Asian Journal of Chemistry; Vol. 25, No. 3 (2013), 1687-1690

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.13822

Study on the Cellulase Components β-Endoglucanase Purification by Chromatography

XIANG NONG[1], HONG WU[2,*], CHEN-HAO XU[1], CHUN-LIN FANG[3], TIAN-XING LIN[1], ZI LIANG[1], YAN-YUN WANG, TIAN-YING QIN[2] and QIANG HU[4]

[1]College of Chemical and Life Science, Leshan Normal University, Le'shan 614000, P.R. China
[2]College of Life Science, Southwest University for Nationalities, Cheng'du 610041, P.R. China
[3]Chengdu Qiankun Veterinary Pharmaceutical Company, Cheng'du 611130, P.R. China
[4]Leshan Bureau of Quality and Technical Supervision, Le'shan, P.R. China

*Corresponding author: Tel: +86 131 11889729; E-mail: wuhong_0203@126.com

(*Received*: 3 April 2012; *Accepted*: 26 September 2012) AJC-12190

Two active components, EgI and EgII, of β-endoglucanase were purified by utilizing salting out method, as well as twice Sephadex G-75 and DEAE-Sephadex A-50 chromatography from the fermentation broth of *Trichoderma reesei* QM9414. The relative molecular weights of EgI and EgII were 65.47 and 57.04 kDa, respectively, as detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The optimal temperatures of EgI and EgII were 50 ºC and 55 ºC, respectively and the optimal pH levels were 4.8 and 5.0. The experiment on the effect of different metal ions showed that: K^+ could activate EgI and EgII activities and Fe^{3+}, Cu^{2+}, Zn^{2+}, Ca^{2+} and Mn^{2+} were the inhibitors of EgI and EgII. EgI and EgII with good thermal stability, in temperature range of 30-60 ºC with 0.5 h, EgI and EgII can maintain more than 78 % enzyme activity, EgI and EgII are heat-resistant. The K_m of EgI and EgII was detected using the Lineweaver-Burk plot method, which showed that their Michaelis constants values were 0.238 g/L and 0.266 g/L, yEgI = 0.6078x + 2.5558, yEgII = 0.6665x + 2.5046, respectively. EgI and EgII are new discoveries according to their characterization. Apparently, the present research built a foundation for the further study of β-endoglucanase in *T. reesei* QM9414.

Key Words: Purification, Characterization, Cellulase components, *Trichoderma reesei* QM9414.

INTRODUCTION

Cellulose, the major polysaccharide of plant photosynthesis, is the earth's most abundant renewable resources. Globally, a new cellulose can be obtained annually at approximately 4.0×10^{10} tons through photosynthesis, which is about 40 % of the earth's total biological resources[1]. Optimal utilization of this vast resource is an enigma. Utilizing cellulase hydrolysis is an efficient, pollution-free and an ideal method to break down cellulose[2-4].

The cellulase from the fermentation by different fungal has different properties. Nowadays the biochemical method is considered as the main methods in the separation and purification processes of cellulase components. These processes were divided into two categories by researchers. One is the less expensive tannins-polyethylene glycol(PEG) method and the other is the classical method, which includes salting out, gel filtration and ion exchange chromatography. Compared with the classical method, the tannin-PEG method is simpler with a higher yield. However, the separation of the cellulase enzyme complex into a single enzyme component using this method is quite difficult. Some scholars also examined other methods to study cellulase. Woodward *et al.*[5] used concanavalin A-agarose to separate β-glucosidase from *Aspergillus niger*. Bao *et al.*[6] screened and characterized cellulose with endocellulase and exocellulase activity from yak rumen metagenome. Whereas Watanbe *et al.*[7] purified β-glucosidase using chromatography. There were also other studies by Zhou *et al.*[8], Yin *et al.*[9] and Colussi *et al.*[10]. For evaluation the characterization of the β-endoglucanase from the fermentation of *T. reesei* QM9414, we purified the β-endoglucanase components, EgI and EgII utilizing the chromatography method.

Trichoderma reesei QM9414 was presented by the Chinese Academy of Sciences. And the procedure of ferment was performed according to the previous optimized conditions for the production crude enzyme in our laboratory.

