Antioxidant and Anticholesterol Activity of Chitooligosaccarides Hydrolyzed of Golden Apple Snail (Pomacea canaliculata) Shell Using Various Types of Enzyme

Dedin Finatsiyatull Rosida[1,2(✉)], Jariyah[1,2], Sekar Ayu Larasati[1,2], Teeradate Kongpichitchoke[3], Anugerah Dany Priyanto[1,2], and Andre Yusuf Trisna Putra[1,2]

[1] Department of Food Technology, Faculty of Engineering, University of Pembangunan Nasional Veteran Jawa Timur, Jl Raya Rungkut Madya, Surabaya 60294, East Java, Indonesia
dedin.tp@upnjatim.ac.id

[2] Center Innovation of Appropriate Technology for Lowland and Coastal Food, Surabaya, East Java, Indonesia

[3] Faculty of Biotechnology, Assumption University, Bangkok, Thailand

Abstract. Chitosan (β1-4 N-acetyl D-glucosamine) is a chitin derivative compound which is obtained through the deacetylation stage, namely the process of releasing the acetamide group in chitin (CH3CONH) so that it becomes an amine group (NH2) in chitosan. The manufacture of chitosan is carried out through 3 stages, namely the process of deproteination, demineralization, and deacetylation. Chitosan has many functional properties because it has a reactive amine group (-NH2), some of its functional properties are antioxidant and anti-cholesterol. However, chitosan is a water insoluble compound, so its use is limited, especially in the food and medical fields. Therefore, chitosan needs to reduce its molecular weight by hydrolysis. Enzymatic hydrolysis is an alternative way that is safe and can be easily controlled. Completely Randomized Design (CRD) was used as the study design with two factors, namely the type of enzyme (cellulase, α-Amylase, and lipase) and concentration (0.1%; 0.5%; and 0.9%). The research showed that there were significant differences in the factors used on the molecular weight, enzymatic activity, antioxidant activity, and anticholesterolemic activity. The best treatment based on the results of this study was sample A1B3, namely chitooligosaccharide samples hydrolyzed using cellulase enzymes, with a concentration of 0.9%, a yield of 85.33% was obtained, with a molecular weight of 13.58 kDa, enzyme activity of 0.95 U/ml, antioxidant activity of 15.97%, and total cholesterol of 44.55 mg/dl (percentage of cholesterol reduction of 55.45%).

Keywords: Chittin · Chitosan · Deacetylation · Golden Snail

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1 Introduction

Golden apple snail (Pomacea canaliculata) is one of the main pest of rice fields, it is able to attack rice plants in a short period of time [1]. The use of golden apple snail in Indonesia is used as a mixture of animal feed or as an or-namental animal, so its utilization is not optimal [2]. The shells are simply thrown away, where they decompose into vile garbage that harms the environment [3]. Shells of golden apple snail itself contains 20–50% chitin content [4].

Chitin (C8H13O5N) or ((1-4)-N-asetyl-D-glukosamine) is a polysaccharide that provides as a structural component for the shells of arthropods (crustaceans, mol-lusks, and insects) [5]. Chitin is first transformed to chitosan in order to be used because the level of acetylation is inadequate and still relatively low. Structurally, chitosan contains primary and secondary hydroxyl groups for each repeating unit, and an amine group for each deacetylated unit, which makes chitosan more reactive [6].

Chitosan, a biopolymer produced by deacetylating chitin, is a chitin derivative product [7]. The acetamide group (-NHCOCH3) in chitin is converted to an amino group (-NH2) to produce chitosan. Chitosan, has the chemical formula of (C6H11NO4)n, it's molecule linked together by (1-4)-glycosidic bond [8]. Unfortunately, chitosan is not water soluble makes it difficult to use in culinary and medicinal applications [5]. Therefore it is necessary to make efforts to increase the solubility of chitosan. The solubility of chitosan can be increased by lowering its molecular weight. One way to reduce the molecular weight of chitosan is by hydrolyzing it using enzymes [9]. This method is easier to monitor and the product is obtained without additional modifications [10]. One of the chitosan derivative products is chitooligosaccharide (COS).

