ISSN : 1410-5217
Accredited : 83/Kep/Dlkti/2009

Original Paper

PURIFICATION AND CHARACTERIZATION OF *Aeromonas media* KLU 11.16 CHITOSANASE ISOLATED FROM SHRIMP WASTE

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Received : August, 10, 2011 ; Accepted : September, 26, 2011

ABSTRACT

Our previous study found that KLU 11.16, isolated from shrimp waste secreted chitinolytic enzymes. The crude enzyme was interesting since their chitooligosccharide was able to inhibit some pathogenic bacteria. In this study we report a purification and characterization of the chitosanase enzyme produced and the identification of the KLU 11.16. Purification of the enzyme was done two steps by ion exchange chromatography followed by gel filtration. Two out of 4 peaks from Gel Filtration step, i.e. fraction 16 and 33 were capable of hydrolyzing 100% deacetylated chitosan, indicating that both fractions contained chitosanase enzyme. The enzyme from fraction 16 had approximate molecular weight of 98.3 kDa. The enzyme worked optimally at temperature of 30^0C, and pH 6. Addition of Ca^{2+}, Fe^{2+}, K^+, Na^+ ions in the form of Cl_2 salt and detergent Triton X-100 increased the enzyme activity, while Co^{2+}, Mn^{2+} and Zn^{2+} ions in the same concentration decreased the enzyme acitivity. Addition of EDTA and SDS significantly decreased the enzyme activity. Molecular based identification revealed that KLU 11.16 was 99% similar to Aeromonas media.

Keywords : purification; characterization; *Aeromonas media* ; chitosanase

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INTRODUCTION

Chitosanase (EC 3.2.1.132) is one of chitinolytic enzymes group, a member of Glycoside Hydrolase (GH) which catalyze a hydrolysis process of β-1,4 linkages between N-acetyl-D-glucosamine and D-glucosamine residue in a partly acetylated chitosan and between D-glucosamine residue of 100% deacetylated chitosan. Chitosanases are categorized in three main classes based on their substrate specificities, i.e. the first class is the chitosanases that degrade chitosan upon recognizing a GlcNAc-GlcN bond, those that recognize both the GlcNAc-GlcN and the GlcNGlcN bond, and those that are specific to the GlcNGlcN bond only. However, the ability to hydrolyze 100% deacetylated chitosan was used to differentiate this enzyme from other enzyme degrading 1,4-β-glucosidic linkage (Park *et al.*, 1999; Chulhong *et al*., 2011).

There is growing attention to the chitinolytic enzyme especially-chitosanase enzymes since the chitoologyosaccharides produced by this enzyme are useful for the application of various industries. In the form of oligomer (chitooligosaccharide), the chitosan has better solubility in water while maintaining and even better performing biological activities such as antimicrobial and protective effects against infection with some pathogens (Jeon & Kim, 1998; Tsai *et. al*., 2000; Kumar *et. al*., 2005, Chasanah *et al*., 2006b; Yan Wang *et al*, 2007; Liang *et al*., 2010), antitumor activity and immune-enhancing effects (Murao *et al.*, 1992; Suzuki, 1996; Nam *et al*., 1999; Kendra and Kim,1998, Jeon *et al.*, 2000; Wahyuni *et al*., 2007). Enzymatic process of chitooligosaccharide production is more preferable compared to chemical and physical process due to safety reason. Among the enzyme

ISSN : 1410-5217
Accredited : 83/Kep/Dlkti/2009

degrading chitosan such as cellulose, pectinase and lysozyme, chitosanase has ability to hydrolyze and produce specific oligosaccharide from chitosan (chitooligosacharide) with high degree of deacetylation.

