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ISOLATION AND PURIFICATION OF THERMOSTABLE CHITINASE *Bacillus licheniformis* Strain HSA3-1a FROM SULILI HOT SPRINGS IN SOUTH SULAWESI, INDONESIA

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ABSTRACT

Thermostable chitinase is produced by *B. licheniformis* HSA3-1a from sulili hot springs in Pinrang, South Sulawesi which had been isolated and purified. Purification through ammonium sulphate precipitation step, dialysis, hydrophobic interaction chromatography with butyl sepharose FF matrix and gel filtration with sephadex G-75. The prepurification with ammonium sulphate precipitation results the highest chitinase activity on fraction 60-70% saturation. The results of hydrophobic interaction chromatography has two active fractions with highest activity on 40th and 43rd fractions, and the results of gel filtration chromatography to have an active fraction with the highest activity on 29th fraction. The results of purification were analyzed by SDS-PAGE showed a protein band at 97 kDa molecular weight estimates.

KEY WORDS: ***purification, characterization, chitinase, Bacillus licheniformis***

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INTRODUCTION

Chitinase is an enzyme that can degrade chitin in bond β-1,4-glycoside, this chitinase can hydrolyze chitin to oligomers then to dimers. Chitinase and the hydrolysis result widely used in agricultural biotechnology, health and environment[1,2]. Generally, chitinase produced from microorganisms is inducible in nature. Extracellular chitinase production is reported to be influenced by medium components such as carbon sources, nitrogen sources and agricultural residues[3]. The main source of the waste chitin is on seafood such as shrimp, lobster, crab shells and shellfish as well as the cell walls of fungi and bacteria [4,5.] Chitin is the structural constituent of insect exoskeleton, crustaceans, certain green algae and fungal cell walls. This polymer of N-acetylglucosamine (GlcNAc) unit is the second most abundant biopoly-saccharides in nature after cellulose [6,7,8]. Purification of chitinase from the thermophilic bacteria generraly uses column chromatography method such as ion exchange chromatography, hydrophobic interaction, and gel filtration. Related to protein purification, some researchers previously did the purification with different chromatographic methods, purifies chitinase from *Bacillus* sp. NCTU2 by hydrophobic interaction chromatography stages continued with gel filtration chromatography[9], purifies chitinase from *Bacillus* sp. 13:26 by phases of ammonium sulphate precipitation, dialysis, heating 60°C for 30 minutes, continued with ion exchange chromatography[10], and purifies chitinase from *B. licheniformis* MB2 with the staps of ammonium sulphate precipitation, dialysis, hydrophobic interaction chromatography, ion exchange chromatography followed by gel filtration chromatography[11]. Based on information from the different purification methods, the method chosen in this research is more simple by combining those methods, namely ammonium sulphate precipitation, dialysis, hydrophobic interaction chromatography with butyl sepharose matrix, followed by gel filtration sephadex G-75. The resulting pure chitinase was applied in chitin hydrolization.

MATERIALS AND METHODS

Materials

***B**. licheniformis* strain HSA3-1a was thermophilic bacteria isolated from the hot springs Sulili South Sulawesi, Indonesia, chitin sigma (EC.215-744.3,) glycol chitin, NaOH, HCl, CH_3COOH, NaCl, H_2SO_4, $(NH_4)_2SO_4$, K_2HPO_4, $MgSO_4.7H_2O$, C_2H_5OH, NaH_2PO_4, Na_2HPO_4, Na_2CO_3, $CuSO_4.5H_2O$, $K_3[Fe(CN)_6]$, yeast extract, peptone, bacto agar, N-acetyl-D-glucosamine, Bovine Serum Albumin (BSA), Schales reagent, butyl sepharose Fast Flow, sephadex G-75, polyethylene glycol-6000, acrylamide, methylene-N'N-bisacrylamide, APS, *sodium dodecyl sulphate*, TEMED, Tris-HCl, bromophenol blue, $Na_2S_2O_3$, and $AgNO_3$.

Inoculum preparation

The culture stock of *B. licheniformis* strain HSA3-1a was cultured again few times in LA medium + 0.5% colloidal chitin in order to obtain fresh isolates. Then performed on the preparation of the inoculum production medium by medium composition: 0.7% ammonium sulphate, 0.05% yeast extract, bacto tripton 0.1%, 0.1% NaCl, 0.1%, K_2HPO_4, 0.01% and $MgSO_4.7H_2O$ 0.5% colloidal chitin as substrate [11].