EXPERIMENTAL

Determination of the activity of carboxymethyl cellulose Eg: Acetate buffer (0.5 mL) was added in a test tube and then a certain amount of diluted crude enzyme solution was added at 50 ºC for 0.5 h, DNS reagent (2 mL) was added and the solution was diluted with distilled water up to 10 mL at 540 nm metering density. Measurement of carboxymethyl cellulose

TABLE-1
PURIFICATION RESULT OF EgI AND EgII

Purification steps	Total volume (mL)	Total protein (μg)	Total activity (IU)	Specific activity (μ/mg)	Yield (%)	Fold
Crude cellulase solution	200	1967110.1	19080.82	9.7	100	1
80 % salting	4	666160.67	10830.42	16.26	56.76	1.68
First G-75	40	382800	8420.84	22.02	44.16	2.27
Second G-75	48	155160.22	6790.52	43.79	35.6	4.51
First DEAE	80	73890.33	4500.94	61.02	23.62	6.29
Second DEAE (EgI)	14	13430.11	1760.88	131.7	9.27	13.58
Second DEAE (EgII)	12	12800.22	1580.42	123.74	8.3	12.76

was according to the international units, defined as 1 mL enzyme per min solution, which yields 1 μmol glucose hydrolysis, *i.e.*, an enzyme activity unit in IU (μmol/min/mL)[11]. Protein content was detected using the Bradford methods[12].

Crude enzyme cellulase: Fermented cellulose with a small amount of acetate buffer extract was filtered through an 8-layer gauze and centrifuged at 5000 rpm for 10 min. The supernatant was stored.

Crude enzyme liquid ammonium sulfate salting: Crude enzyme solution (200 mL) was placed inside a clean 500 mL beaker. The solution was magnetically stirred. Next, a full dry 10.6 g ammonium sulfate was slowly added until the ammonium sulfate reached 20 % saturation. The solution was stored overnight at 4 ºC. Then, it was centrifuge at 5000 rpm for 5 min. Ammonium sulfate (51.6 g) was added to the supernatant until a saturation of 80 % was reached. The solution was placed inside a freezer at 4 ºC and chilled overnight. Then, it was centrifuged at 10000 rpm for 10 min with a 2 mL buffer dissolved by precipitation. The solution was placed in 3.5 kD dialysis bag overnight. Afterward, 10 % $BaCl_2$ was used until no white precipitate was formed.

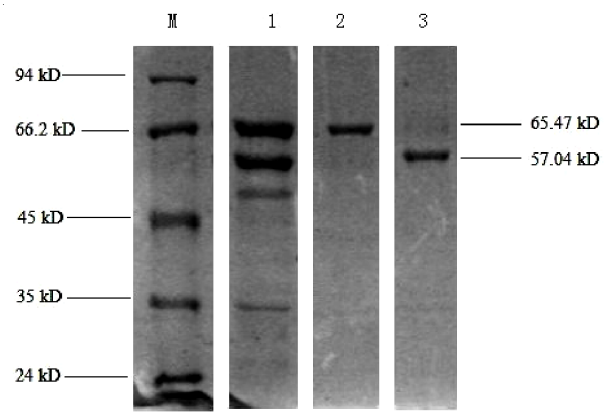
Sephadex G-75 chromatography: A 6 g Sephadex G-75 dry rubber dissolved in 200 mL of distilled water at room temperature after standing overnight. The clear water was suctioned, then, 0.2 mol/L NaOH was slowly added under stirring for 10 min. The gel was washed with water, repeated thrice. The gel was placed into a 1.7 × 40 cm gel column using pH 4.8 acetate buffer balance columns. Then, 4 mL enzyme samples were loaded into the columns for elution with a flow rate of 1 mL/min. Each tube collected 2 mL samples.

DEAE-Sephadex A50 ion exchange chromatography: A 4 g DEAE-Sephadex A50 rubber was dissolved in 200 mL distilled water, soaked overnight, mixed with 0.2 mL HCl for 0.5 h and then washed repeatedly with distilled water. Acetate buffer balance columns (pH 5.8) were prepared and 2 mL samples mixed with 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 M NaCl were loaded for the elution stage, at 0.5 h for each concentration elution. The flow rate was 1 mL/min and each tube to collected 2 mL samples. The second DEAE A-50 ion exchange chromatography eluted salt concentration was 0.25 M. Proteins were collected after each elution peak, with concentrated PEG20000.

Enzymatic properties of EgI and EgII: Utilizing the Lineweaver-Burk plot method, the K_m of EgI and EgII were detected. The optimal pH was determined by 0.1 M acetate buffer. EgI and EgII were incubated at different temperatures at a certain time. Then, the activity were measured at different pH levels to detect the optimal temperature and pH of the two components. The experiment detected the activator and inhibitor metal ions for EgI and EgII.