Worldwide annual recovery of chitin from the processing of marine crustaceans is approximated by 37,300 metric tons (MT), while potency of shrimp and crustacean waste in Indonesia is estimated $\pm$ 76,657 – 114,986 MT of shrimp shell and 3,643 – 4,128 MT of crab shell equals to 12,045 – 17,867 MT a year chitin (Fawzya, 2009). The used of Crustacean waste for chitin production by proteolytic enzymatic methods has been reported (Risjani and Yunianta, 2008; Liang *et al*., 2010). Commercial interest in the utilization of chitin and its derivatives has led to the need for inexpensive and reliable sources of enzymes. Production of a suitable and stable enzymes is an important element in the utilization of shellfish wastes, not only solving environmental problems but also promoting the economic value of marine products.

Our group has isolated chitinolytic bacteria from various Indonesia marine environment/and traditional fishery products , *i.e*. from several sponges (Uria *et al*., 2005, and Chasanah *et al*., 2007), *terasi* (Zilda *et al.*, 2006 and Noviendri *et. al.,* 2006) and from shrimp waste (Chasanah *et al*., 2009a). One isolate from shrimp processing waste, KLU 11.16, was further studied due to its potential. Previous result showed that chitooligosaccharide produced by the crude enzyme was capable of inhibiting *Pseudomonas aurogenosa* and *V. parahaemolyticus* (Chasanah *et al*., 2009b). This paper will report purification and characterization of the KLU 11.16 and the isolate identification.

MATERIALS AND METHODS

Production and extraction of enzyme.

Bacterial cells were cultivated in the Minimal Synthetic Medium (MSM) supplemented with 0.5% colloidal chitin as the sole carbon source (Chasanah *et al*., 2006a). The medium consisted of 0.1% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, 0.1% NaCl, 0.7% $(NH_4)_2SO$, 0.05% yeast extract. One full loop of the fresh bacterial cells grown on an agar plate was picked up and inoculated into the 20-ml starting medium. After 15 hours of incubation in the shaking water bath (37^{o}C, 100 rpm), 10% (v/v) of the resulting culture was transferred into the 80-ml production medium, followed with an one-day incubation at the same temperature and shaking as above. Crude chitosanase enzyme was obtained by centrifuging the resulting culture at 9,000 *x g* (4^{o}C, 25 min).

Purification of the enzyme.

Purification was initiated by concentrating the enzyme extract by ammonium sulphate precipitation technique. A series concentration of 20% - 80% (saturation) ammonium sulphate was`added slowly to enzyme extract and kept overnight at 4^{o}C. The mixture was then centrifuged at 12.000 *x g* (4^{o}C, 6 min). The precipitate obtained was suspended with 15 ml 0.05 M phosphate buffer, pH 7.0 and the suspension was dialyzed with the same buffer to remove the salt. Purification was conducted by Ion exchange and Gel Filtration chromatography. A 1 ml of dialysed sample, diluted with 4 mL milliQ water, was loaded in chromatography colomn containing DEAE Sepharose Hi Trap FF matrix (Amersham) previously equilibrated with Tris-Cl 0.02M buffer (pH 8). Targeted enzyme was eluted with gradient buffer of 0 -1 M 0.02M NaCL Tris-Cl pH 8 buffer. Peaks showing chitosanase activity was checked for purity by SDS-PAGE. A 5 mL of unpure fraction resulted from previous purification above, showing chitosanase activity was loaded in chromatography containing Sephadex G-100. Before loaded with the sample, the coloumn was previously equilibrated with enzyme buffer (0.05M phosphate buffer pH 6.0). The targeted enzyme was eluted with the same enzyme buffer with elution speed of 0.5 ml/h

Assay of chitosanase activity.

Assay of chitosanase activity was done based on the method described by Yoon, *et al.* (2000) with modification. The substrate, 0.1% soluble chitosan (100 µl), was added with phosphate buffer (0.05M,) pH 6.0 in an eppendorf tube. Then 100 µl of enzyme solution was added into the tube followed with a 30-min incubation at 37^{o}C. The enzyme reaction was stopped by

ISSN : 1410-5217
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immersing the tubes for 5 minutes at boiling water to stop the reaction. The resulting mixture (200 µl) was put in a reaction tube filled with 800 µl water. After adding with 1 ml of Schales reagent, it was boiled for 15 min. and then centrifuged at 8.000 rpm for 10 min. The absorbance of the supernatant was measured at 420 nm using spectrophotometer. One unit of chitosanase activity was defined as the amount of enzyme that produce reducing sugar corresponding to 1 µmol of D-glucosamine per minute.