Production of chitinase

Chitinase production conducted in 500 mL erlenmeyer: 10-15% active inoculum of spores cultured *B. licheniformis* HSA3-1a was shaked at 55°C with aeration at 180 rpm for 72 hours, then crude enzyme was assay by using schales method[12,13], and the protein was assay by using Bradford method[14].

Determination of chitinase
Chitinase activity measurement principle is based on the amount of reducing sugars resulting from hydrolysis of colloidal chitin [11,12].The reaction mixture consisted of 50 μL enzyme, 100 μL colloidal chitin, and 150 μL buffer solution were incubated at 55°C for 30 minutes, heated at a temperature of 100°C for 5 min and centrifuged at 3000 rpm for five minutes. Furthermore, the enzyme mixture is added Schales reagent. The result of the reaction was measured at a wavelength of 420 nm. These measurements use the GlcNAc (N-acetyl-D-glucosamine) as a standard solution. One unit of chitinase activity is defined as the amount of enzyme that produces 1 mol of N-acetyl-D-glucosamine (reducing sugars) per minute on certain conditions.

Purification of chitinase
Chitnase purification crude extract is started by 0-80% ammonium sulphate precipitation dialysis with a cellophane membrane[15], hydrophobic interaction chromatography column with butyl sepharose Fast Flow 0.05 M phosphate buffer pH 7, the next purification of the matrix is used sephadex G-75 and 0.05 M phosphate buffer pH 7.0 [16]. The approximate molecular weight (MW) of pure enzyme chitinase is used by SDS-PAGE stained with silver staining [16, 17] .

RESULTS AND DISCUSSION

There are four steps in doing this chitinase purification,The first is precipitation by using ammonium sulphate which is continued by centrifugation, the second is separation by using butyl sepharose FF, and the last gel filtration chromatography. The results of these staps are described as follows:

Precipitation with ammonium sulphate
Precipitation by using salt ammonium sulphate aims to increase the concentration of the enzyme and as the first step chitinase purification process from thermophilic bacteria *B. licheniformis* strain HSA3-1a. Deposition performed at saturation levels range from 0 to 80% and chitinase activity assays performed on each sediment. Chitinase activity data at each level of the precipitate obtained by ammonium sulphate saturation of the highest activity levels in the range of 60-70% ammonium sulphate saturation is 0.805 U/mL as shown in Figure 1. The results of this research are similar to chitinase from *Bacillus* sp. K-29 which is fractionated with ammonium sulphate at 70% saturation level[19] and chitinase from *B. licheniformis* MB2 which is fractionated with ammonium sulphate at 80% saturation level[11]. Based on this phenomenon, it is suggested that chitinase produced from the termophilic bacteria genus *Bacillus* can be fractionated with ammonium sulphate at a rate of about 60-80% saturation. Phenomena relevant with chitinase fractionation using ammonium sulphate salts showed that the addition of ammonium sulphate to 60-80% saturation level will be salting out (decreased protein solubility). This event indicates that at a certain saturation level, there was a water jacket surrounding the withdrawal of surface proteins by salt ions in solution increases, and then the protein interaction , aggregate, and precipatate [18, 19].

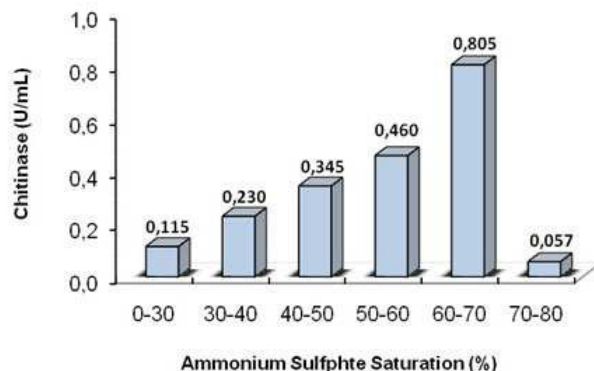

Figure 1
Effect of the level of saturation $(NH_4)_2SO_4$ to the activity of chitinase fromB. licheniformis HSA3-1a

Dialysis.

In the previous step, an excess salt was still remaining in the mixture. However, the salt was not expected to be in the further purification. Therefore, dialysis was conducted to eliminate the salt and other affecting enzyme activity. Dialysis was performed by using a cellophane membrane as passed by the solute molecular weight <10 kDa. Buffer solution used in the outer membrane has a lower concentration than the concentration inside a cellophane membrane. Thus small molecules such as salt or other nuisance ions exit through the pore membrane to the concentration inside and outside the membrane balanced. Furthermore, the buffer solution get into the membrane to replace the small molecule. The results of dialysis chitinase ammonium sulphate fractionation showed the decrease of protein levels of ammonium sulphate precipitation from of 0.75 mg/mL to 0.70 mg/mL with a decrease in protein value of 0.05 mg/mL. This occurs because the volume after dialysis increased from 15 mL to 17 mL, but the chitinase activity of dialysis increased to 1.15 U/mL. This phenomenon indicates that the loss of several metal ions cause the increased chitinase activity of 1.03 U/mL to 1.15 U/mL.

