Purification of chitosanase from *Stenotrophomonas maltophilia* KPU 2123 and *Micromonospora* sp. T5a1 for chitooligosacharide production

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Abstract. *Stenotrophomonas maltophilia* KPU 2123 and *Micromonospora* sp. T5a1 are bacterial strains isolated from shrimp waste capable of hydrolyzing chitosan to produce chitooligosacharides (COSs). Their COS products showed antimicrobial activity. The aim of this study was to purify chitosanase from those bacteria and used for COSs production. Chitosanase from both strains were produced using 0.5% colloidal chitin as inducer. The chitosanase was then purified by ultrafiltration, DEAE Sepharose ion exchange and Separacryl S-300 gel filtration chromatography. The specific activity and the yield of chitosanase KPU 2123 increased 2.35-fold and 30.90% after three steps of purifications, whereas chitosanase T5a1 increased 3.32-fold and 22.79%, respectively. The molecular weight of both chitosanases KPU 2123 and T5a1 were about 76 and 20 kDa, respectively. The COS products generated by chitosanase KPU 2123 contained N-acetyl-D-glucosamine, Di-N-acetyl-D-glucosamine, Tri-N-acetyl-D-glucosamine, Tetra-acetyl-D-glucosamine and Penta-N-acetyl-D-glucosamine while by chitosanase T5a1 contained N-acetyl-D-glucosamine, Di-N-acetyl-D-glucosamine. Based on their COS products, chitosanase KPU2123 can be categorized as endo-type chitosanase. Further study is needed to analysis the bioactivity of COSs obtained from the pure of chitosanases KPU 2123 and T5a1.

Keywords: chitooligosaccharides, chitosanase, *Micromonospora* sp., purification, *Stenotrophomonas maltophilia*

1. Introduction

Chitin and chitosan have been developed and used in various industries as biomaterials for food, pharmaceutical, textile, and other sectors (Choi *et al* 2004). Their derivative products, chitin oligosaccharides (CTOs) and chitosan oligosaccharides (COSs) are known more valuable than their original material because of their biological activities (Wang *et al* 2008). Transforming chitosan to COS increases physiochemical properties such as water solubility and nontoxicity (Lodhi *et al* 2014). Furthermore COSs were reported having biological properties for decreasing cholesterol, enhancing calcium absorption, and improving antihypertensive, antioxidant, antimicrobial and antitumor effects (Aam *et al* 2010, Je and Kim 2012, Yang *et al* 2019).
Conventionally, COSs are produced by chemical processes. This method however, produced a small number of oligosaccharides, which contained short chain of glucosamine units, and secondary compounds that are difficult to separate in the purification process (Mourya et al 2011, Wang et al 2008). Acid or alkaline hydrolysis is also considered not environment-friendly because of its waste pollution (Choi et al 2002). Furthermore, the chemical residues make COS not applicable for human consumption (Je and Kim 2012). An alternative to replace chemical method is enzymatic process, which is eco-friendly, easy to control and generate COSs without any harmful side molecules (Liang et al 2018, Su et al 2006). Chitosanase is one of the enzymes that hydrolyze chitosan to COSs (Lodhi et al 2014).

Chitosanases (EC 3.2.1.132) are enzymes catalyzing hydrolysis of β 1-4 glycosidic bond of the chitosan releasing mixture of N-glucosamine (Rodriguez-Herrera et al 2008, Pechsrichuang et al 2018). These enzymes are mostly found in microorganisms, including bacteria and fungi (Choi et al 2004, Wang et al 2008). Chitosanases are classified into seven families based on their action of glycoside hydrolase (GH). The mechanism actions of chitosanases are different depending on their sources. Bacteria chitosanases are often grouped in GH family 46, while fungi chitosanases are in family 75 (Qin et al 2018). The isolation and development of chitosanases have been attracting researchers and industry, especially cold adaptive and thermostable chitosanases (Qin et al 2018, Zhou et al 2019).