Measurement of protein content.

The protein content of the crude enzyme was estimated according to the Lowry method with Bovine Serum Albumin (BSA) as standard (Bollag and Edelstain, 1991). Samples (100 µL) was mixed with Lowry reagent (900 µL), incubated for 15 minutes, and followed by addition of 3 mL Follin reagent. After vortexing and incubating for 45 minutes, the absorbancy to of the mixture was read using spectrophometry within 540 nm wavelength. Blank was aquadest to replace the samples, while standard curve was set at concentration range of 0.05 – 0.2 mg/mL of BSA.

Determination of optimum pH and temperature.

The optimal pH of chitosanase activities was determined at 37^{o}C by using overlapping pH ranges. To generate such pH ranges, the following buffer used were 0.2 M citrate buffer (pH 4.0-6.0), 0.2 M phosphate buffer (pH 6.0-8.0), 0.2 M borate buffer (pH 8.0-9.0), 0.2 M glycine buffer (pH 9.0-10), 0.2 M NaOH-borate buffer (pH 10). The optimal temperature of the enzyme activity was measured at different temperatures (30, 37, 50, 60, 70^{o}C) at optimum pH. The measurement procedure and conditions followed the standard assay as described previously.

Effect of metals.

The effects of divalent cations as chlorides metals on the enzyme activity were measured at the final concentration of 1 mM. The tested divalent cations included Mg^{2+}, Mn^{2+}, Li^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Ba^{2+} and Ca^{2+}. As control, the enzyme solution without addition of any metals was used. Monovalent cations (K^{+}, Na^{+}, $NH4^{+}$) as well as a trivalent cation (Fe^{3+}) were also tested in this experiment. The effect of EDTA on the enzyme activity was determined in the same concentration.

SDS-PAGE and detection of the enzyme by zymogram.

SDS-PAGE and zymogram were performed using 10% (w/v) polyacrylamide gel. The substrates used for zymogram were 0.1% of 85% deacetylated degree soluble chitosan. Samples were run without boiling with addition of gliserol and 2.5% SDS in 125 mM Tris-HCl (pH 6.7). Electrophoresis was run at constant current of 50 and 25 mA, 100 volt at 40^{o}C. After separation, the gel was renatured following procedure of Chasanah (2004) for chitosanase enzyme.

RESULTS AND DISCUSSION

Identification of KLU 11.16

KLU 11.16 was gram negative, rod (basil) bacteria. Based on 16S rRNA, the bacteria was closed to *Aeromonas media* as shown in phylogenetic tree (**Fig. 1**). The isolate has been isolated from exoskeleton of shrimp which has been sampled at one of the shrimp industries at Muara Baru, Jakarta. The pH and temperature of the waste was 8 and 20^{0}C. It was likely that the shrimp has been treated with water containing chlorine. Therefore, it was suspected that the microbial flora contained in the shrimp waste was the one which can withstand with chlorine and low temperature. From the results (**Fig. 1**), the microbe has been closely identified as *Aeromonas media.* The microbe could be from the water where the shrimp has been cultivated or could be from contamination. Janda & Duffey (1988) and Altwegg & Geiss (1989) reported that *Aeromonas* spp. has been isolated from water, but some of them were implicated as a cause of diarrheal on man. *Aeromonas media* has been isolated from environment (water), infected fish and some of them have been found at diarrhea patient stools. Further study on this bacteria found that this bacteria was potentially enterotoxigenic (Singh, 2000).

