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Physicochemical properties of chitooligosaccharide prepared by using chitosanase from *Stenotrophomonas maltophilia* KPU 2123

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Abstract. Study on the physicochemical properties of chitooligosaccharide (COS) prepared by hydrolysis of chitosan using chitosanase from *Stenotrophomonas maltophilia* KPU 2123 has been carried out. Hydrolysis process was conducted by reacting the soluble chitosan with 8 $U \cdot g^{-1}$ chitosan of chitosanase for 0; 8; 16 and 24 h incubation and stopped by addition of 0.25 M NaOH until reached pH 7. The COS was obtained as supernatant after being centrifugation. The liquid COS were then freeze-dried and analyzed their physicochemical properties, which comprised yield, viscosity, moisture and ash content, the degree of deacetylation (DD), as well as lead (Pb), arsenic (As) content and analyses of COS by Thin Layer Chromatography (TLC). The optimum hydrolysis time was found to be 16 h with the COS viscosity was 8.50 ± 0.87 cPs. The high COS yield was related to high ash content, i.e. 251.70 ± 77.97 % and 50.45 ± 3.19 % (db), respectively. There was lead (Pb) and arsenic (As) metals detected, i.e. 4.4 ppm and 0.1 ppm, respectively. However, they still met the requirement of Pb and As content in a commercial COS referred. Based on the COS properties, desalination process should be applied in the preparation of COS by enzymatic method.

1. Introduction

Chitooligosaccharides (COS) or Chito-oligomers are the hydrolysates of chitosan, mainly composed of β-(1→4)-linked D-glucosamine and partially of N-acetyl-D-glucosamine. In the last few decades, COS has been the subject of increased attention particularly in pharmaceutical and medicinal applications, due to their wide bio activities, such as antibacterial [1], antifungal [2], antitumor activity [3] and radical scavenging [4]. In addition, COS is non-toxic substance, water soluble and low in viscosity which makes them absorbable in the in vivo systems.

COS is generated by depolymerization of chitosan using acid, enzyme or physical hydrolysis. Enzymatic degradation is commonly preferable because the processes are relatively mild, eco-friendly and also produce more homogeneous products. Enzymes involved in COS preparation are particularly chitinolytic enzymes. In Indonesia, research on exploration of chitinolytic enzymes have been conducted in several research institutions, including isolation and screening of microbial chitinolytic enzymes, application of the enzymes for COS preparation and determination of COS bioactivities [2, 5, 6, 7, 8, 9, 10]. Most of them have been done on a laboratory scale with only a few grams of chitosan used as raw material.

Our research center has carried out studies on preparation and bioactivities of COS using chitosanases from our laboratory's bacterial collection since the last decade. The studies were done on a minimal scale (grams of chitosan as raw material) due to the exploratory research purposes and the availability of equipment and instrumentation. One of chitosanases, which is produced by *Stenotrophomonas maltophilia* KPU 2123, has been characterized and used to prepare COS. The resulting COS has been tested for antibacterial, antifungal and antitumor [2, 7]. The result showed that the COS seems to be potential for antifungal. In order to obtain other properties of COS which related to it's specification, preparation of COS at a larger scale should be carried out. This research aimed at production COS using chitosanase *Stenotrophomonas maltophilia* KPU 2123 at a larger scale and characterization of its physicochemical properties.

2. Materials and Methods

2.1. Materials

Materials used in this study were chitin and chitosan from crab shell (Sigma, with chitosan degree of deacetylation or DD more than 85 %), chitosan obtained from Bogor Agricultural University (IPB), glucosamine (Sigma) and standard COS (1–6 units) from Seikagaku Corp., Japan. *Stenotrophomonas maltophilia* KPU 2123 isolated from shrimp waste was used to produce chitosanase. Other chemicals and microbiological media components used were in analytical grade.

2.2. Methods

The research was done in four steps, including the production of chitosanase enzyme from *S. maltophilia* KPU 2123, preparation of soluble chitosan, production of COS and analysis of COS's product.

2.3. Production of chitosanase enzyme

Chitosanase was produced in a 5 L fermentor with working volume of 3 L minimal synthetic medium (MSM) contained of 0.1 % K_2HPO_4; 0.01 % $MgSO_4.7H_2O$; 0.1 % NaCl; 0.7 % $(NH_4)_2SO_4$; 0.05 % yeast extract and 0.5 % colloidal chitin as inducer [6]. The enzyme was harvested after 72 h incubation at 30 °C, 120 rpm by centrifugation at 10,000 × g, 4 °C for 15 min. The enzyme was then subjected to ultrafiltration using a 10 kDa MWCO membrane (UFP-10-E-4MA) for further concentration by 10 × of volume and assayed its activity by Schales method with glucosamine as standard [6].