In the previous studies, our group has isolated chitinolytic bacteria from marine environments and shrimp waste. A total of 106 isolates were found including Stenotrophomonas maltophilia KPU 2123 and Micromonospora sp. T5a1 (Chasanah et al 2011a, Chasanah et al 2011b, Uria and Chasanah 2005, Zilda et al 2006). Chitosanases from both isolates have been produced, characterized as well as applied for COSs production (Chasanah et al 2011b, Fawzya et al 2009, Fawzya et al 2018, Zilda and Fawzya 2006). The COSs from both enzymes showed antimicrobial activity (Chasanah et al 2011b, Chasanah et al 2013). The production of those COSs were conducted using crude enzyme. Therefore, in this study, we reported the purification of chitosanases from Stenotrophomonas maltophilia KPU 2123 and Micromonospora sp. T5a1, and production of COSs using the purified enzymes.

2. Materials and methods

2.1. Materials

The strains of Stenotrophomonas maltophilia KPU 2123 and Micromonospora sp. T5a1 were a collection of Biotechnology laboratory at RCMFPPB, isolated from shrimp waste and shrimp paste, respectively (Chasanah et al 2007, Zilda and Fawzya 2006). Chitosan was obtained from Bogor Agricultural University (IPB University). COSs standard (1-6 units) was purchased from Seikagaku Corp., Japan, while glucosamine from Sigma. Other chemicals and microbiological media were in analytical grade.

2.2. Methods

2.2.1. Production of chitosanase. The production of chitosanase was conducted by growing S. maltophilia KPU 2123 and Micromonospora sp. T5a1 in 4×250 mL of liquid medium (Erlenmeyer flask 1000 mL) containing of 0.1% K2HPO4, 0.01% MgSO4·7H2O, 0.1% NaCl, 0.7% (NH4)2SO4, 0.05% yeast extract and 0.5% colloidal chitin as inducer (Fawzya et al 2018). The cultures were grown in an orbital shaking incubator for 48 hours at 30°C (S. maltophilia KPU 2123) and 24 hours at 37°C (Micromonospora sp. T5a1). After incubation, broth cultures were centrifuged at 10,000g for 10 min at 4°C. The supernatants were used for further purification.

2.2.2. Purification of chitosanase. The chitosanase purification consisted of three stages. The supernatants were firstly subjected to ultrafiltration using membrane with MWCO 10 kDa (GE
Healthcare) until reached concentration of ±10 times. The concentrated enzymes were applied into ion exchange chromatography using DEAE Sepharose TM Fast Flow (GE Healthcare) column in AKTA Purifier (GE Healthcare). The enzymes were eluted using gradient concentration of 0.02 M Tris-CI buffer pH 9 and 1 M NaCl. The active fractions were further purified using HiPrep Separacyl S-300 (GE Healthcare) column in the gel filtration chromatography system. As a mobile phase, 0.02 M of Tris-CI buffer pH 9 was used. The chitosanase activity and total soluble protein of each purification steps were assayed. The purity of each step was analyzed using 10% SDS-PAGE.

2.2.3. Chitosanase activity. The activity of chitosanase was determined based on Schales method as reported by Yoon et al (2000). Soluble chitosan 1% was used as a substrate prepared according to Choi et al (2004), while glucosamine was used as a standard.

2.2.4. Protein content. The protein content of the enzyme was measured using Lowry method (Bollag and Edelstein 1991). A serial concentration of bovine serum albumin (BSA) from 0 to 0.2 mg/mL was used as a standard.

2.2.5. Production of chitooligosaccharides. Chitooligosaccharides were produced using chitosanase at a concentration of 8 U/gram of chitosan. The mixture was incubated for 16 hours. The reaction was inactivated by boiling the mixture for 10 minutes. The mixture then was centrifuged at 9,000 g for 15 minutes to separate the COSs and the residue. The COSs content was analyzed using HPLC. A Shodex Asahipak NH2P-50 column was used. A mixture of monomer to hexamer chitooligosaccharides was used as a standard (Seikagaku Corp., Japan).