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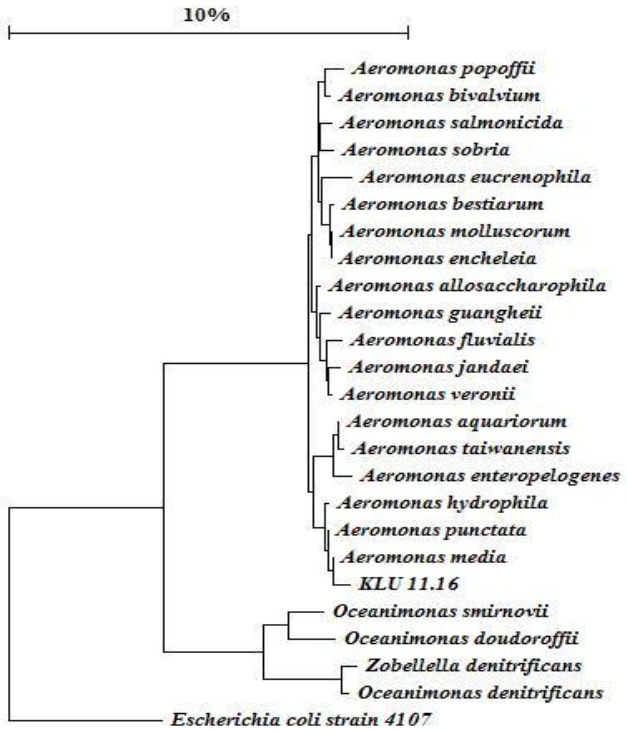

Fig. 1. Phylogenetic tree of KLU 11.16 isolate

Aeromonas sp producing chitinolytic enzymes has been reported by Mitsutomi (1990), Huang (1996), Choi *et al.,* (2003), Kojima (2005), Kuk (2005 and 2006), and Sun *et al.,* (2009). *Aeromonas hydrophyla* was reported to produce 94 kDa chitinase while *Aeromonas schubertii* has been reported to produce 30 and 38 kDa chitinase. Charles-Rodrigues *et al.,* (2008) has isolated *Aeromonas* sp and produced a 20 kDa chitosanase from the bacteria, while Sun *et al.,* (2009) reported a 70 kDa chitosanase from *Aeromonas* sp screened from soil. This report is likely the first report on chitosanase from *Aeromonas media.* This isolate secreted not only chitinolytic enzymes but also proteolytic enzyme (Chasanah *et al*., 2009b) when cultivated in chitin medium.

Purification and characterization of chitosanase

Ammonium sulphate precipitation method was used to concentrate the extracellular enzyme produced by the *Aeromonas media* KLU 11.16 before entering purification step. **Fig. 2** shows that the enzyme could be concentrated by addition of 50% (saturation) ammonium sulphate. The extract was then dialysed using 10,000 Da molecular weight cut off (MWCO) membrane .

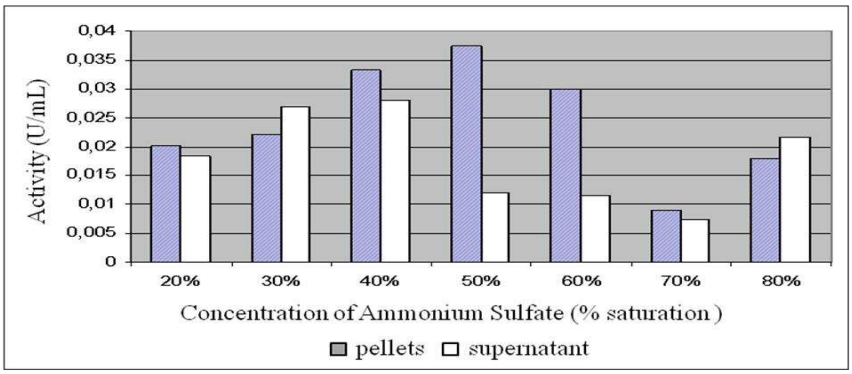

Fig. 2. Optimization of ammonium sulphate addition to concentrate the enzyme

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Fig. 3 shows result of first step purification. There were 2 peaks of chitosanase at fraction no. 8 and 9. Result on SDS-PAGE showed that both fraction had 3 bands, *i.e.* 22,4 kDa, 28,6 kDa dan 98,3 kDa. Therefore, both fractions were mixed and runned at second system of purification, *i.e.* Gel filtration.