2.4. Preparation of soluble chitosan

Soluble chitosan was prepared by dissolving of 50 g chitosan in 1 M CH_3COOH, stirred manually, followed by addition of 1 L of aquadest and stirred at 100 rpm. The mixture was then added with 1 M CH_3COONa until pH 6 and the volume was made 2.5 L by addition of 0.05 M CH_3COONa. The preparation of soluble chitosan was done in a 5 L beaker glass on a plate equipped with a speed adjustable agitator.

2.5. Production of COS

Production of COS was done by mixing the soluble chitosan with chitosanase at a concentration of 8 $U \cdot g^{-1}$ chitosan [2]. The mixture was then incubated for 0; 8; 16 and 24 h at room temperature. Soon after incubation, the solution was measured its viscosity using a viscometer (Brookfield Synchro-electric) with spindle no. 2 [11]. The enzymatic reaction was stopped by addition of 0.25 N NaOH until pH 7. The mixture was then centrifuged at 2,000 rpm, room temperature for 20 min. The hydrolysate containing COS was then freeze-dried.

2.6. Analyses of COS

The dried product was analyzed for its physicochemical properties, including yield, moisture content [12], ash content [13] and degree of deacetylation using Fourier Transform Infrared

Spectroscopy or FTIR [14]. The selected COS (based on the best properties) was determined for its Pb and As content [15] and analyzed the mixture by TLC using silica gel 60 F 254 chamber. Chitooligosaccharides (1–6 units) from Seikagaku Corp., Japan; D-glucosamine. HCl, as well as N-acetylglucosamine from Sigma, were used as standards.

3. Results and Discussion

The activity of crude chitosanase produced from *S. maltophilia* KPU 2123 was 0.191 ± 0.018 $U \cdot mL^{-1}$, increased to 0.459 ± 0.032 $U \cdot mL^{-1}$ after being concentrated 10 × volume by ultrafiltration using a 10 kDa MWCO membrane (UFP-10-E-4MA). This method will separate the enzyme from other substances which have a molecular size lower than 10 kDa MWCO. They will pass through the membrane, whereas the chitosanase enzyme retained inside the membrane, and concentrated. Hence, the activity of the enzyme will increase because most of the residue which can lower its activity has been removed [7]. Crude chitosanase produced by *S. maltophilia* KPU 2123 has an activity of 0.1314 $U \cdot mL^{-1}$ and 1.0049 $U \cdot mL^{-1}$ after being concentrated using ultrafiltration [2].

Preparation of COS was carried out by addition of chitosanase into soluble chitosan conducted at pH 6 related to the optimum pH of enzyme work [7]. Hydrolysis process rapidly occurred at the beginning, indicated by a very sharp drop viscosity of soluble chitosan (table 1). The initial viscosity was high, i.e. 1,537.50 cPs which corresponded with the large molecular mass of chitosan [16]. It drastically decreased to 30.25 cPs soon after mixing with chitosanase, and then gradually declined in slow rate until 24 h incubation. This might be due to the decrease of β-(1, 4) glycosidic bond existed within chitosan as well as enzyme activity to support the degradation reaction. The similar result was investigated by other researchers [17], who studied on preparation of low molecular weight chitosan by complex enzymes hydrolysis. They further stated that the slow rate reaction was caused by inhibition of enzyme activity by the increase of oligomeric sugar concentration at a certain level. The decrease in viscosity showed that chitosan was already converted to COS's.

Chitosan used in this study is categorized as high viscosity chitosan, i.e. in the range of 800–2,000 cPs [18]. The viscosity reduction reached 98.03–99.54 %, resulted in COS viscosity 7.00 cPs after 24 h incubation. Statistical analysis indicated that incubation time 0 h gave a significant difference in viscosity with 8; 16 and 24 h incubation. Meanwhile, the 3 h incubation time gave similar product viscosity or did not show any difference in their viscosity. A commercial COS product specification required viscosity less than 10 cPs [19]. If we refer to this specification, chitosan hydrolysis in such conditions takes 16 h. GlcNAc which was produced using a crude enzyme from *Paenibacillus illinoisensis* KJA-424 reached the maximum production after 24 h of incubation [20]. At the same time, N-acetyl chitooligosaccharides were also produced in a lower concentration.

Table 1. The viscosity of soluble chitosan and chitosan hydrolysate containing chitooligosaccharides.