Chitosan that has undergone hydrolysis or had its glycosidic linkages broken is known as COS. Enzymes can be used to hydrolyze chitosan. The enzymatic treat-ment can be used in controlled environments without any further modifications [5]. The specific enzyme needed to hydrolyze chitosan is chitosanase, however it is an expensive and difficult enzyme to get [11]. However, several non-specific enzymes, namely glucanase (cellulase, amylase, lyso-zyme), protease (pronase, pepsin, and papain) and lipase were found to be effective in breaking the -1,4-glycosidic bond in chitosan. Chitosan that has been hydrolyzed and has a low molecular weight is called chitooligosaccharide (COS). COS has many functional properties, some of which are antioxidant and anti-cholesterol properties.

Antioxidant compounds will stabilize free radicals by completing the lack of electrons possessed by free radicals [12]. COS is able to stop free radical chain reactions because COS can provide donors to free radicals and convert free radicals into more stable products [13]. COS can effectively reduce levels of DPPH free radicals and protect human embryonic liver cells from oxidative stress [14]. COS also has the ability to act as an anticholesterol by binding to dietary cholesterol that enters the body. The binding of fat by COS involves the attraction of two opposite charges, COS which has a positive group on NH2 will attract negatively charged fatty acids [15].

Therefore, this study aims to reduce the molecular weight of chitosan and make it COS, and measure the functional properties of COS, namely its antioxidant and anti-cholesterol activities, which are seen based on molecular weight, by treating non-specific

enzymes (cellulase, α-amylase, and lipase). and the concentration (0.1%; 0.5%; and 0.9%).

2 Research Methods

2.1 Material

The materials used is golden snail shell obtained from East Jakarta, Indonesia, Cellulase (Novoenzymes), α-Amilase (Sigma Aldrich), Lipase (Novoenzymes), Hydrocloric Acid 37% (Smart-Lab A 1050), NaOH (Merck 106498), Acetic Acid 100% (Smart-Lab A 1001), Potassium Bromide, Phosphate Buffer pH 6, Schales Reagent, Aquades, DPPH obtained from Sigma Aldirch (St. Louis, MO, USA) and Cholesterol Oxidase Peroxsidase Aminoantypirin (CHOD-PAP) reagent obtained from Dialab (Wr. Neudorf, Austria).

2.2 Tools

The tools used in this research are crusher (wooden pestle), blender (Philips), 100 mesh sieve, magnetic stirrer, beaker glass, funnel, filter paper (Whatman), measuring flask, erlenmeyer glass, stirring rod, spoon, scales, pH paper (Merck), ostwalt viscometer (Iwaki), and drying oven (Memmert), drip plate, micro pipette, vortex, spectrofotometer UV-Vis.

2.3 Research Design

There are two factors used in this study. The first factor is the type of enzyme (cellulase, α-amylase, and lipase) and the concentration (0.1%; 0.5%; and 0.9%) of the enzyme used.

2.4 Test Parameters

Molecular Weight. Molecular weight testing was carried out using an Ostwald viscometer, a COS solution (which was dissolved in 1% acetic acid) was prepared with a concentration of 0.5%; 1%; 1,5%; 2%; up to 2,5%. After that, the solution is pumped through the viscometer's pipe as the flow rate is calculated. The same thing was done with 1% acetic acid (without COS). Determination of the molecular mass of COS was carried out by entering the intrinsic viscosity data obtained from the curve between reduced viscosity and concentration into the Mark-Houwink equation, given [16]:

$$[\eta] = \text{k.M.va}$$

Descriptions:
η = intrinsic viscosity, obtained from the curve (intercept value)
k = 3.5 x 10–4.
M = Molecular Weight.

a = 0.76.