RESULTS AND DISCUSSION

Purification of EgI and EgII: After utilizing the methods of Sephadex G-75 and DEAE-Sephadex A-50 chromatography, we isolated the single composition of EgI and EgII. Then the samples from the previous purification steps were stored for further testing, SDS-PAGE detected the molecular of EgI and EgII by 5 % stacking gel and 12 % separation gel, when the sample was tested into the stacking gel with a voltage intensity was of 60 V and after the sample was examined into the separation gel pressure at 100 V, the results were showed in Fig. 1.



Note: Lane 1 was marker, Lane 2 was the crude enzyme after liquid ammonium sulfate salting, Lane 3 and 4 were the EgI and EgII after purification

Fig. 1. SDS-PAGE detection of the molecular EgI and EgII

Purification of EgI and EgII: Purified EgI and EgII were obtained from the 200 mL crude cellulase solution with a total activity of 19080.82 IU. The specific activities were 131.70 and 123.74, respectively, while the purification fold was 13.58 and 12.76, respectively (Table-1).

Optimal temperature of EgI and EgII: The setting temperatures of the water pot were regulated at 40, 45, 50, 55 and 60 ºC, respectively. The carboxymethyl cellulose (CMC) substrate concentration was 1 %, which required 30 min to react (Fig. 2).

The optimal temperature of EgI is 50 ºC, whereas that of EgII is 55 ºC. The two enzymes components have high activities between 50 and 55 ºC.

Optimal pH of EgI and EgII: Buffers with various pH levels, namely, 4, 4.5, 4.8, 5.0, 5.2 and 5.6, were used to configure 1 % substrate. The EgI reaction temperature was 50 ºC, whereas that of EgII was 55 ºC, with the other reaction

conditions. From the reaction shown in Fig. 3. EgI required an acidic pH, with an optimal pH of approximately 4.8, whereas that of EgII is approximately 5.0. Both components exhibited high, activities when the pH was between 4.8 and 5.6.

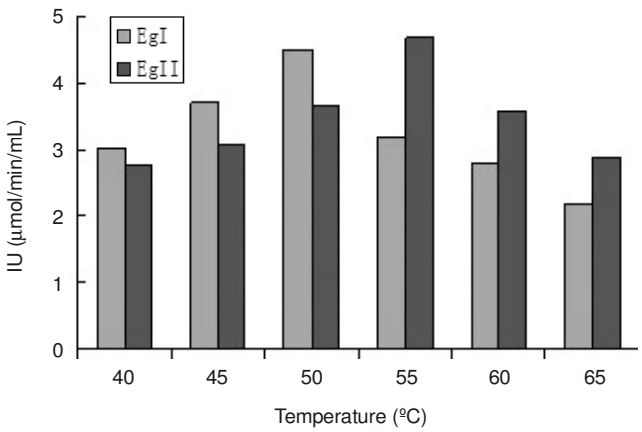


Fig. 2. Comparison of the optimal temperature of EgI and EgII

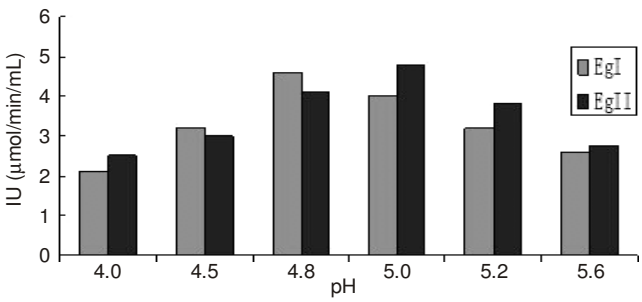


Fig. 3. Comparison of the optimal pH of EgI and EgII

Influence of metal ions on EgI and EgII activities: The results of the experiment which metal ions influence of the activities of EgI and EgII showed that K^+ could activate the activities of EgI and EgII and that, Fe^{3+}, Cu^{2+}, Zn^{2+}, Ca^{2+} and Mn^{2+} could inhibit the activities of EgI and EgII (Table-2).

