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The Cytotoxicity of Agaro-Oligosaccharides and Neoagaro-Oligosaccharides on Macrophage Cells

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Abstract: Agarose is a polysaccharide from red algae. Enzymatic hydrolysis of agarose can produce Agaro-Oligosaccharidess (AOS) and Neoagaro-Oligosaccharidess (NAOS). Different times of enzymatic hydrolysis can produce different types of AOS and NAOS based on their degree of polymerization (DPs). This study aims to examine the cytotoxicity of AOS and NAOS with different hydrolysis times on the macrophage cell line RAW 264.7. The parameters used were the percentage of cell viability and IC50 value. The cytotoxicity test using MTT assay and One-way ANOVA were used as statistical tests. The results showed that AOS-0 at a concentration below 125 µg/mL was not toxic and showed moderate toxicity up to a high concentration of 1000 µg/mL, while AOS hydrolyzed for 24 h (AOS-24) was not toxic to RAW 264.7 cells at all concentrations tested. The different results were shown in all NAOS samples, which were highly toxic to RAW 264.7 cells in the 125 to 1000 µg/mL, indicating that it was concentration dependent. The results showed that different hydrolysis times caused differences in the structure of AOS and NAOS compounds and influenced the toxicity level. Research development for further studies on antioxidants and anti-inflammatory needs more attention to the sample type and hydrolysis time.

Keywords: AOS; NAOS; Enzymatic hydrolysis; Cytotoxicity

INTRODUCTION

Agarose is a polysaccharide that can be obtained from red algae (Rhodophyta). Red algae have differences in the composition of cell wall polysaccharides and intercellular matrix when compared to other plants.[1] Polysaccharides contained in red algae are polymers that can exhibit different properties depending on their monomer composition. It makes the use of polysaccharides sourced from red algae applicable in various industries such as food, pharmaceutical, and biotechnology.[2] Agarose is commonly used for gel electrophoresis, but in recent decades, it has been used by researchers as a material for making biocompatible and biodegradable products that are non-toxic.[3]

Agarose can be hydrolyzed enzymatically or chemically to produce agaro-oligosaccharides (AOS) and neoagaro-oligosaccharides (NAOS). Chemical hydrolysis can be done using acidic solutions or Hydrogen Peroxide (H_2O_2). The advantage of chemical hydrolysis is that it can produce high concentrations of monomers. The disadvantages of chemical hydrolysis are that the oligosaccharides produced are not specific and produce residues in the form of 5-hydroxy-methyl-furfural (HMF), which is toxic.[4,5] Enzymatic hydrolysis is carried out using agarase enzymes. There are 2 types of agarase enzymes there are α-agarase and β-agarase. The α-agarase enzyme will form AOS with 3,6 anhydro-L Galactose (AHG) at the reducing end, while

the endo β-agarase enzyme will produce NAOS with β-D-galactose at the reducing end. Hydrolysis using enzymes can produce specific oligosaccharides and does not produce toxic residues. The disadvantages of enzymatic hydrolysis are the relatively long reaction time and the enzyme easily loses its activity. In addition, enzymes are also relatively expensive.[4]

The stages of agarose hydrolysis can be divided into two stages liquefaction (using chemical compounds or enzymes) and saccharification (using certain enzymes). The liquefaction process hydrolyzes agarose to produce oligomers. Furthermore, the saccharification process converts agarose oligomers into monomers through enzymatic reactions. The use of enzyme types or chemical compounds can affect the structure of oligomers and the formation of monomers. The use of endo-type β-agarase enzyme will hydrolyze agarose into NAOS by breaking the β-1,4 bond. Next, NAOS will be hydrolyzed into neoagarobiose using exo-type β-agarase. L-AHG and D-Gal monomers are then formed by breaking the α-1,3 bond using neoagarobiose hydrolase.[6]

Different hydrolysis times can produce different types of AOS and NAOS based on their degree of polymerization (DPs). With the longer hydrolysis time, the AOS and NAOS formed will have a simpler degree of polymerization. Research on the potential of AOS and NAOS with various DPs has been widely reported, especially in the food, cosmetic, and pharmaceutical industries, such as antioxidants, antidiabetics, and anti-inflammatories, and they also play a role in skin whitening.[7–9] Cytotoxicity test is an initial parameter that is often carried out to analyze the safety of a compound or natural product that has potential as a drug and is used to analyze the effect of the compound on cell viability.[10,11]