Enzymatic Activity. Mix 100 µl of each chitooligosaccharide sample, 100 µl of 0.05 M phosphate buffer pH 6, and 100 µl of enzyme as sample. Mixing 100 µl 0.05 chitooligosaccharide sample, and 100 µl 0.05 M phosphate buffer pH 6 as a control. Samples and control were incubated in a water bath at 70 °C for 15 min, and then put in the freezer for 15 min. A total of 200 µl of incubated sample was mixed with 800 µl of distilled water and 1000 µl of Schales reagent. As a control, 133 µl of incubated control solution was taken and mixed with 67 µl of enzyme, 800 µl of distilled water, and 1000 µl of Schales reagent. As a blank, 1000 µl of Schales reagent was taken and mixed with 1000 µl of distilled water. Samples, controls, and blanks were boiled for 15 min in a water bath. Put in the freezer until the temperature reaches 4 °C. Centrifuged for 10 min. The supernatant obtained was then measured for its absorbance at a wavelength of 420 nm. The absorbance values of the samples, controls, and blanks were entered into the standard curve, the intercept value being the amount of glucosamine contained in the sample. After obtaining the value of glucosamine, to determine the value of enzyme activity is entered in the formula, as follows:

$$\text{Enzymatic Activity (U/ml)} \ : \ \frac{300 \times \text{Glc} \times 1000}{200 \times \text{BM} \ \times 100 \times 30}$$

Descriptions:
300 = Sample volume resulting from the enzymatic reaction
200 = Sample volume for the schales coloring
Glc = The amount of glucosamine in the sample (µg/ml)
BM = The Molecular Weight of Glukosamine (215,6 g/mol)
1000 = µl to ml conversion factor
100 = Initial sample solution volume
30 = Incubation time (minutes)

Antioxidant Activity. Determination of antioxidant activity was carried out by the DPPH method. The first is to make a DPPH solution with a concentration of 50 µM. Control absorption was carried out by taking 3.8 ml of 50 µM DPPH solution and adding 0.2 ml of distilled water. Determination of sample absorption was carried out by making a sample with a concentration of 100 ppm, namely by taking 0.2 ml of each 100 ppm chitooligosaccharide test sample. Add 3.8 ml of 50 µM DPPH solution, and vortex until homogeneous. The mixture was then incubated at 35 °C in a dark room for 30 min. The absorbance measurement of each test sample and control sample used a UV-Vis spectrophotometer at a wavelength of 516 nm. The percentage of chitooligosaccharide antioxidant activity was measured using the formula:

$$\text{Antioxidant Activity (\%)} = \frac{A0 - A1}{A0} \times 100\%$$

Description: A^0 = control absorbance.

A^1 = sample absorbance

Cholesterol-Lowering Activity. Determination of cholesterol lowering activity was carried out using CHOD-PAP method. Preparation of standard cholesterol solution with a concentration of 100 μg/mL in a tube covered with aluminum foil. Take 2 ml of standard cholesterol solution and add it to 2 ml of sample solution. The mixture was homogenized using a vortex, and incubated at 37 °C for 60 min. The mixture was then centrifuged at 4000 rpm for 5 min. Take 10 μl of each sample and then add 1000 μl of CHOD-PAP cholesterol reagent kit. The mixture was incubated at 37 °C for 10 min. Absorbance measurement by spectrophotometry at a wavelength of 510 nm. Cholesterol levels can be calculated using the formula, as follows:

$$\text{Cholesterol Levels (mg/g)} = \frac{\text{Sample Absorbance}}{\text{Standart Absorbance}} \times \text{Cholesterol Standart Concentration}$$

Functional Group Analysis (FTIR) for Best Treatment. First, as much as 0.02 ml sample of the best treatment mixed with 200 mg KBr until homogeneous. The mixture is put into a pellet to be compacted and vacuumed. The pellet then inserted into the cell placement chamber, then shooted with an IR beam. The FTIR histogram recordings are produced on the monitor during detection using the detector button. The histogram displays the peak data of a sample's functional groups. To gather both qualitative and quantitative data, the histogram was first obtained and then examined.

Data Analysis. Research data were analyzed using ANOVA (Analysis of Variance), if there is a real difference between samples, then proceed with DMRT test (Duncan Multiple Range Test) at 5%.