TABLE-2
EFFECT OF DIFFERENT METAL IONS ON EgI AND EgII ACTIVITIES

Metal ions	EgI	EgII
	Related cellulase activity (%)	
K^+	110	109
Fe^{3+}	21	23
Cu^{2+}	25	22
Zn^{2+}	57	61
Ca^2	81	78
Mn^{2+}	29	31

Thermal stability of EgI and EgII: The results showed that, EgI and EgII with good thermal stability (Table-3). In 30-60 ºC temperature range with 0.5 h, EgI and EgII can maintain more than 78 % enzyme activity, as the temperature rises to 80 ºC, the relative enzyme activity is very low. Therefore, EgI and EgII are heat-resistant, shows a good application prospect in the production.

Michaelis constants value of EgI and EgII: The K_m of EgI and EgII were determined using the Lineweaver-Burk plot method, wherein the substrate was a different concentration of carboxymethyl cellulose. The results showed that their Michaelis constants value were 0.238 g/L and 0.266 g/L, yEgI = 0.6078x + 2.5558, yEgII = 0.6665x + 2.5046 respectively. The double reciprocal curve is shown in Fig. 4.

From Fig. 4, the K_m of EgI was less than the K_m of EgII, indicating that the substrate affinity of EgI is larger than EgII.

TABLE-3
THERMAL STABILITY OF EgI AND EgII

Temperature (ºC)	EgI	EgII
	Related cellulase activity (%)	
30	100	100
40	100	100
50	100	100
60	78	88
70	40	43
80	10	8
90	0	0
100	0	0

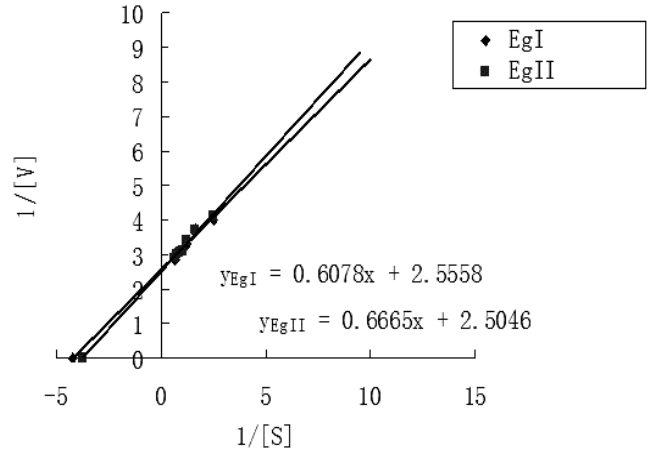
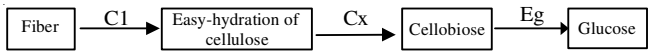


Fig. 4. Comparison of the K_m of EgI and EgII

Cellulase is composed of numerous highly synergistic hydrolytic enzymes, which can be divided into three main components[13]. First is the exo-glucanase (EC 3.2.1.91), also called C1 enzyme, 1.4-β-D-glucan cellobiohydrolase or exo-1,4-β-D-glucanase, which comes from a fungus. This component is also known as CBH. Second is endo-glucanase (EC 3.2.1.4, Endoglucanase), also termed as C_x enzyme, 1,4-β-D-glucan giucanohydrolase or end-1,4-β-D-glucanase, which also comes from a fungus. This component is also known as Eg. Last is β-glucanase (EC 3.2.1.21), also called cellobiase and β-1,4-glucosidase, also known as Bg. The C1 enzyme mainly damages the crystalline structure and hydrates the cellulose. This enzyme acts on the surface of insoluble cellulose. The cellulose crystal chain cracks, leading to the release and exposure of the terminal portion of long-chain cellulose molecules resulting in the easy hydration of the cellulose. The C_x enzyme is adsorbed above the cellulose molecules, cut at any position within the β-1, 4-glycosidic bonds of the cellulose molecules break and fiber for the three-cellobiose. Finally, β-glucanase decomposes the cellobiose and other low molecular weight cellotriose fiber dextrin[14-18]. The synergy of the three enzymes is as follows:



Using the anion exchange media as an example, the classical ion exchange reaction is as follows:

$$Exch^+ + X^- \Leftrightarrow Exch^+ + X^-$$

$$Exch^+ + A^- \Leftrightarrow Exch^+A^-$$

$$Exch^+ + B^- \Leftrightarrow Exch^+B^-$$

There $Exch^+$ is the anion exchange medium and X^-, B^- and A^- are representative anions. Based on the formula, X^-, B^-, A^- and other anions exhibit a competitive adsorption relationship. Considering that this absorption depends on the coulomb electrostatic adsorption, the anion and ion-exchange media $Exch^+$ adsorption strength depends on their volume. Thus the larger the density of the ion exchange, the more solid the exchange media and the more difficult the ion exchange.