There are many methods to measure cell viability. One commonly used method is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. MTT assay has even become the "gold standard" for measuring cell viability and cell proliferation. MTT assay is a toxicity test method based on the metabolic activity of living cells. The tetrazolium salt contained in the MTT reagent will react with dehydrogenase and reducing agents in active mitochondria in living cells and form formazan crystals. The intensity of formazan crystals formed can be measured using a microplate reader with a wavelength of about 570 nm. The more formazan crystals formed, the more cells are still alive.[12,13] This study aims to analyze the toxicity level of enzymatically hydrolyzed AOS and NAOS with different hydrolysis times against macrophage cells RAW 264.7.

MATERIAL AND METHOD

Agaro-oligosaccharides (AOS) and Neoagaro-Oligosaccharides (NAOS) preparation

Agarose (Promega) was enzymatically hydrolyzed using agarase and glucosidase enzymess. AOS was obtained from prehydrolysis agarose than enzymaticrolysis with β-glucosidase, while NAOS was obtained from hydrolysis agarose using β-agarase. 500 µL of 4 mg/mL agarose was reacted with 50 µL of 1M Tris-HCl (pH 8), 5 µL of 1M $MgCl_2$-$6H_2O$ and 50 µL of agarase enzyme, and diluted with H_2O until the total reaction volume became 1 mL. The enzymatic reaction was carried out at 55°C with different reaction times for AOS (0 and 24 hours) and NAOS (3, 6, 12, and 24 hours). The reaction was stopped by vortexing and spindown, and then the enzyme reaction was stopped by heating at 100°C for 3 minutes.

Cell Culture

RAW 264.7 cells were cultured in a 75 cm^2 flask using DMEM medium with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. Confluent RAW 264.7 cells were then plated onto 96 well plates (50,000 cells/well) for 24 hours. After 24 hours, cells were induced with AOS and NAOS at various concentrations (1000; 500; 250; 125; 62.5; 31.25; and 15.625 µg/mL) for 24 hours.

Cytotoxicity Test

The cytotoxicity test was performed using the MTT method. RAW 264.7 cells that have been confluent in 96 well plates and induced by AOS or NAOS for 24 hours were washed with PBS and given 0.5 mg/mL MTT reagent as much as 100 µL to each well, then incubated for 4 hours. After 4 hours, 100 µL of stop reagent was added to each well and incubated overnight in a dark room. Absorbance was measured using a microplate reader with a wavelength of 595 nm.[14]

Data Analysis

Cytotoxicity test data in the form of absorbance values were analyzed using Ms. Excell to calculate the percentage of cell viability and obtained a linear regression equation to determine the IC_{50} value. One

Way ANOVA statistical analysis was performed using IBM SPSS Statistics version 23 to determine the significance of among the research groups ($p<0.05$). All data from at least three independent experiments with duplicates of each point.

Ethical Consideration

This research does not require ethical approval because it does not use human or animal subjects.

RESULT

Based on Figure 1, the viability of RAW 264.7 cells is still above 50% after the administration of AOS-0 and AOS-24 up to a concentration of 500 µg/mL. These results indicate that both AOS-0 and AOS-24 are not toxic to RAW 264.7 cells at these concentrations. AOS-0 at concentrations of 62.5 and 125 µg/mL can increase the proliferation of RAW 264.7 cells, indicated by an increase in the viability of RAW 264.7 cells after being given AOS-0 at these concentrations, then back down after giving AOS-0 concentrations of 250, 500, and 1000 µg/mL. RAW 264.7 cells experienced a decrease in viability to 64.15% after being given AOS-0 at the highest concentration (1000 µg/mL), while RAW 264.7 cell viability was still around 80% after being given AOS-24 at a concentration of 1000 µg/mL. It shows that AOS-0 is moderately toxic to RAW 264.7 cells at a concentration of 1000 µg/mL, while AOS-24 is still non-toxic at a high concentration (1000 µg/mL). The treatment of AOS-0 and AOS-24 significantly affected the viability of RAW 264.7 cells ($p<0.05$).

