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Full Length Research Paper

Chemical modification of *β*-endoglucanase from *Trichoderma viridin* by methanol and determination of the catalytic functional groups

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***β*-Endoglucanase from *Trichoderma viride* was modified by methanol to explore the catalytic functional groups of cellulase. Crude cellulase was produced, and the conditions of saturation and pH by salting out with ammonium sulfate were optimized. Under optimal conditions, crude cellulase was isolated and purified. The pure cellulase was modified by excess methanol, and it was found that the carboxyl in the side-chain radical of cellulase proved to be the catalytic functional group by analysis of the infrared spectrum of modification of cellulase. Modification of cellulase with different concentrations of methanol was carried out and results show that the modified side-chain radical lies at the active site of cellulase or on essential groups. Sodium carboxymethyl cellulose (CMC-Na) which protected the cellulase from inactivation by methanol indicated that the carboxyl modified by methanol is not only a structural radical but also lies at the active site. The inactivation degree of cellulase modified by methanol could be decreased by glucose and it showed that the modification occurred in the active site of cellulase. The kinetic analysis according to Keech and Farrant equation demonstrated that one carboxyl was an essential group for cellulase activity.**

Key words: Chemical modification, cellulase, methanol, carboxyl.

INTRODUCTION

Cellulose, both the most widely distributed and the most abundant of carbohydrate forms in nature, represents 35 to 50% of biomaterials in the world (Ragauskas et al., 2006). It is also the largest number of the renewable substance for humans; and its degradation is the key link of carbon recycles in nature (Wang et al., 2004). The use of cellulases in cellulose' hydrolysis is an ideal way of high efficient and non-pollution, which can make large cellulose resources into the required material of humans (Wu, 2008).

Cellulases are enzymes that hydrolyze cellulose (beta-1,4-glucan or beta D-glucosidic linkages) resulting in the formation of glucose, cellobiose, cellooligosaccharides, and the like (Su et al., 2010). Cellulases have been traditionally divided into three major classes: endo-glucanases (EC 3.2.1.4) ("EG"), exoglucanases or cellobiohydrolases (EC 3.2.1.91) ("CBH") and beta-glucosidases (EC 3.2.1.21) ("BG") (Lynd et al., 2002; Sinegania and Hosseinpour, 2006). The complete cellulase system needs different types of these various cellulases, by which the enzymatic process to hydrolyze cellulosic materials could be accomplished. Now, cellulases have a great application prospect in many fields, such as medicine, textile, chemical engineering, paper engineering, food and fermentation industries, commercial detergent, tobacco, waste water treatment, feed additive etc (Howard et al., 2003; Su et al., 2010). The application of cellulase has very important significance in solving the problems of the raw materials of industry and agriculture, the energy crisis, and the environmental pollution and so on.

Unfortunately, cellulases, like all other enzymes, become unstable and lose activity in storage or catalytic process, due to the effect of external conditions. Therefore, scientists focus more on the stability of cellulase.

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There are several major and principally different routes to obtain cellulases with improved stability properties to date: (1) by selection from organisms living in appropriate extreme environments (Kashima et al., 2005; Huang et al., 2005; Kubartova et al., 2007), (2) by gene recombination and genetic engineering (Valenzuela et al., 2006; Feng et al., 2007; Kim et al., 2007; Brune, 2007; Schmidt, 2007; Liu et al., 2006), (3) by immobilization (Wu et al., 2005; Sinegania et al., 2005; Dong et al., 2008), and (4) by chemical modification (Kwinam et al., 2002; Yang et al., 2009). The main strategies of aforementioned ways are based on changing the cellulases' molecule structure or living environments. So, further study on the amino acid composition at the active site of cellulase and the catalytic functional groups of cellulase could provide scientific basis research on the stability of cellulase. In the past decade, great progress has been made on the research of the amino acid composition at the active site of cellulase, which confirmed that they were mostly tryptophan, glutamic acid and aspartic acid (Liu and Xia, 2008). However, the research of the catalytic functional groups of cellulase by methanol modification has not been reported in detail to date.

Chemical modification, especially by small molecular chemistry, has an important role in determining the catalytic functional groups of enzyme (Yan and Gao, 1997; Ackers and Smith, 1985). It is the process of covalent attachment of special group of modifiers to the side-chain group of certain amino-acid residue in enzyme. In this paper, chemical modification by using small organic molecular methanol as modifier was carried out to explore the catalytic functional groups of the cellulase from *Trichoderma viride*. The main objective of this present study was to verify the relation between the activity of cellulase and the catalytic functional groups. This, to our best knowledge, is the first report on cellulase modification by using methanol as the modifier, which may provide a theoretical reference for better understanding of the catalysis mechanism of cellulase.

MATERIALS AND METHODS

Strain, media and chemicals

T. viride used to produce *β*-endoglucanase was provided from the institute of microbiology, Chinese academy of sciences.