2.5 Methods

Preparation of golden apple snail shell powder. The golden snail shells are first sorted and cleaned thoroughly under running water to remove any dirt or dust. To make the snail shells easier to destroy, they are then dried at 60 °C for 2–3 h. After that the shells were crushed using a crusher (wooden pestle) and pulverized using a blender. The shell powder was then sieved using a 100 mesh sieve. The sifted powder is ready to be processed into chitosan.

Production of Chitosan. The process of making chitosan refered to [9], with three stages which are demineralization, deproteination, and deacetylation. The shell powder was weighed for the demineralization process, which involved reacting it with 1 M HCl in a ratio of 1:15 (w/v), stirring it with a magnetic stirrer for three hours at ambient temperature (25–30 °C), the dry demineralized residue was then weighed and reacted with 4% NaOH solution, with a ratio (w/v) 1:10, heated for two hours at 65 °C. The residue obtained is chitin. The deacetylation process was carried out by mixing the chitin obtained with NaOH solution with a concentration of 60%, and stirring at 120 °C for 3 h. Each stage of the process, the residue obtained is filtered using filter paper and rinsed

using distilled water until a neutral pH (±7) is obtained. The neutral residue is then put into the dryer cabinet at 60 °C for 2–3 h or until dry.

Production of Chitooligosaccarides. The procedure for making chitooligosaccharides uses a modified method from, [17]. Chitosan was dissolved in 1% acetic acid with a w/v ratio (1:10) stirred until homogeneous, and the pH was conditioned to 5. The addition of enzymes and their concentrations varied according to predetermined factors. The solution was heated in a water bath for 5 h at 55 °C. Enzymes were inactivated at 100 °C for 10 min. Wait for the solution to cool to room temperature, before centrifuging it for 20 min. The supernatant obtained was a COS hydrolysate.

3 Results and Discussion

3.1 Chitooligosaccarides (COS) Characteristics

The characteristics of chitooligosaccharides were measured based on the molecular weight of chitosan after being hydrolyzed and the activity of enzymes involved in the hydrolysis process. The average molecular weight and enzyme activity can be seen in Table 1.

3.2 Molecular Weight

Molecular weight is one of the important parameters used to see the effectiveness of each treatment enzyme in degrading chitosan. The chitosan obtained had an initial molecular weight of 640.83 kDa. The average value of COS molecular weight can be seen in Table 2. The results showed that the molecular weight of chitooligosaccharides ranged from 13.52 to 19.18 kDa. The sample with the highest molecular weight was a chitooligosaccharide hydrolyzed using α-amylase enzyme with an enzyme concentration of 0.1%, while the

Table 1. Average molecular weight and enzymatic activity of chitooligosaccarides

COS Treatment	Research Parameter	
	Berat Molekul (kDa)	Enzymatic Activity (U/ml)
Cellulase 0.1%	15.23 ± 0.22^{c}	0.88 ± 0.009^{b}
Cellulase 0.5%	14.32 ± 0.37^{b}	0.92 ± 0.013c
Cellulase 0.9%	13.58 ± 0.45^{a}	0.95 ± 0.007d
Amylase 0.1%	19.18 ± 0.37^{d}	0.87 ± 0.008a
Amylase 0.5%	14.37 ± 0.10^{b}	0.91 ± 0.006c
Amylase 0.9%	13.82 ± 0.15^{a}	0.95 ± 0.003d
Lipase 0.1%	14.72 ± 0.14^{b}	0.95 ± 0.002d
Lipase 0.5%	13.60 ± 0.20^{a}	1.97 ± 0.007e
Lipase 0.9%	13.52 ± 0.32^{a}	2.03 ± 0.003f

sample with the smallest molecular weight was a sample hydrolyzed using lipase enzyme with an enzyme concentration of 0.9%. Data analysis of variance showed that there were significant differences (interaction) ($p \leq 0.05$) between the types of enzymes and the concentrations of the enzymes used.

