covalent bonds.

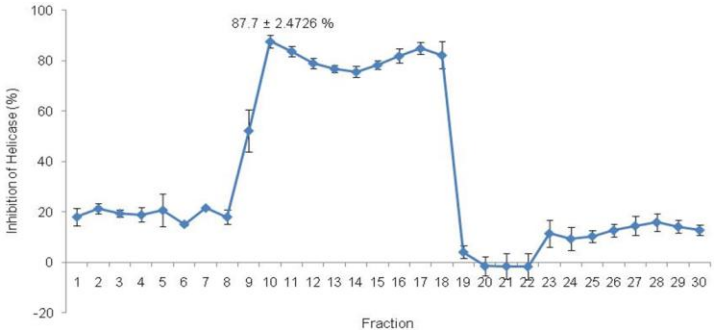


Figure 3. ATPase inhibition activity of polysaccharide fractions against RNA helicase

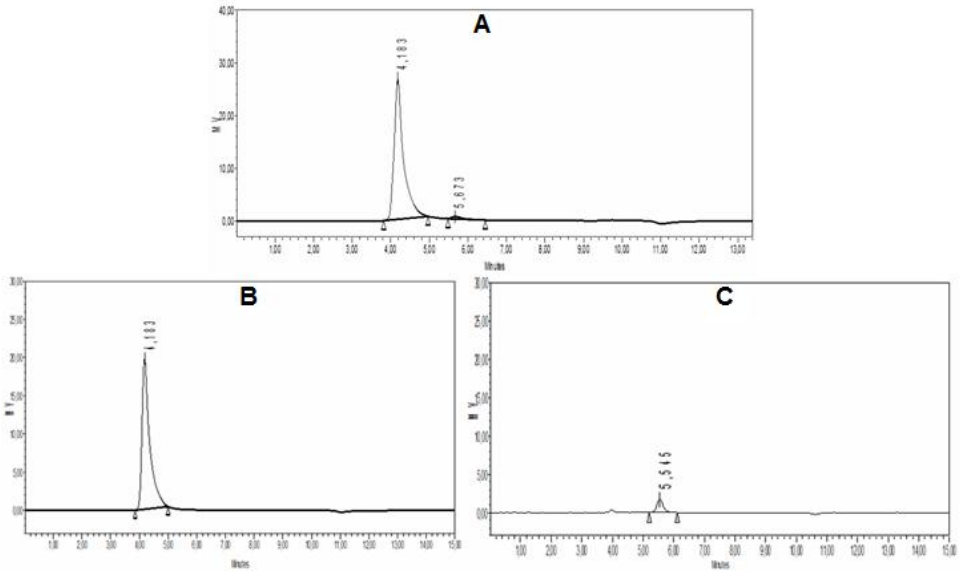


Figure 4. Chromatogram of fraction 10 using HPLC. (A). polysaccharide microalgae BTM 11. (B) Standard of maltopentose. (C) Standard of glucose

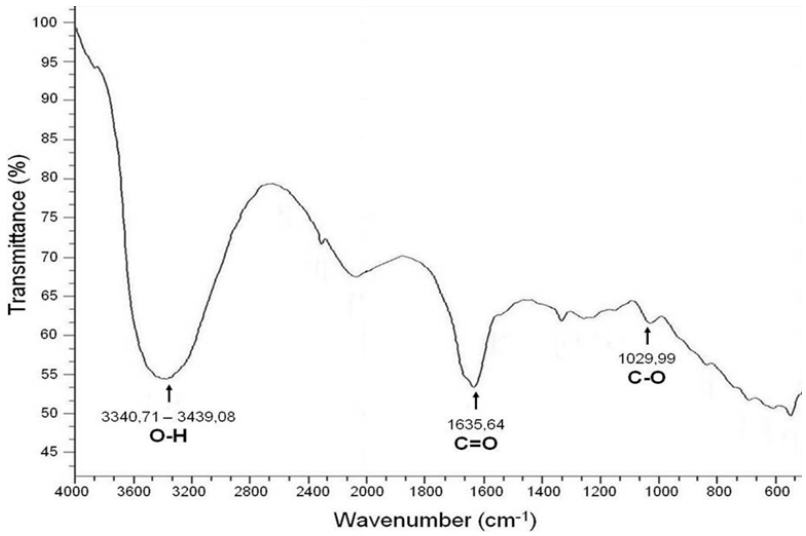


Figure 5. FT-IR spectra of the polysaccharide from fraction 10

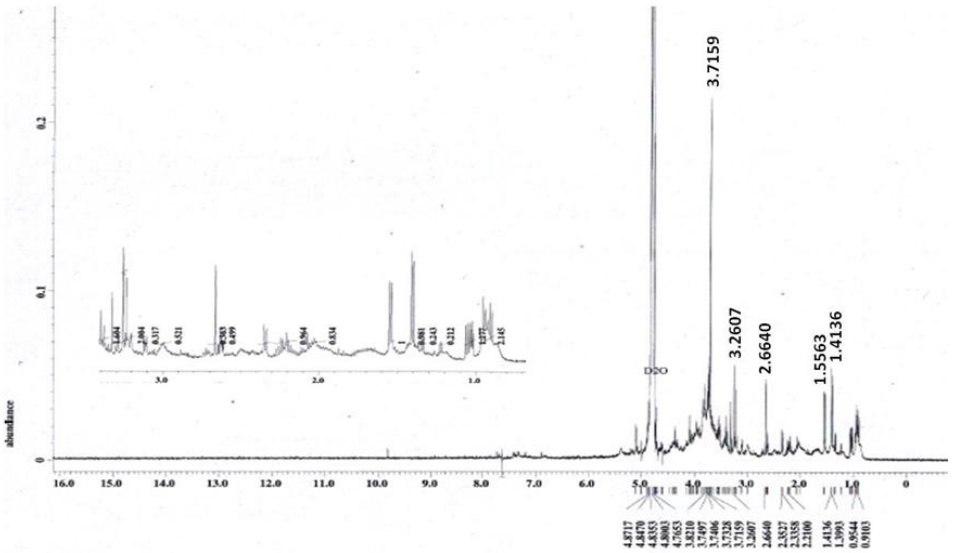


Figure 6. $^{1}$H NMR spectra of the polysaccharide from fraction 10

## CONCLUSION

We have succesfully obtained purified polysaccharide from microalgae BTM 11 that shown inhibition of HCV through ATPase activity of helicase protein. Furthermore, this study can be used as a tool to develop anti-HCV agents based on tropical marine bioresources.

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