Nutrient solution of $(NH_4)_3P0_4$ contained (w/w): $(NH_4)_3P0_4$, 2.06%; $MgS0_4 \cdot 7H_20$, 0.05%; KH_2P0_4, 0.01%. Citrate buffer at pH of 4.0, 50 and 6.0 correspondingly contained (mol/l): 0.1 mol/l citric acid, 13.1, 8.2 and 3.8 ml; sodium citrate, 6.9, 11.8 and 16.2 ml. Solid fermentation medium contained: rice straw powder, 4.0 g; wheat bran, 1.0 g; nutrient solution of $(NH_4)_3P0_4$, 12.5 ml. 3,5-Dinitrosalicylic acid (DNS) solution contained (g/l): potassium-sodium tartrate, 200 g; DNS, 10.0 g; sodium hydroxide, 10.0 g; phenol, 2.0 g; sodium sulfite, 0.5 g. Disodium hydrogen phosphate (pH 5.0) contained (g/l): $Na_2HP0_4 \cdot 12H_20$, 1.4755 g; citric acid, 0.4076 g. Sodium carboxymethyl cellulose (CMC-Na) solution contained (g/l): CMC-Na, 6.25 g; disodium hydrogen phosphate (pH 5.0), 31.25 ml. Chemicals of the mentioned solution or medium and other chemicals were of analytical grade and purchased from Shanghai Sangon Co. Ltd, PR China. The Sephadex G-100 column and DEAE-Sepharose-FF anion exchange chromatography were purchased from Amersham Pharmacia.

Production of crude cellulase

The *T. viride* strains preserved in a 4°C were incubated in an Erlenmeyer flask containing 20 ml nutrient solution of $(NH_4)_3P0_4$ and glass beads. The culture was grown at 30°C with vigorous shaking (200 rev/min) for 1 h and then 2.5 ml suspension was mixed into the solid fermentation medium equably. The mixture used to produce crude cellulase was then incubated at 30°C for 120 h. After solid state fermentation, 100 ml citrate buffer at pH of 4.0, 50 and 6.0 was added to each mixture, and incubated at 30°C for 1 h. The fermentation liquor was filtrated with two layers of sterile gauze and centrifuged at 8500 rev/min for 30 min at 4°C. The fermentation supernatant of crude cellulase was collected, respectively at the end.

Assay of cellulase activity

Cellulase activity was measured by the DNS method (Miller et al., 1960), through the determination of the amount of reducing sugars liberated from CMC solubilized in 50 mM Tris-HCl buffer, at pH 8.0. Briefly, this mixture was incubated for 20 min at 50°C and the reaction was stopped by the addition of DNS solution. The treated samples were boiled for 10 min, cooled in water for color stabilization, and the optical density was measured at 550 nm (Lee et al., 2008). The cellulase activity was determined by using a calibration curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute.

Optimization of conditions of salting out with aluminum sulfate

To determine the optimum saturation degree and pH of salting out with ammonium sulfate, 7 crude cellulase of 15 ml each at pH of 4.0 was salted out, respectively by relative saturation of ammonium sulfate at 20, 30, 40, 50, 60, 70 and 80%, left at 4°C for 12 h, and each centrifuged at 8500 rev/min for 20 min at 4°C. The supernatant and precipitate were collected from each mixture, respectively, and the precipitate of each was dissolved with the corresponding pH buffer to the original volume. The content of protein of each was determined by measuring the ultraviolet (UV) absorbance at 280 nm, and the activity of cellulase was measured as previously described. Crude cellulase at pH of 5.0 and 6.0 were processed as described at pH of 4.0. The precipitation curve of enzyme with ammonium sulfate was prepared to determine the optimum of saturation interval X1~X2% and pH of ammonium sulfate.

Production and purification of cellulase

$(NH_4)_2S0_4$ was added into the crude cellulase solutions to X1% saturation with slow stirring. The reaction mixture was at 4°C for 12 h, and centrifuged at 8500 rev/min for 20 min at 4°C. The precipitate was removed and the supernatant was brought to 90% saturation with $(NH_4)_2SO_4$, left at 4°C for 12 h, and centrifuged. The precipitate was dissolved in citric acid buffer solution (pH 6.0), and then was passed through a Sephadex G-100 column (2.2 $\times$70 cm) at a flow rate of 1.0 ml/min. The fractions collected were filtered through a 0.22 µm membrane filter, and those desalted enzyme were subjected to 1 ml DEAE-Sepharose-FF anion exchange

chromatography to remove large amounts of acidic protein. The results of separation were recorded by using a fast protein liquid chromatography system (FPLC). Active fractions were collected, combined and stored at 4°C. The purity and relative molecular mass of the purified *β*-endoglucanase were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of side-chain radical modified by methanol

The modification of pure cellulase was carried out by adding excess methanol and adequate hydrochloric acid (0.02~0.1 mol/l) that the reaction required (Zhou and Wang, 1998). The reaction mixture was at 20°C for a few hours, and made into solid by evaporation of low temperature. The Fourier transform infrared (FTIR) spectra of cellulase modified and unmodified were gotten by using Bruker EQUINOX 55 remote sensing FTIR spectrometer and OPUS/IR software control system. The side-chain radical of cellulase modified by methanol was confirmed by analysis of these spectra (Zhu, 2000).