Hydrophobic interaction chromatography

Chitinase purification process is then performed by hydrophobic interaction chromatography. In this research, the results of chitinase purified by dialysis in butyl sepharose FF matrix which is nonpolar as type sepharose such as phenyl or butyl sepharose. Purification process is conditioned with 0.05 M phosphate buffer pH 7.0 and equilibrated with 30% ammonium sulphate. The separation column showing the elution profile of chitinase butyl sepharose FF HSA3-1a isolates contains two peaks of protein which have chitinase activity, namely the peak-1 (fractions 4-5) and peak-2 (fractions 40-43). Fractions which have the highest chitinase activity is the fraction of 40-43 as shown in Figure 2. The first peak (fractions 4-5) is a mixture of proteins that is not bound to the matrix in a hydrophobic state, while the second peak (fractions 40-43) is a mixture of proteins that is bound strongly to the matrix and released during the ammonium sulphate concentration of zero percent. Based on the phenomenon Figure 2 indicates that at high salt concentration interactions between protein and matrix occure. The more hydrophobic the protein or enzyme, the stronger its binding in the matrix. Proteins bound to the matrix can be detached if eluted with the eluent ionic strength decreases.

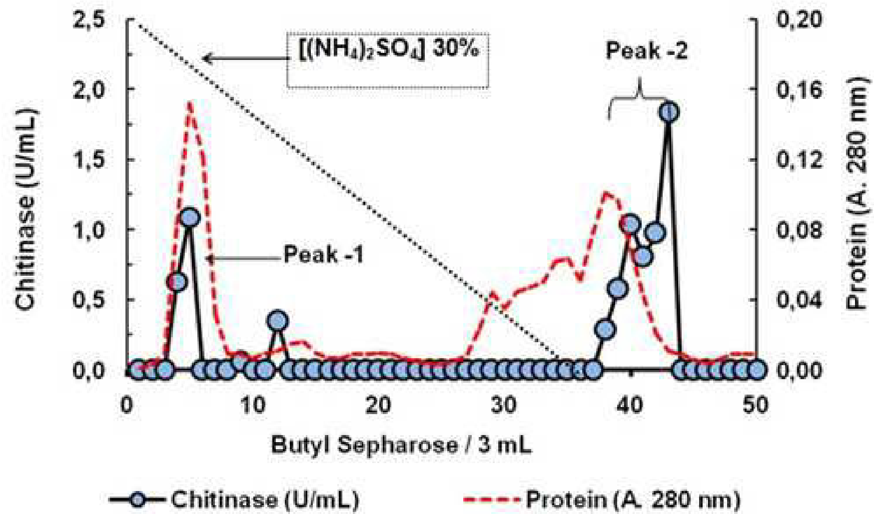

Figure 2
Purification of chitinase from B. licheniformis strain HSA3-1a with butyl Sepharose FF.

Gel filtration chromatography

Further purification using the protein chitinase enzyme derived from a mixture of fractions 40-43 (12 mL) butyl sepharose FF the separation matrix. Based on the results of the purification, chitinase was concentrated with PEG 6000 to 6 mL, then loaded onto the column containing the matrix sephadex G-75 gel filtration chromatography. The results of purification of gel filtration chromatography elution profile of chitinase HSA3-1a isolates contains two peaks of protein has chitinase activity of the fractions-22 and fraction-29 (Figure 3).