The decrease in the molecular weight of chitooligosaccharides when compared to chitosan is due to the cutting of the glycosidic bonds in the chitosan chain, which is caused by enzyme hydrolysis. Based on [18] the enzymes hydrolyzing chitosan act in a certain way, namely by breaking down the molecule into oligomers at reactive sites, and in the end act releasing monomers or dimers sequentially. Based on [19] there is high enzyme activity and the incubation that is carried out can provide an opportunity for enzymes in the process of breaking polymer bonds and cleaving into monomers. Based on Table 2, the higher the concentration of the enzymes used in hydrolyzing chitooligosaccharides, the lower the resulting molecular weight, this is in accordance with the statement from [20] that increasing the concentration of enzymes during the hydrolysis of chitosan causes more enzymes to catalyze the termination glycoside bonds in chitosan, the more glycosidic bonds that are cut, the lower the resulting molecular weight.

The results of research on COS were hydrolyzed using lipase enzymes with a concentration of 0.1%; 0.5%; and 0.9% has a molecular weight of 14.72 kDa; 13.60 kDa; and 13.52 kDa. The results of the study showed that chitooligosaccharides hydrolyzed using lipase enzymes had the lowest molecular weight, this was due to the enzymes depolymerizing chitosan with the combined action of endo and exo, this was based on [21] in Lee et al., (2008) that lipase hydrolyzes chitosan with a double action at the beginning of the hydrolysis time, after glucosamine begins to form lipase with constant hydrolysis with exo action.

Cellulase and amylase enzymes are enzymes that hydrolyze chitosan non-specifically with the endo mode of action [15, 22] cellulase and amylase are enzymes that hydrolyze chitosan non-specifically, because they are one enzymes that can hydrolyze two different types of substrates, namely the specific substrate cellulose and amylose and the non-specific substrate chitosan. The mode of action may be the same or different depending on the enzyme used. Based on [11] and [22]enzymes with endo action tend to hydrolyze chitosan with a rapid decrease in molecular weight at the beginning of the hydrolysis time, then followed by a linear trend afterwards, cellulase enzymes decrease the molecular weight rapidly during the first 10 min of hydrolysis, followed by a slower decrease during the next 2 h, and constant after 3–4 h, while the results of hydrolysis using the enzyme amylase the molecular weight tends to decrease during the 1–3 h of hydrolysis, and is constant after 4–5 h.

Based on the results of research on the molecular weight of chitooligosaccharides which were hydrolyzed using cellulase enzymes at a concentration of 0.1%; 0.5%; and 0.9% is 15.23 kDa; 14.32 kDa; and 13.58 kDa. Tsai et al., (2004) in [15] hydrolyzed chitosan using cellulase enzymes to produce chitooligosaccharides with a molecular weight of 12 kDa. Similar results were stated by [15] chitosan has a degree of deacetylation of 84%, which was hydrolyzed using cellulase at a concentration of 1%, and hydrolyzed for 4 h has a final molecular weight of 11.8 kDa.

Based on the results of research on the molecular weight of chitooligosaccharides which were hydrolyzed using the α-amylase enzyme at a concentration of 0.1%; 0.5%; and 0.9% is 19.18 kDa; 14.37 kDa; and 13.82 kDa. The results of research from [9] hydrolyzed rice snail shell chitosan using the α-amylase enzyme with a concentration of 0.1% ranging from 12.85–18.65 kDa. Similar results were found in a study belonging to [9] who also used rice snail shell chitosan that the molecular weight of chitooligosaccharides hydrolyzed using the α -amylase enzyme at a concentration of 0.1% was 17.12 kDa.

3.3 Enzymatic Activity

Enzyme activity was carried out to quantitatively measure the performance of the enzymes used on the substrate used, namely chitosan. The average value of the enzyme activity of the research results is presented in Table 2. The activity value of the chitooligosaccharide enzyme ranged from 0.87–2.03 U/ml, the smallest activity of 0.87 U/ml was the activity of the amylase enzyme with a concentration of 0.1%, while the greatest activity was lipase enzyme activity, which was 2.03 U/ml. Based on the results of the analysis of variance, there was a significant difference (interaction) between the types of enzymes and the concentrations of the enzymes used ($p \leq 0.05$), the results of the analysis were then continued with the DMRT follow-up test. The higher lipase activity can be attributed to the way the enzyme depolymerizes the chitosan chains. Cellulase and amylase enzymes depolymerize chitosan by endo action [9, 23], while lipase depolymerizes by a combined endo and exo action.