Incubation time (h)	Viscosity (cPs)	Viscosity reduction (%)
0	$30.25^{a} \pm 7.25$	98.03
8	$11.50^{b} \pm 2.60$	99.25
16	$8.50^{b} \pm 0.87$	99.45
24	$7.00^{b} \pm 0.00$	99.54
Soluble chitosan	1,537.50	-

The yield of chitosan hydrolysate containing COS is presented in figure 1. It is shown that the yield of COS increased with the time of incubation/enzyme reaction. The yield calculation was based on the weight of chitosan in soluble chitosan reacted with chitosanase. The high yield (> 100 %) was probably due to impurities derived from salts of enzymatic reaction results as well as inactivation using NaOH solution which was related to the high ash content produced in hydrolyzate containing

COS (table 2). Whereas, the moisture content of COS were found to be less than 10 % which meet with commercial chitosan oligomer requirement [19].

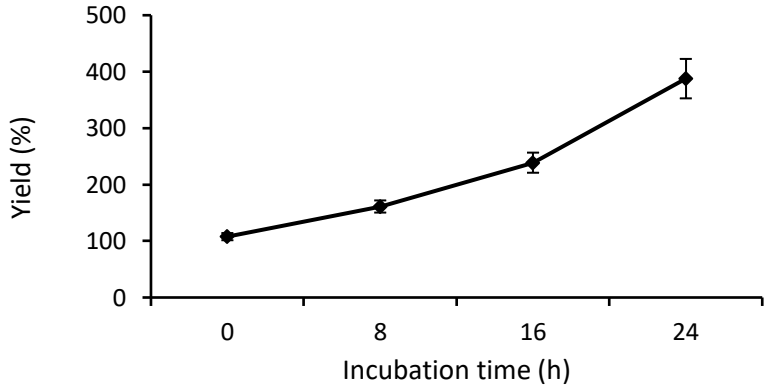


Figure 1. The yield of hydrolysate containing COS.

Ash content of COS was much higher than that of commercial COS specification (table 2). This was probably caused by salts produced during the process, such as in the preparation of soluble chitosan, enzyme addition as well as in the activation process which used NaOH solution. The salt content of COS was contributed by a hydrolysis reaction, and need a desalting process to obtain a pure oligosaccharide [21]. To reduce the salt content, desalination of COS was conducted using dialysis and gel filtration [10]. They found that those dialysis and gel filtration chromatographies were effectively removed the salt from COS with the efficiency of desalting up to 100 %.

Table 2. Moisture and ash content of chitosan hydrolysate containing COS.

Samples	Moisture content (%)	Ash content (% db)
COS – 0*	5.05 ± 2.53	58.85 ± 0.29
COS – 8*	2.50 ± 0.60	53.52 ± 5.93
COS – 16*	2.30 ± 0.54	50.45 ± 3.19
COS – 24*	3.75 ± 1.15	56.42 ± 1.38
Chitosan	11.46 ± 0.51	2.19 ± 0.12
Soluble chitosan (2 % w/v)	92.30 ± 0.02	48.46 ± 0.49
Chitosanase enzyme	98.96 ± 0.02	38.10 ± 0.36

*The number shows time (h) of hydrolysis.

The degree of deacetylation as quality parameter indicates acetyl group which can be removed from chitosan. The higher DD value, the less acetyl group contained within chitosan. The result of DD measurement showed that the DD value of chitosan hydrolysate containing COS ranged from 43.24 ± 0.88 % to 53.02 ± 3.08 %. The chitosan, as the raw material, had DD value of 42.35 ± 1.65 %. These values were much lower than that of commercial COS specification, due to the different method applied in DD measurement. To make sure that different methods result in different DD values, DD measurements have been done on Sigma chitosan. The DD value of chitosan from Sigma as listed on the label was > 85 %, however, the value based on FTIR method showed 45.05 ± 3.26 % (figure 2). Thus, the DD value of chitosan hydrolysate containing COS from 16 and 24 h hydrolysis was probably > 85 %, if they were measured as Sigma chitosan method.