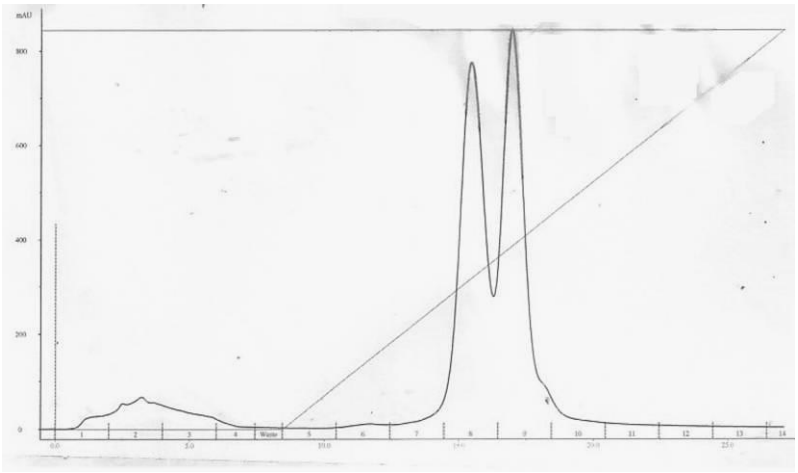

Fig. 3. Purification profile of the enzyme using DEAE *Sepharose*

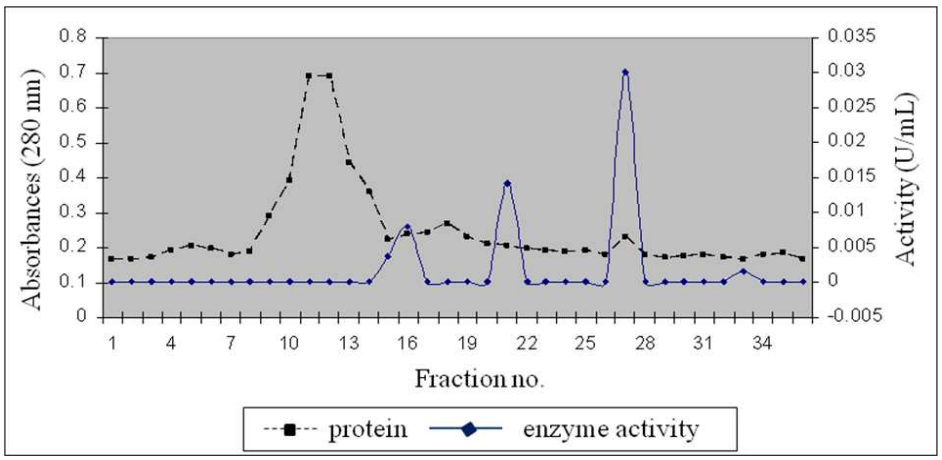

Fig. 4. Gel Filtration profile using *Sephadex* G-100

Using Sephadex G-100, there were 4 peaks of chitosanase, *i.e* fraction16, fraction 21, fraction 27 and fraction 33. Activity and zymogram based assay using 100% deacetylated soluble chitosan as substrate found that among the 4 fractions only 2 fractions, *i.e* fraction 16 and 33 were capable of degrading 100% deacetylated chitosan, assuring that both fractions contains chitosanase enzyme. The ability to degrade 100% deacetylated chitosan have been used to differentiate chitosanase among other chitinolytic enzymes such as chitinase (Chasanah, 2004; Park *et al*, 1999). Result of SDS-Page (**Fig. 6**) and Zymogram (not presented) of the fraction 16 was a single band of protein enzyme. The ,molecular weight was around 98.3 kDa. This fraction was used further for characterization study.