The enzyme activity of the research results is still relatively small when compared to the commercial chitosanase enzyme activity in the study of [13] which is equal to 31 U/ml which was isolated from the bacterium Aeromonas sp. However, it is still larger when compared to research from [17] from the bacterial isolate Klebsiella sp. Based on [24], the efficiency and activity values of enzymes vary depending on the type of substrate, environmental conditions and ionic strength. Enzymes obtained from different sources will have different properties, especially the enzyme response to environmental conditions, such as temperature and pH.

3.4 Antioxidant Activity of COS

Chitooligosaccharides are substances with antioxidant properties, namely substances with the ability to bind or scavenge free radicals. The DPPH method was used in this study to evaluate antioxidants by determining the effectiveness of chitooligosaccharide antioxidants at scavenging free radicals. Table 2 provides information on the average outcomes of research on antioxidant activity.

Based on Table 2, the average value of chitooligosaccharide antioxidant activity ranged from 12.51%–16.54%. Sample treated with the enzyme amylase at an enzyme concentration of 0.1% showed the lowest activity, namely 12.51%, while the treatment with lipase enzyme at a concentration of 0.9% showed the highest activity, namely 16.54%. The results of the analysis of variance showed that the type of enzyme and concentration of the enzyme produced a significant difference in the antioxidant activity of chitooligosaccharides ($p \leq 0.05$), then continued with the DMRT follow-up test.

Table 2. Average antioxidant activity of chitooligosaccarides

COS Treatment	Antioxidant Activity (%)
Cellulase 0.1%	$15{,}27 \pm 0{,}076^{c}$
Cellulase 0.5%	$15{,}66 \pm 0{,}076^{d}$
Cellulase 0.9%	$15{,}97 \pm 0{,}076^{e}$
Amylase 0.1%	$12{,}51 \pm 0{,}076^{a}$
Amylase 0.5%	$13{,}91 \pm 0{,}131^{b}$
Amylase 0.9%	$15{,}53 \pm 0{,}076^{d}$
Lipase 0.1%	$15{,}00 \pm 0{,}076^{c}$
Lipase 0.5%	$15{,}57 \pm 0{,}330^{d}$
Lipase 0.9%	$16{,}54 \pm 0{,}131^{f}$

The type of enzyme and the concentration of the enzyme used in hydrolyzing chitosan affect the antioxidant activity of the resulting chitosan. The greater the concentration of enzymes used, the higher the antioxidant activity. The results showed that chitosan hydrolyzed using lipase enzyme at a concentration of 0.9% (A3B3) had the highest antioxidant activity. Based on the research of Lee et al., (2008) in [25] reported that lipase can produce oligomers and chitosan monomers due to its endo and exo specificity for chitosan, because lipase shows a strong affinity and higher activity for chitosan which has a moderate degree of deacetylation, namely 70–85%.

According to [26], the amine group (NH2) of chitooligosaccharides absorbs hydrogen ions (H+) from free radicals, which later form an ammonia group (NH +). Based on Xie et al., (2001) Regarding the OH groups in chitooligosaccharides, the free radical scavenging mechanism is related to the fact that the OH groups react with active hydrogen atoms in free radicals, and form stable macromolecules, this is because the OH group is a reactive group. which can interact with amino acids or DNA.

Research from [27] suggested that chitooligosaccharide powder with a molecular weight ranging from 5–10 kDa had an antioxidant activity of 17.61%. Je, et al., (2004) suggested that chitosan with a molecular weight of 1–5 kDa had the highest radical scavenging activity. This was proven by [13] who reported that chitosan oligomers with low molecular weights (2.30; 3.27; and 6.12 kDa) had better antioxidant activity than chitosan oligomers with lower molecular weights. Higher (15.25 kDa).

Based on Table 2, the higher the concentration of enzymes used, the higher the antioxidant activity, this is closely related to the molecular weight of the chitooligosaccharides produced, that the lower the molecular weight of chitooligosaccharides, the higher the antioxidant activity. Based on [11] chitooligosaccharides that have a lower molecular weight have tenuous intramolecular bonds and make radical scavenging activity higher, because tenuous intramolecular bonds raise hydroxyl and amine groups and produce many electrons donated to free radicals.