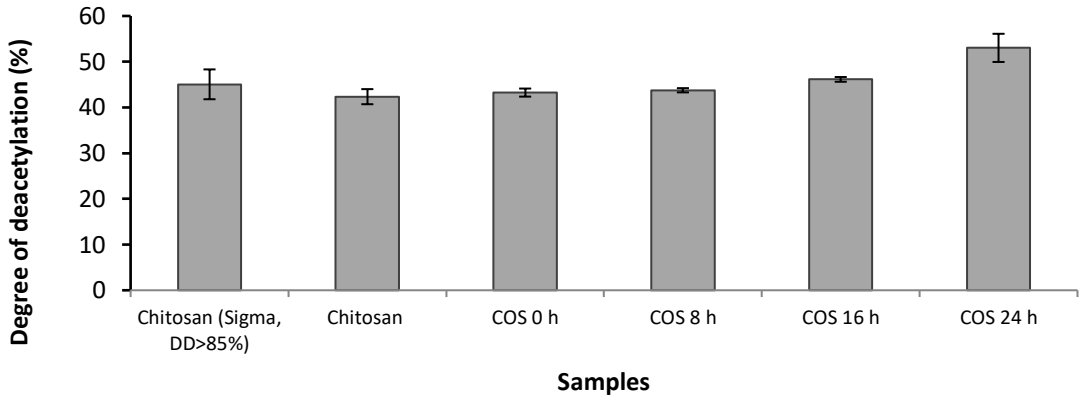


Figure 2. The degree of deacetylation of chitosan and chitosan hydrolysate containing COS.

Heavy metals may a cause of environmental pollution and cause a detrimental human or environmental effect. These metals include lead (Pb), cadmium (Cd), mercury (Hg), arsenic (As), chromium (Cr), copper (Cu), selenium (Se), nickel (Ni), silver (Ag) and zinc (Zn) [22]. They are toxic at higher concentrations. Excessive levels can be damaging to the organism. Since commercial chitosan are mostly produced from crustacean shell waste which its habitat may be subject to heavy metal contamination, the chitosan industry specifies the requirements for heavy metal content, e.g. Pb and As.

The analyzed heavy metals were lead (Pb) and arsenic (As) of soluble chitosan and chitosan hydrolysate from 0 h and 16 h (table 3). It was shown that Pb was not detected on soluble chitosan as raw material, whereas As content in this sample was 0.017 $\mu g \cdot g^{-1}$. In addition, chitosan hydrolysate was found to be positive in the two heavy metals content. The initial time of chitosan hydrolysis was found to be higher, i.e. 2,700 $\mu g \cdot g^{-1}$ and 4,400 $\mu g \cdot g^{-1}$ for Pb and As, respectively, however at 16 h hydrolysis the Pb and As content was 0.072 $\mu g \cdot g^{-1}$ and 0.102 $\mu g \cdot g^{-1}$, respectively. It was predicted that the heavy metals were generated from colloidal chitin as well as aquadest. However, the samples still meet the specification of commercial COS reported previously [19] related to Pb and As i.e. < 5 ppm and < 3 ppm, respectively. Pb may be found naturally in surface water [23]. Fresh or river water usually contains 1–10 $\mu g \cdot L^{-1}$ Pb, but this metal content in seawater is commonly lower. Industries that potentially cause Pb contamination include battery industry, foundry, fuel, cable, etc. Some arsenic compound as methylarsenic may be found in water as biological activity product. This compound will oxidize to methylarsenic acid. Organic arsenic compounds can be found in marine life and are very resistant to chemical degradation [24].

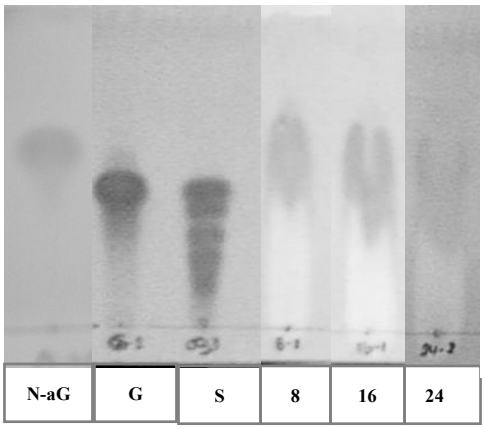
Table 3. Lead (Pb) and arsenic (As) content of chitosan and chitosan hydrolysate containing COS.

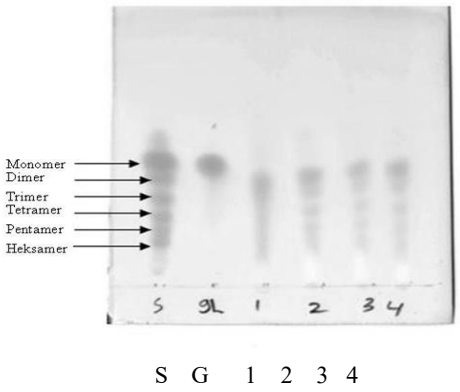
Samples	Heavy metal content ($\mu g \cdot g^{-1}$)	
	Lead (Pb)	Arsenic (As)
Soluble chitosan	nd	0.017
COS – 0*	2,700	0.072
COS – 16*	4,400	0.102

*The number shows time (h) of hydrolysis;nd not detected.
Limit detection for Pb: 0.005 ppm; for As: 0.0005 ppm.