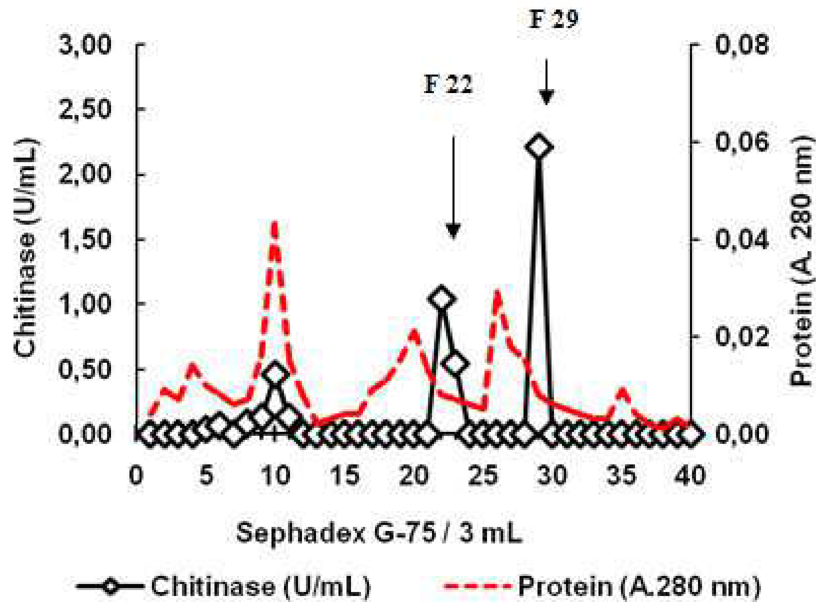

Figure 3
Purification of chitinase from B. licheniformis strain HSA3-1a with sephadex G-75.

The results of purification showed that ammonium sulphate fractionation results chitinase 60-70% had a specific activity of 1.38 U/mg with a purity of the enzyme 1.2-fold, and the results of dialysis has a specific activity of 1.64 U/mg with a purity of 1,4-fold from crude extract. The results of fractionation with butyl sepharose (fractions 40-43). The specific activity of 24.74 U/mg with a purity of 21.1-fold from crude extract. The results of fractionation sephadex G-75 showed that the fraction 29 has a specific activity of 39.18 U/mg enzyme with a purity of 33.4-fold from crude extract (Table 1). The results obtained showed that the purity of the separation process thermostable chitinase is larger than the other studies. Purified chitinase from *Bacillus* sp. NCTU2 through hydrophobic interaction chromatography steps followed by gel filtration chromatography with a purity of chitinase produced 15.6-fold [9]. Purified chitinase from *B. licheniformis* MB-2 through the steps of ammonium sulphate precipitation, dialysis, hydrophobic interaction chromatography with phenyl sepharose, ion exchange with DEAE-Sephacel and gel filtration chromatography matrix sephadex G-75 with a purity of chitinase produced 15.3-fold purity of the extract endokitinase[11]. The high purity obtained from this research due to differences in sequence and matrix method is used.

Table 1
Purification of chitinase from B. licheniformis strain HSA3-1a

Purification Steps	Total Volume (mL)	Total Protein (mg)	Chitinase Activity (U/mL)	Total Unit Chitinase	Specific Activity (U/mg)	Purification (Fold)
Crude extract	2000	587.84	0.34	689.66	1.17	1
Ammonium sulphate precipitation (60-70 %)	15	11.22	1.03	15.52	1.38	1.2
Dialysis	17	11.92	1.15	19.54	1.64	1.4
Butyl Sepharose (F 40-43)	6	0.45	1.84	11.03	24.74	21.1
Sephadex -G-75 (F-22)	3	0.21	1.03	3.09	14.71	12.5
Sephadex G-75 (F-29)	3	0.17	2.18	6.55	39.18	33.4

Moleculer weight determination of chitinase

Results of purity test and molecular weight determination of chitinase using SDS-PAGE method shown on Fig. 4. Lane-1 is the standard protein or marker LMW (Low Molecular Weight). lane-2 is a chitinase protein crude extract showing five protein bands are: 22, 43, 48, 71 and 79 kDa. Lane-3 is protein resulted butyl sepharose FF (F 40-43) which is showing two protein bands, namely 71 and 79 kDa. Lane-4 is protein result of sephadex G-75 (F-29) showing one protein band that is 79 kDa. The results of chitinase purification by butyl sepharose (fraction 43) showed the presence of two protein bands which indicate that chitinase is not pure yet, whereas the results of further purification with sephadex G-75 (fraction 29) showed a single protein bands at 79 kDa molecular weight range. This shows that chitinase (fraction 29) obtained a pure chitinase (Fig. 4). Based on the purification of chitinase resulted from thermophilic bacteria HSA3-1a, pure chitinase is found on fraction 29. The pure chitinase (fraction 29) has different BM with some thermostable chitinase from previous researchs such as chitinase from *Bacillus* MH-1 which is isolated from the compost with molecular weight of 71, 62, and 53 kDa [5], chitinase from *Bacillus* sp K-29 is isolated from crater Kamojang West Java has a molecular weight of 67 kDa[19] and *B. licheniformis* MB-2 is isolated from the hot springs Manado, North Sulawesi has a molecular weight of 67 kDa and 102 kDa[11]. Based on these phenomena, it is indicated that the chitinase obtained from different sources can produce different chitinase enzymes.