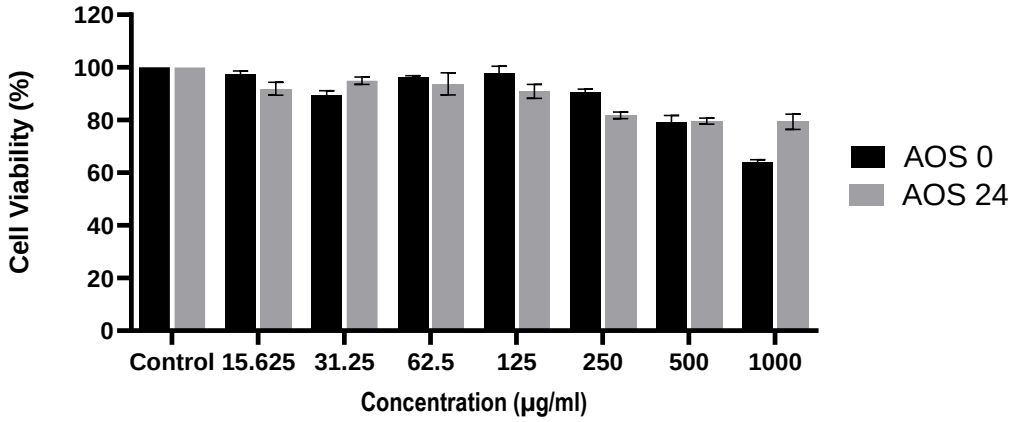

Figure 1. Percentage of RAW 264.7 cell viability with AOS-0 and AOS-24 treatment ($p<0.05$)

The results of NAOS cytotoxicity at different enzymatic hydrolysis times are shown in Figure 2. Sample NAOS 3 showed a hydrolysis time of 3 hours, NAOS 6 showed a hydrolysis time of 6 hours, NAOS 12 showed a hydrolysis time of 12 hours, and NAOS 24 showed a hydrolysis time of 24 hours. The viability of RAW 264.7 cells with NAOS 3, 6, 12, and 24 treatments all showed percentages above 50% at the lowest concentration of 15.625 µg/ml. NAOS 3, 6, 12, and 24 treatments at a concentration of 62.5 µg/ml reduced RAW 264.7 cell viability by more than 50% when compared to the control. The cell viability values were 34.75±2.85, 41.16±5.46, and 40.61±2.63%, respectively. All samples were highly toxic to RAW 264.7 cells in the treatment of NAOS concentrations of 125 to 1000 µg/ml. NAOS treatment of NAOS 3, 6, 12, and 24 had a significant effect on RAW 264.7 cell viability ($p<0.05$).

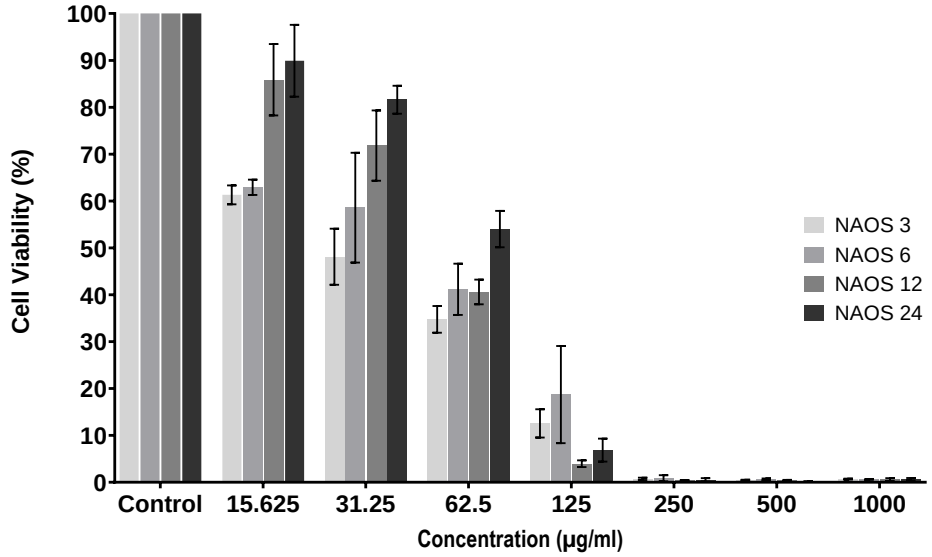

Figure 2. Percentage of RAW 264.7 cell viability with NAOS (3, 6, 12, and 24 hours) treatment ($p<0.05$)

In addition to the viability percentage, the toxicity level can be classified based on the IC50 value. The IC_{50} values of each AOS and NAOS with different hydrolysis times can be seen in Table 1.