The result of COS analysis is presented in figure 3. It is shown that there was no COS product detected in TLC result. It may be due to the low concentration of COS's under the detection limit of

TLC and the high salt or ash content which interferes with the reaction occurred in analyses of COS using TLC. Oligosaccharides move according to its molecular weight. Thin irregular spots were observed, which were predicted that these spots were generated from 1–2 degree of polymerization of COS. Crude chitosanase from *Bacillus licheniformis* MB2 produced monomer and dimer when incubated with 100 % DD chitosan, while the purified enzyme produced pentamers to hexamers [5]. It was estimated that the crude enzyme consisted of mixed enzyme both endo- and exo-type of chitosanase which are able to work synergistically. In other research, at least six COSs (monomer to hexamer) produced from hydrolysis of chitosan using 8 $U \cdot g^{-1}$ chitosan of chitosanase from *S. maltophilia* KPU 2123 [2]. The experiment was done using 51.4 % DD chitosan and 1.004 $U \cdot mL^{-1}$ chitosanase which were relatively higher than that used in this research. This fact showed that the specification of raw materials used will affect the quality of the product.





Note :
N-aG: N-acetyl glucosamine
G: Glucosamine
S: COS standard (DP 1–6)
8: COS from eight hydrolysis
16: COS from 16 h hydrolysis
24: COS from 24 h hydrolysis

G : Glucosamine
S : COS standard (DP 1–6)
1 : COS from 1 h hydrolysis
2 : COS from 2 h hydrolysis
3 : COS from 3 h hydrolysis
4 : COS from 4 h hydrolysis

Figure 3. TLC profiles of COS produced in the hydrolysis of soluble chitosan by crude chitosanase from *S. Maltophilia* KPU 2123 (left) compared to [2] result on COS hydrolyzed by the same chitosanase with different activity's stock, i.e. 0.459 $U \cdot mL^{-1}$ (left) and 1.0049 $U \cdot mL^{-1}$ (right). Chitosan's DD used was 42.4 % (left) and 51.4 % (right). Hydrolysis process was conducted on 2 % soluble chitosan using 8 $U \cdot g^{-1}$ chitosan of chitosanase *S. maltophilia* KPU 2123.

A good result of TLC with clear spots was found in some studies on COS production using purified enzyme or further treatment of COS to purify it, as reported on COS production using pure cellulase from *Trichoderma reesei* [7], and purified COS from chitosan using crude enzyme of *Bacillus cereus* D-11 [25].

Based on the results, related to the high ash content; ultrafiltration membrane with 5 kDa MWCO was used to reduce ash content of COS from 16 h-hydrolysis. The sample was mixed with a 25 mM acetic buffer with the same volume and passed through the membrane until equilibrium was reached (the same volume was obtained). The process was repeated in three cycles. Sampling was done for ash content analyses at each cycle (table 4). It was shown that ultrafiltration reduced ash content drastically from 50.45 % (db) to less than 1 % (db). Thus ultrafiltration can be regarded as one of the purification steps [26]. Other techniques for separation and purification of COS had also been reported, such as gel filtration [10, 27], ion exchange [28] and metal affinity chromatography [29].

Table 4. Ash content of chitosan hydrolysate containing COS before and after ultrafiltration.

No	Sample	Ash content (% db)
1	Before ultrafiltration	50.45
2	After ultrafiltration (1st cycle)	0.79
3	After ultrafiltration (2nd cycle)	0.60
4	After ultrafiltration (3rd cycle)	0.35

Commercial products of chitooligosaccharides are commonly expressed as being the salt of acetic, lactic or citric acid of the oligosaccharides [21]. In addition, glucosamine is available in the form of a hydrochloric or sulfuric salt. This is because of the expensive and complicated process of purification to reduce or remove the salt. For that reason, scaling up production of COS should be done by using: high-quality chitosan (e.g. high-DD of chitosan) and crude enzyme with high activity as well as high-quality chemicals (such as acid for preparing soluble chitosan) to minimize salt content as well as cost production of the COS.

4. Conclusion

Preparation of COS by using crude chitosanase from *S. maltophilia* KPU 2123 with the ratio enzyme/substrate of 8 $U \cdot g^{-1}$ chitosan produced COS which high in ash content and poor visualization of TLC. The enzymatic hydrolysis for 16 h produced COS with viscosity < 10 cPs which met the requirement of a commercial COS. The COS was also eligible for the specification of commercial COS related to Pb and As content. Desalination process should be done to reduce ash content which passes for a commercial specification.

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