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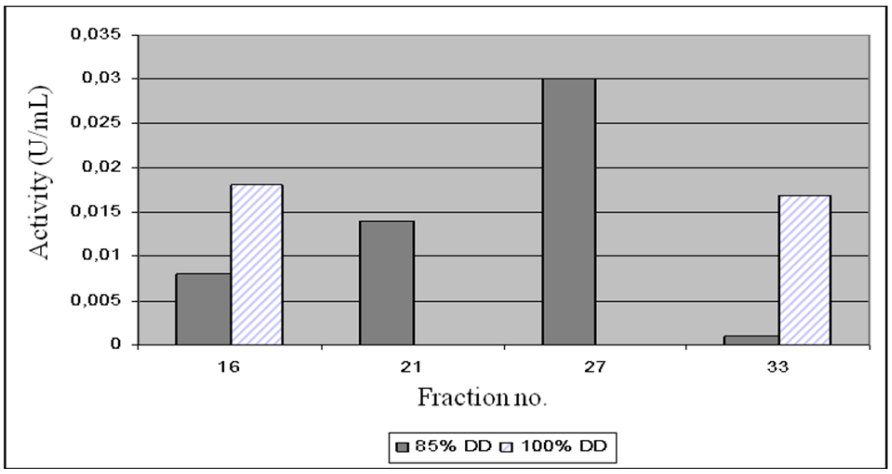

Fig. 5. Enzyme assay of 4 fractions resulted from Gel Filtration chromatography using 85% and 100% deacetylated chitosan

Pure enzyme performed best at 30^0C (**Fig. 7**) and at pH 6 (**Fig. 8**). Activity of the pure enzyme was increased by 1 mM Ca^{2+}, Fe^{2+}, K^+, Li^+ dan Na^+ ion (in the form of Cl_2 salt), ranging from 148% to 340%. While addition of Co^{2+}, Mn^{2+} dan ion Zn^{2+} in the same

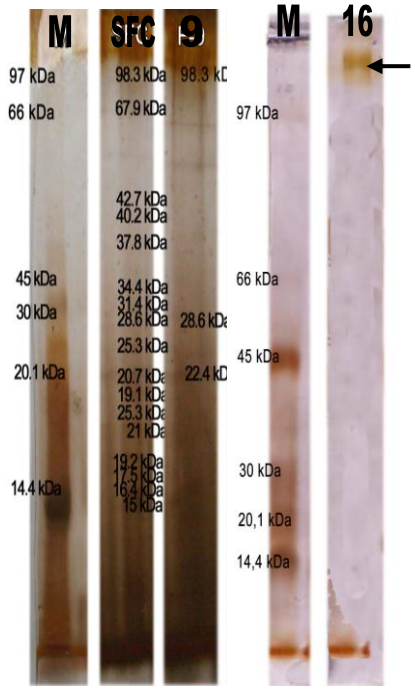

Fig. 6. SDS- PAGE of the protein enzyme from purification step

Note : SDS-PAGE (left) : M = LMWMarker ; SFC = crude enzyme, 9 = fraction 9 of DEAE Sepharose FF, 16 = fraction 16 of Gel Filtration Sephadex G-100

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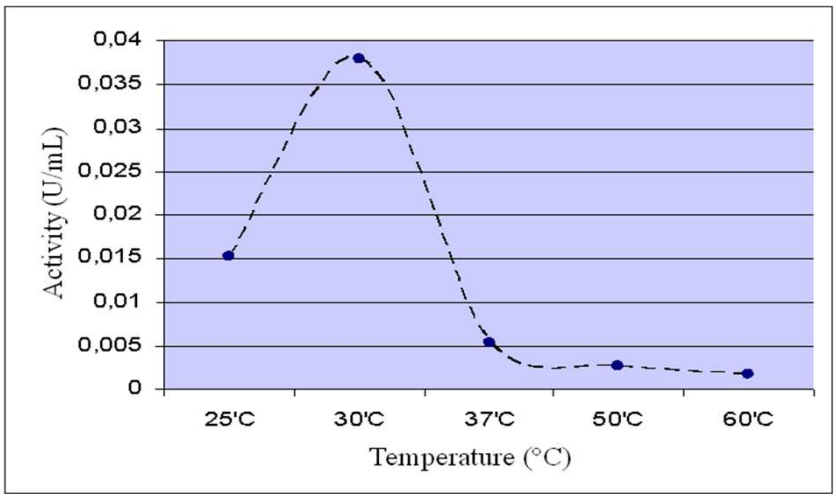