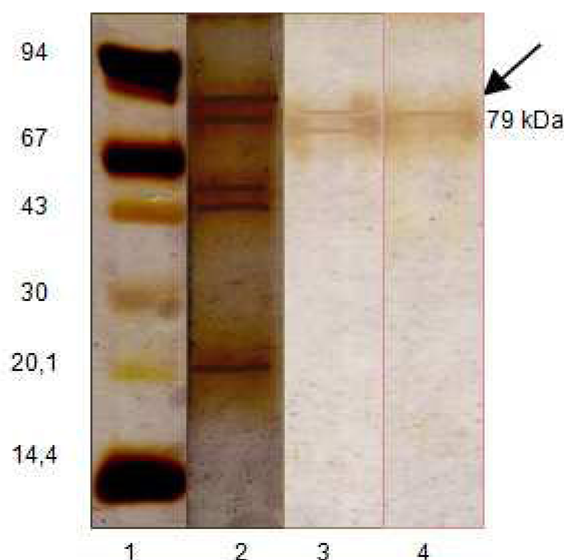

Figure 4
Molecular mass determination of chitinase (10% SDS- PAGE visualized by silver staining: lane-1 standar molecular marker proteins in kDa; lane-2, crude extract chitinase; lane-3, purified chitinase with butyl sepharose FF; lane-4, purified chitinase with sephadex G-75.

CONCLUSION

Pure chitinase (fraction 29) from *B. licheniformis* HSA3-1a obtained through the steps are 60-70% ammonium sulphate precipitation, dialysis, butyl sepharose FF, and sephadex G-75 has 33 fold the level of purity of enzyme crude extract, with an estimated molecular weight of 79 kDa.

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REFERENCES

1. Muzzarelli, R. A. A. and Peter, M. G. (1997), Atec. Grottamare Italy, 165-174, 305-311.
2. Patil, S. R., Ghormade, V., and Deshpande, M. V. (2000) *J. Enzyme and Microbiol. Tech.* 26, 473-483.
3. Ravikumar, V., and Meignanalakshmi, Sundaram, (2013), *Int. J. Pharm. Bio Sci.*, 4,2: 238 - 248.
4. Cohen-Kupiec, R. and Chet, I. (1998), *Curr Opin Biotechnol.,* 9, 270-277.
5. Sakai, K., Yokota, A., Kurokawa, H., Wakayama, M., and Moriguchi, M. (1998), *J. Appl. and Env. Microbiol.,* 9, 3397-3402.
6. Jholapara, R. J., Metha,R.S., and Sawant, Ch.S., (2013), *Int. J. Pharm. Bio Sci.,* 4,2: 464 - 471.
7. Gooday, G. W., (1990), *Adv. and Biotehcnol.,* 34, 715-719.
8. Jang, M. S., Lee, Y. M., Cho, Y. S., Choi. Y. L., Kim, C. H., and Lee, Y. C. (2005),

Indian J. Biochem. and Biophys. 42, 339-344.

9. Wen, C. M., Tseng, C. S., Chen, C. Y., and Li, Y. K., (2002), *J. Biotechnol. Appl. Biochem.,* 35, 219-321.
10. Yuli, E. P., Suhartono, M. T., Rukayadi, Y., Hwang, J. K., and Pyun, Y. R. (2004) *J. Enzym and Microbial. Technol.,* 13-26
11. Toharisman, A, Suhartono M T, Spindler, B. M., Hwang, J. K., and Pyun, Y. R. (2005), *World J. Microbiol. Biotechnol* . 5, 730-738.
12. Natsir, H., Chandra, D., Rukayadi, Y., Suhartono, M. T., Hwang, J. K., and Pyun, Y. R., (2002), *J. of Biochem. Molecular Biol. and Biophys.,* 4, 279-282.
13. Natsir, H., Patong, A. R., Suhartono, M. T., and Ahmad, A., (2010), *Indo. J, of Chem.,* 2, 256-260.
14. Bradford, M. M., (1976), *J. Anal. Biochem.*, 72, 248-254.
15. Harris, E. L. V., (1989), Protein Purification Methods, 123-161.
16. Scopes, R.K., 1987, Protein Purification Principles and Practice, Sec. Edit., Springger-Verlag, New York.
17. Bollag, D.M., and Edelstein, S.J., 1991, *Protein Methods*. New York.
18. Laemmli, U. K., (1970), Nature, 227, 680-685.
19. Rahayu, S., Tanuwijaya, F., Suhartono, M. T., Hwang, J. K, dan Pyun, Y. R., (2004), *J. Microbiol. And Biotech.,* 4, 647-652.