3.5 Anticholesterol Activity of COS

Hypocholesterolemic characteristics, or the ability of chitooligosaccharides to bind to fat and cholesterol and reduce them in the body, are some of their other excellent properties. Although the lipid component cholesterol serves a variety of purposes in the body, having too much of it in the blood can also be dangerous. This property of chitosan and its derivatives, including chitooligosaccharides, has been proven by lowering total cholesterol using the CHOD-PAP method in vitro. Table 3 provides information on the average decline in total cholesterol for each test sample.

Based on Table 3, total cholesterol chitooligosaccharide values ranged from 34.05 mg/dl–47.89 mg/dl. Treatment with the amylase enzyme at an enzyme concentration of 0.1% showed the highest cholesterol-lowering activity, with total cholesterol of 34.05 mg/dl, while the treatment with the lipase enzyme at a concentration of 0.9% showed the lowest cholesterol-lowering activity. ie with total cholesterol of 47.89 mg/dl. The results of the analysis of variance showed that the type of enzyme and concentration of the enzyme produced a significant difference in the cholesterol-lowering activity of chitooligosaccharides ($p \leq 0.05$), then continued with the DMRT follow-up test.

Based on Table 3, the higher the enzyme concentration used, the higher the total cholesterol contained in the test sample, which indicates that the lower the anti-cholesterol activity. This is related to the molecular weight of each chitooligosaccharide sample. According to [15, 28] in [15] one of the mechanisms for the ability of chitosan and its derivatives to reduce cholesterol increases is related to the increase in the molecular weight possessed by these compounds, the molecular weight the greater indicates the longer the chitooligosaccharide chain it has, the more cholesterol is bound to the sample, and the greater the cholesterol-lowering activity.

The results showed a higher cholesterol-lowering activity when compared to study, namely blue crab shell chitooligosaccharides with a molecular weight of 4.51 kDa, and had a cholesterol-lowering activity of 4.06%–7.48%, indicating that Molecular weight is

Table 3. Average cholesterol lowering activity of chitooligosaccarides

COS Treatment	Total Cholesterol (mg/dl)	Cholesterol Reduction Percentage
Negative Control	100	-
Cellulase 0.1%	$41{,}69 \pm 0{,}28^{b}$	58,31
Cellulase 0.5%	$42{,}64 \pm 0{,}55^{c}$	57,36
Cellulase 0.9%	$44{,}55 \pm 0{,}28^{d}$	55,45
Amylase 0.1%	$34{,}05 \pm 0{,}55^{a}$	65,95
Amylase 0.5%	$41{,}37 \pm 0{,}28^{b}$	58,63
Amylase 0.9%	$44{,}71 \pm 0{,}28^{d}$	55,29
Lipase 0.1%	$45{,}66 \pm 0{,}28^{e}$	54,34
Lipase 0.5%	$46{,}94 \pm 0{,}28^{f}$	53,06
Lipase 0.9%	$47{,}89 \pm 0{,}28^{g}$	52,11

one of the determining factors for the anti-cholesterol activity of chitooligosaccharides. The results of research have a small molecular weight, so that little cholesterol is bound to the sample. Another study was the study of [29] which compared chitosan samples with a molecular weight of 30 kDa, 890 kDa, and 2100 kDa. The results showed that the sample with a molecular weight of 2100 kDa had the highest fat absorption activity. This is because chitosan polymers with high molecular weight have high fat trapping properties which are dispersed along the chitosan chains.

3.6 Functional Group Analysis (FTIR) of the Best Treatment

Based on the analysis, the research conducted resulted in the best treatment of enzyme types and enzyme concentrations used in terms of the characteristics of chitooligosaccharides, antioxidant activity, and anti-cholesterol activity. Determination of the best treatment was carried out based on the effectiveness test method which was carried out based on all the parameters of the chitooligosaccharide test. The sample with the highest total effectiveness value was chitooligosaccharide hydrolyzed using the cellulase enzyme with a concentration of 0.9% (A1B3), namely the sample with the best effectiveness value. The samples were then tested using FTIR to determine the functional groups. The infrared spectrum of chitooligosaccharides can be seen in Fig. 1 (Table 4).