Fig. 7. Optimum temperature of fraction 16 KLU 11.16 chitosanase

concentration have decreased the enzyme acitivity (**Fig. 9**). Addition of additives, *i.e.* EDTA and SDS in the concentration of 1mM decreased significantly the chitosanase activity, while addition of β-mercaptoethanol decreased enzyme activity by 68%, but detergent Triton X-100 increased the enzyme activity (**Fig. 10**). Addition of additives, *i.e.* EDTA and SDS in the concentration of 1mM decreased significantly (100%) the chitosanase, while addition of β-mercaptoethanol decreased enzyme activity by 68%. Detergent Triton X-100 increased the enzyme activity. Compared to other chitosanases studied so far, this chitosanase is considered unusual due to its high molecular weight (98.3 kDa). Another *Aeromonas* chitosanase having high molecular weight has been reported by Sun *et al.,* (2009). *Aeromonas* sp. HG08 isolated from soil secreted the chitosanase (named AsChi) with molecular weight of 70 kDa. The enzyme performed well at optimum pH of 6.0 and temperature of 55°C. On the contrary of *Aeromonas media* KLU 11.16, the activity of *Aeromonas* sp HG08 chitosanase was markedly enhanced by Mn^{2+} and inhibited by Fe^{3+}, Cu^{2+}, Ag^{+} and Hg^{2+} Recent report on chitosanase produced by marine bacterium *Bacillus subtilis* CH-2 has been isolated from the intestine of *Sebastiscus marmoratus* (scorpion fish) (Chulhong *et al*., 2011). The molecular mass of the purified chitosanase was detected as 29 kDa, and the enzyme was performed well at the pH and temperature of 5.5 and 60°C, respectively. The purified chitosanase was continuously thermostable at 40°C.

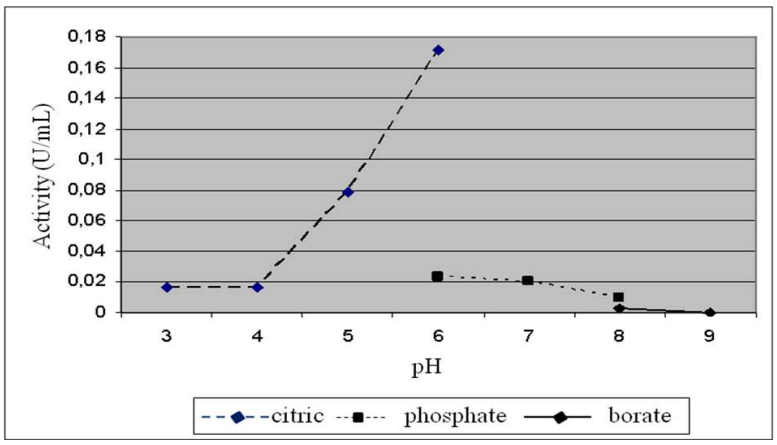

Fig.8. Optimal pH of fraction 16 KLU 11.16 chitosanase

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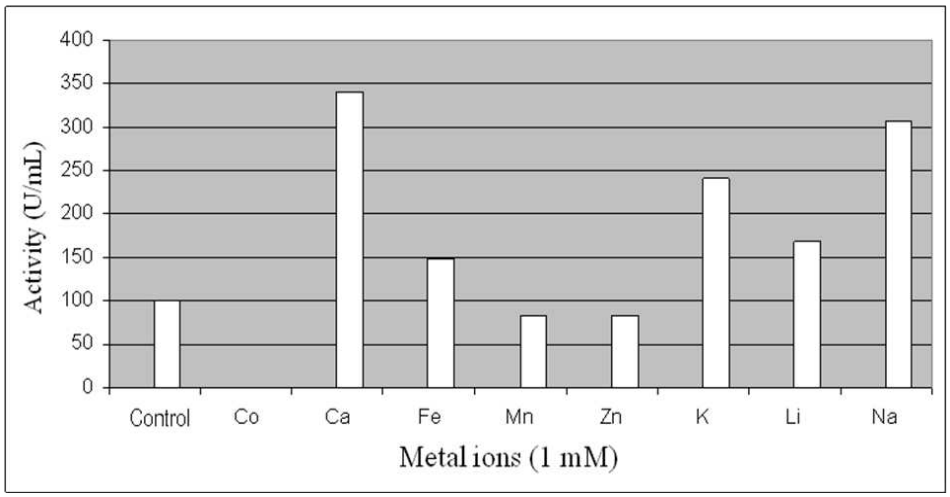