Table 1. IC_{50} value of AOS dan NAOS with different hydrolysis times in RAW 264.7 cell

Compound	Hydrolysis time (Hours)	IC_{50} (µg/ml)
Agaro-Oligosaccharides (AOS)	0	1431.45
	24	1084151
Neoagaro-Oligosaccharides (NAOS)	3	22.73
	6	32.56
	12	49.59
	24	64.28

DISCUSSION

This study used Agaro-Olligosaccharidess (AOS) and Neoagaro-Olligosaccharidess (NAOS) samples, which are the result of enzymatic hydrolysis of agarose using β-glucosidase and β-agarase enzymes respectively, with different hydrolysis times. Different hydrolysis times can produce different types of AOS and NAOS based on the Degree of Polymerization (DPs). AOS and NAOS formed from longer hydrolysis time will produce compounds that have various types of polymers with various DPs.[8,14] Previous research mentioned that agarose hydrolysis for 2 hours can produce NAOS with relatively high DPs such as Neoagarododecaose (DP 12), Neoagarodecaose (DP 10), Neoagarooctaose (DP 8), and Neoagarohexaose (DP 6) while Neoagarotetraose (DP 4), and Neoagarobiose (DP 2) were only detected after 24 hours of hydrolysis. Another research conducted by Qu et al. mentioned that agarose hydrolysis with β-agarase enzyme for 100 minutes produced NAOS with a more complex composition (NA4, NA6, NA8, NA10, and NA12) than agarose hydrolyzed with a shorter time.[9,15] Similar results were also shown by Chen et al, who mentioned that agarose hydrolyzed using HCl with a longer time can produce more complex AOS with a more complex composition than agarose hydrolyzed with a shorter time.[16]

The toxicity test in this research used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method to determine the concentration threshold of AOS and NAOS at various hydrolysis times that are safe to use. The MTT assay is a toxicity test method based on the intensity of formazan crystals formed as an indicator of the metabolic activity of living cells.[17] The MTT test was conducted on RAW 264.7 cells that AOS and NAOS had induced with various concentration variations to obtain the IC_{50} value of each compound. In addition to the percentage of viability, the level of toxicity can be classified based on the IC_{50} value. Results of the cytotoxicity test based on IC_{50} values showed that AOS-0 and AOS-24 were in the non-toxic category

(Table 1), while all NAOS were in the moderately toxic category. The toxicity level based on the IC_{50} value is grouped into 4 categories, namely highly toxic (≤20 µg/mL), moderately toxic (21-200 µg/mL), weakly toxic (201-500 µg/mL), and non-toxic (>500 µg/mL).[16] From these results, it can be seen that NAOS is more toxic to RAW 264.7 cells. The difference in toxicity properties possessed by AOS and NAOS can be attributed to differences in the structure of their compounds. The AOS contains a 3,6 Anhydro-L-Galactose (L-AHG) structure, while NAOS contains a complex composition of the polymer.[18] The differences in the structure of the compounds in AOS and NAOS may indicate that there are differences in the ability of biological activity possessed.[19]

Based on Table 1, both AOS and NAOS produced with longer hydrolysis time will be safer for cells. The results obtained show the same thing as the research of Zou et al. (2019), which states that the AOS fraction from chemical hydrolysis with low DPs is relatively safer than the AOS fraction from chemical hydrolysis with high DPs. Meanwhile, the crude chemical hydrolyzed AOS is more toxic than the high DPs AOS fraction. Based on this description, the decreasing toxicity of AOS and NAOS, along with the length of hydrolysis time, can be caused by the combination of low DPs AOS or NAOS, which are non-toxic, with high DPs AOS or NAOS, which are more toxic.[20–22]

CONCLUSION

The conclusion is that AOS and NAOS hydrolyzed at different times had different levels of toxicity. The longer the hydrolysis time is, the AOS and NAOS compounds produced will be relatively safe for RAW 264.7 cells. The differences in the toxicity properties of AOS and NAOS are related to differences in the structure of the compounds.

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CONFLICT OF INTEREST

We declared that there is no conflict of interest among the authors.

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