Based on the data from the tests conducted, there were 2 peaks in the infrared spectrum of chitooligosaccharides, namely at a wavelength of 3421.72 cm^{-1} and 3446.79 cm^{-1}. This wavelength indicates that there is stretching of the overlapping OH and NH groups, the OH group which usually appears at a wavelength of 3200–3600 cm^{-1} and the NH group which appears at 3300–3500 cm^{-1}. There is also a weak absorption at a wavelength of 1610–1680 cm^{-1}, which indicates the presence of the C=C alkene functional group, or indicates the presence of a bending of the NH group. The results

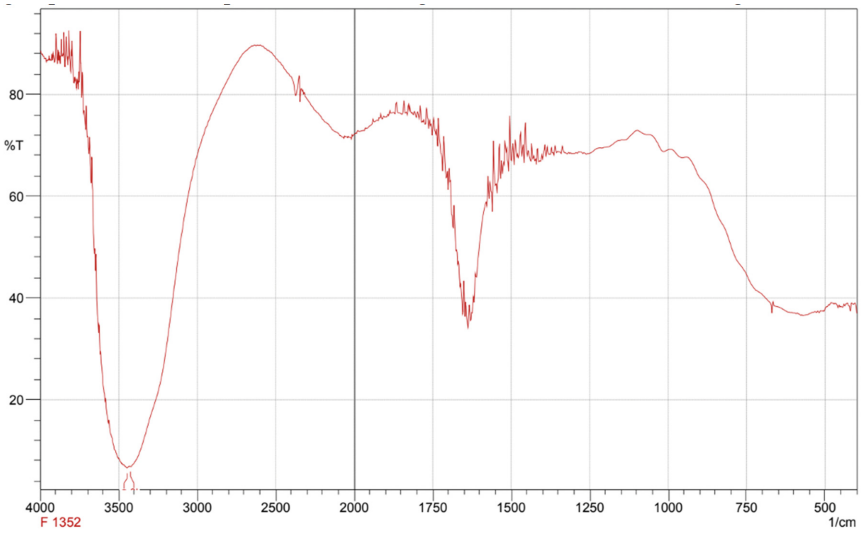

Fig. 1. Best Treatment Sample of Chitooligosaccharide Infrared Spectrum

Table 4. FTIR spectrum characterization of the best treatment chitooligosaccharide

	Wavelength (cm^{-1})	
	Analysis Result	References (Ni'mah et al., 2019)
OH (Hydroxyl) dan NH (Overlap)	3421,72 3446,79	3464 (Squid pen) 3455 (Crab)
NH (Bending)	1610–1680	1629 (Squid pen) 1636 (Crab)

of the FTIR analysis carried out in this study are in accordance with research belonging to [30], which isolated water-soluble chitosan from several different types of materials. There is a peak at the wavelength of 3400 which is absorption from the overlapping OH and NH groups, and there is also a weak absorption caused by bending of the NH groups at wavelengths 1610–1680. Based on Liu (2001) in [16] the chemical structure of chitooligosaccharides is almost the same as the chemical structure of chitosan.

4 Conclusions

Enzymatic hydrolysis of chitosan can result in the production of chitooligosaccharides, a chitosan derivative product. The two types of actions that enzymes use are endo and exo. The molecular weight, enzyme activity, antioxidant activity, and anti-cholesterol activity of chitooligosaccharides were significantly affected by the type of enzyme (cellulase, -amylase, lipase), enzyme concentration (0.1%, 0.5%, and 0.9%). According to the findings of this study, sample A1B3, which consisted of chitooligosaccharide samples hydrolyzed with cellulase enzymes, provided the best results. Its yield was 85.33 percent, its molecular weight was 13.58 kDa, its enzyme activity was 0.95 U/ml, its antioxidant activity was 15.97%, and its total cholesterol level was 44.55 mg/dl (percentage of cholesterol reduction of 55.45 percent).

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