Fig. 9. Effect of ion metal to the fraction 16 KLU 11.16 chitosanase activity

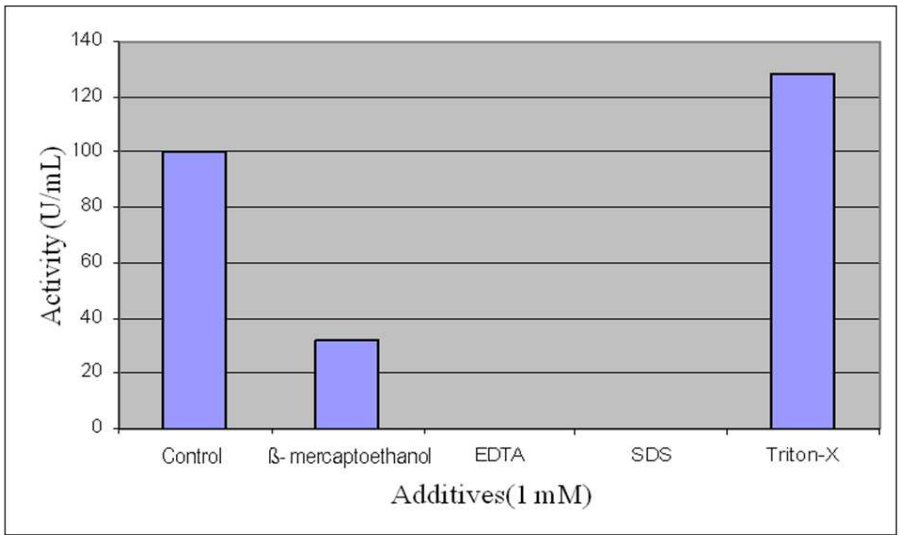

Fig. 10. Effect of additives to fraction 16 KLU 11.16 chitosanase

CONCLUSION

Chitosanase from *Aeromonas media* KLU 11.16 isolated from shrimp waste has been successfully purified by two steps purification. One of the two chitosanases (fraction 16) had approximate molecular weight of 98.3 kDa. The enzyme worked optimally at temperature of 30^0C, and pH 6. Addition of Ca^{2+}, Fe^{2+}, K^+, Na^+ ions in the form of Cl_2 salt and detergent Triton X-100 increased the enzyme activity. The presence of Co^{2+}, Mn^{2+} and Zn^{2+} ions and EDTA and SDS in the same concentration decreased the enzyme acitivity.

The utilization of shellfish (shrimp) waste as a sources of microbes and raw material of chitin, will not only solves environmental problems but also decreases the production cost of microbial chitosanases. The production of inexpensive chitosanase is an important element in the process of chitooligosaccharide production. From this finding, we have an alternative local bacterial chitosanase that can be use for enzymatically chitooligosaccharide production. Since properties of the enzymatically produced chitooligosaccharide, albeit its application, is very depending on the enzyme used, further study on the action of the pure enzyme to hydrolyze chitosanase is very

ISSN : 1410-5217
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urgently needed. Since the chitosanase isolated was quite unique, having high molecular weight, and firstly reported from *Aeromonas media*, further protein characterization such as its N-terminal amino sequence might also be worthed to add scientific data on bacterial chitosanase from *Aeromonas media* KLU 11.16 which has been isolated from Indonesia.

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