During ion exchange, the adsorption performances of the properties for target protein adsorption depend mainly on mass transfer rate, adsorption dynamics, axial diffusion and effects of the different factors in ion exchange chromatography, which include buffer, pH, gradient elution mode, column height to diameter ratio and temperature among others.

In the present study, we used gel filtration and ion exchange chromatography to purify the cellulase components of EgI and EgII. The result is similar to the study by. Olama *et al.*[19], Charara *et al.*[20]. There are several drawbacks in both single method, gel filtration is inefficient in separating proteins with similar molecular weight. Ion-exchange chromatography is impaired when the pH and polarity of some complex proteins have very little difference from the separation protein. Therefore, during the process of separation and purification of the cellulase, both methods are usually utilized simultaneously. In the result of the study, EgI and EgII are new discoveries according to their characterization. Apparently, the present research built a foundation for the further study of β-endoglucanase in *T. reesei* QM9414.

ACKNOWLEDGEMENTS

The authors thank Prof. D.Z. Y of Chinese Academy Sciences for his assistance and for providing the *Trichoderma reesei* QM9414.

REFERENCES

1. P. Tomme, A.J. Warren and N.R. Gilkes, *Microb. Physiol*., **37**, 1 (1995).
2. D.J.T. Hill and T.T. Le, *Polym. Degrad. Stab*., **51**, 211 (1996).
3. C.Y. Wang, Y.R. Hsieh, C.C. Ng, H. Chan, H.T. Lin, W.S. Tzeng and Y.T. Shyu, *Enzyme Microb. Technol.*, **44**, 373 (2009).
4. A.M. de Castro, M.C. Ferreira, J.C. da Cruz, K.C.N.R. Pedro, D.F. Carvalho, S.G.F. Leite and N. Pereira Jr., *Enzyme Res.*, Article ID 854526 (2010).
5. J. Woodward, H.J. Marquess and C.S. Picker, *Prep. Biochem.*, **16**, 337 (1986).
6. L. Bao, Q. Huang, L. Chang, J.G. Zhou and H. Lu, *J. Mol. Catal. B*, **73**, 104 (2011).
7. T. Watanbe, T. Sato, S. Yoshioka, T. Koshijima and M. Kuwahara, *Eur. J. Biochem.*, **209**, 651 (1992).
8. J. Zhou, Y.-H. Wang, J. Chu, Y.-P. Zhuang, S.-L. Zhang and P. Yin, *Bioresour. Technol.*, **99**, 6826 (2008).
9. L.J. Yin, H.H. Lin and Z.R. Xiao, *J. Marine Sci. Technol. Taiwan*, **18**, 466 (2010).
10. F. Colussi, L.C. Textor, V. Serpa, R.N. Maeda, N. Pereira Jr and I. Polikarpov, *Acta Crystallogr.*, **F66**, 1041 (2010).
11. X.Y. Ye and K.J. Cheng, *Int. J. Biochem. Cell Biol.*, **33**, 87 (2001).
12. M.M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
13. A.M. Scrivener and M. Slaytor, *Molec. Biol*., **24**, 223 (1994).
14. H. Chanzy, B. Henrissat, R. Vuong and M. Schuelein, *FEBS Lett.*, **153**, 113 (1983).
15. D.C. Irwin, M. Spezio, L.P. Walker and D.B. Wilson, *Biotech. Bioeng.*, **42**, 1002 (1993).
16. M. Linder and T.T. Teeri, *Proc. Nat. Acad. Sci. USA*, **93**, 251 (1996).
17. Y.-J. Lee, B.-K. Kim, B.-H. Lee, K.-I. Jo, N.-K. Lee, C.-H. Chung, Y.-C. Lee and J.-W. Lee, *Bioresour. Technol.*, **99**, 378 (2008).
18. V.T.O. Santos, P.J. Esteves, A.M.F. Milagres and W. Carvalho, *J. Ind. Microbiol. Biotechnol.*, **38**, 1089 (2011).
19. Z.A. Olama, M.A. Hamza, M.M. El-Sayed and M. Abdel-Fattah, *Food Chem.*, **47**, 221 (1993).
20. C. Chararas, R. Eberhard, J.E. Courtois and F. Petek, *Insect. Biochem.*, **13**, 213 (1983).