3. Results and discussion

Purification results of chitosanases are summarized in table 1. Ultrafiltration increased the specific activity of chitosanase KPU 2123 from 0.83 to 1.46 U/mg (1.76 fold), and specific activity of chitosanase T5a1 from 2.55 to 2.99 U/mg (1.17-fold). In addition, this process produced enzymes with 76.48 and 46.94% of yield respectively. Ultrafiltration with 10 kDa cut off membrane will hold protein above 10 kDa in the reservoir and remove the smaller ones (Fawzya et al 2018).

Table 1. Purification summary of chitosanase from KPU 2123 and T5a1.

| Purification       | Total protein (mg) | Total Activity (U) | Specific activity (U/mg) | Yield (%) | Purification fold |
|--------------------|--------------------|--------------------|--------------------------|-----------|-------------------|
| KPU2123            |                    |                    |                          |           |                   |
| Crude enzyme       | 158.1              | 131.4              | 0.83                     | 100.00    |                   |
| Ultrafiltration    | 68.65              | 100.49             | 1.46                     | 76.48     | 1.76              |
| DEAE Sepharose     | 24.80              | 43.12              | 1.74                     | 32.82     | 2.09              |
| Separacyl S-300    | 20.80              | 40.60              | 1.95                     | 30.90     | 2.35              |
| T5a1               |                    |                    |                          |           |                   |
| Crude enzyme       | 24.00              | 61.20              | 2.55                     | 100.00    |                   |
| Ultrafiltration    | 9.60               | 28.73              | 2.99                     | 46.94     | 1.17              |
| DEAE Sepharose     | 4.95               | 15.45              | 3.12                     | 25.25     | 1.22              |
| Separacyl S-300    | 1.65               | 13.95              | 8.45                     | 22.79     | 3.32              |

Ion exchange chromatography using DEAE sepharose generated six peaks for KPU2123 (figure 1a) and and five peaks for T5a1 (figure 1b). The chitosanase activities of those peaks are shown in figure 2a and 2b. In figure 2a (KPU2123), peak 4 showed the highest chitosanase activity (0.216 U/mL) while in figure 2b (T5a1), the highest activity (0.010 U/mL) was found in peak 3. The elution for both of peaks was started at 0.3 M NaCl gradient. The purity of those peaks was analyzed by SDS-PAGE. The results showed there are more than one bands appeared on acrylamide gel for both peaks (figure
4a– lane 2a and figure 4b–lane 2b). However, the bands for both fractions are reduced compared to the crude enzyme (lane 1a and 1b). Thus, a further purification step is required. This result was similar to the purification of chitosanase from Serratia marcescens, which required gel filtration process after DEAE ion exchange (Wang et al, 2008). DEAE ion exchange is categorized as anion exchange chromatography resin. This resin will bind the negative charge of proteins (Scopes 2013).

Figure 1. DEAE sepharose profile of KPU2123 chitosanase (a) and T5a1 chitosanase (b) derived from ACTA purifier.

The sepacryl S-300 purification profiles of KPU2123 and T5a1 chitosanases are shown in the figure 3. The KPU2123 had three peaks in its profile, while the T5a1 had five peaks. Although the number of peaks for both enzymes were different, the first three peaks had similarity in term of their elution. SDS PAGE results showed that only peak number two for both KPU 2123 and T5a1 had chitosanase activity (figure 4). The size of the bands was about 76 kDa and 20 kDa for KPU2123 and T5a1 respectively. After this Sepacryl purification step, the specific activity of KPU2123 was 1.95 U/mg, 2.35-fold greater than that of the crude enzyme, with the yield was 30.90%. Meanwhile, the specific activity of T5a1 was greater (8.45 U/mg) than that of KPU 2123. This activity increased 3.32-fold from the initial activity. The enzyme yield of T5a1 was 22.79% (table 1).
**Figure 2.** Activity of KPU2123 chitosanase (a) and T5a1 chitosanase (b).

**Figure 3.** HiPrep Separacyl S-300 profile of KPU 2123 chitosanase enzyme (a) and T5a1 (b) derived from ACTA purifier.

The yield of both enzyme after the purification process was comparable to previous reports (Chen *et al* 2005, Choi *et al* 2004). However, the specific activity and the purification fold were lower compared to other studies (Choi *et al* 2004, Pelletier and Sygusch 1990). The differences may be caused by the different in purification and sources of enzyme. The molecular weight of chitosanases was different
depending on the type of enzymes. The molecular weight of chitosanase ChiA from *Aspergillus sp.* was reported 109 kDa, while ChiB from the same sources was 29 kDa. The size of other chitosanases from *Aspergillus spp.* was between 22.5 kDa to 135 kDa where endo-type chitosanase (22.5-40 kDa) has lower molecular weight compared to the exo-type chitosanase (108-135 kDa) (Chen *et al* 2005). The molecular weight of chitosanase from T5a1 was similar to that of chitosahase from *Serratia marcescens* (21 kDa) and chitosanase C from *Bacillus megaterium* (22 kDa) (Pelletier and Sygusch 1990). Whereas the size of chitosanase from KPU2123 was different from other chitosanases that have been reported. The nearest sizes were chitosanases from *Serratia plymuthica* with molecular weight of 60.5 and 95.6 kDa (Wang *et al* 2008).

**Figure 4.** SDS-PAGE profile of chitosanase during purification steps (a) KPU2123 and (b) T5a1. Ma: high molecular weight marker (GE), Mb: low molecular weight marker (GE), 1: ultrafiltration enzyme, 2: DEAE fractions, 3-7: Separacyl S-300 fractions.

**Figure 5.** HPLC analysis of chitooligosaccharides. Standard of chitooligosaccharides (a), KPU 2123 COSs product (b) and T5a1 COSs product.
The pure chitosanases were used to produce chitooligosaccharides. The identification of COS products by HPLC are presented in figure 5. Chitosanase KPU2123 produced N-acetyl-D-glucosamine, Di-N-acetyl-D-glucosamine, Tri-N-acetyl-D-glucosamine, Tetra-acetyl-D-glucosamine and Penta-N-acetyl-D-glucosamine (figure 5b). Chitosanase of KPU2123 produced more types of COS compared to the crude enzyme. In contrast, chitosanase T5a1 only resulted a peak of N-acetyl-D-glucosamine glucosamine with concentration of 0.96 % (figure 5c). Chitosanase is classified into two types, endo-chitosanase and exo-chitosanase. Endo-chitosanase produces COS product which is dominantly by dimer to hexamer, while exo-chitosanase only produced monosaccharide. In addition, endo-chitosanase generally less than 100 kDa in size (Chen et al 2005). In this case, chitosanase KPU 2123 may be classified endotype of chitosanase, while T5a1 is ambiguous, because of different COS product between pure and crude enzyme.

4. Conclusion

The specific activity of chitosanase Stenotrophomonas maltophilia KPU 2123 increased 2.35-fold after three steps of purifications and the yield of the enzyme was 30.90%. Whereas, the final specific activity of chitosanase Micromonaspora sp. T5a1 was 8.45, increased 3.32-fold from the initial activity and the yield was 22.79. The molecular size of both chitosanase KPU 2123 and T5a1 were about 76 and 20 kDa respectively. The characteristic of COS products generated by chitosanase of KPU 2123 contained N-acetyl-D-glucosamine, Di-N-acetyl-D-glucosamine, Tri-N-acetyl-D-glucosamine, Tetra-acetyl-D-glucosamine and Penta-N-acetyl-D-glucosamine, while the T5a1 contained N-acetyl-D-glucosamine, and Di-N-acetyl-D-glucosamine. Further study is needed to analysis the bioactivity of COSs obtained from chitosanase KPU 2123 and T5a1.

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