Effect of methanol on cellulase activity

To evaluate the effect of methanol on cellulase activity, methanol with the definite volume of different concentrations was added into the reaction system containing CMC-Na (0.625 g/l, pH 4.8). Deionized water was chosen as control instead of methanol, and its activity was defined as 100%. The activity of each reaction mixture was measured as previously described.

To analyze the modification degree of methanol on cellulase activity, modification of cellulase was carried out with ratios of methanol to cellulase solution of (v/v): 0.2:0.5, 0.6:0.5 and 1.0:0.5, respectively. Deionized water was also chosen as control instead of methanol, and its activity was defined as 100%.

Effect of substrate on modification

To analyze the effect of substrate on modification, methanol with a ratio of methanol to CMC-Na solution of 1:1(v/v), was added to CMC-Na solution (0.625 g/l) with different concentrations, and cellulase solution was added subsequently. The activity of cellulase was measured after the system had been reacted for 30 min at 50°C. Deionized water was chosen as control instead of methanol and CMC-Na, and its activity was defined as 100%.

Effect of glucose on modification

To analyze the effect of glucose on modification, glucose solution with the definite volume of different concentrations was added to the cellulase solution modified by methanol. The activity of cellulase was measured after the system had been reacted for 30 min at 50°C. Deionized water was chosen as control instead of methanol and glucose, and its activity was defined as 100%.

Analysis of time process of methanol modification

The relative activity of cellulase at different times was measured, respectively from the reaction mixture with a ratio of methanol to cellulase solution of 1/1 (v/v). Deionized water was chosen as control instead of methanol, and its activity was defined as 100%. The time process curve was plotted and $t_{1/2}$ (the time required for 50% decrease in activity) was calculated. The linear equation was established by the curve plotted with logarithmic of activity against time, and the K_I (the deactivation rate constant) was calculated.

Time process curves were plotted, respectively from different reaction mixtures with ratios of methanol to cellulase solution of 0.2:0.5, 0.35:0.5, 0.5:0.5, 0.65:0.5 and 0.80:0.5(v/v). Values of $t_{1/2}$ were calculated from different time process curves. The linear regression equation was established by the standard curve plotted with logarithmic of $1/t_{1/2}$ against logarithmic of concentration of methanol, and values of n (apparent molecule number of inhibiter in the formation of the enzyme-inhibiter complex of inactivation) and k' (apparent first-order inactivation rate constant) were calculated subsequently.

RESULTS AND DISCUSSION

Optimization of saturation degree and pH by salting out with ammonium sulfate

Effect of saturation degree of ammonium sulfate on the activity of cellulase is shown in Figure 1. The activity of cellulase salted out by ammonium sulfate increased rapidly in the 40 to 60% ammonium sulfate saturation range, while the activity in other range remained nearly constant. Changes of the activity of pH 4.0 were similar to that of pH 5.0, and the activity of cellulase over the saturation range of 40% of pH 6.0 were slightly higher than those of pH 4.0 and pH 5.0.

Effect of saturation degree of ammonium sulfate on the content of protein is shown in Figure 2, which showed that small amounts of protein were precipitated at the 40% salt saturation. However, protein precipitated at the saturation of 40% was mostly impurity because the activity of cellulose was very low (Figure 1). Therefore, when proteins precipitated at the saturation of 40% were removed, cellulase could be isolated from the contamination proteins to a great extent. As shown in Figures 1 and 2, although large amounts of protein were salted out when the saturation increased from 60 to 80%, protein precipitated over the saturation range of 60% was mostly impurity because the content of protein and the activity of cellulase remained nearly constant. Then, these results indicate that the optimum process parameters for the precipitation and activity of cellulase salted out by ammonium sulfate were the saturation range from 40 to 60%, and a pH of 6.0.

Isolation and purification of cellulase

After optimization, crude cellulase was isolated and purified according to the described method. Results after each purification stage are as shown in Table 1. The cellulase attained 8.1 fold purification and 7.5% recovery through a series of purification stages: precipitation with ammonium sulphate, Sephadex G-100 gel filtration, and DEAE-Sepharose-FF anion exchange chromatography. The recovery observed in this study is higher than those reported by Saha (2004) and Yang et al., (2004). SDS-PAGE of the purified cellulase demonstrated one band with a mobility corresponding to a molecular weight of 26.4 kDa (Figure 3), which showed the separation and

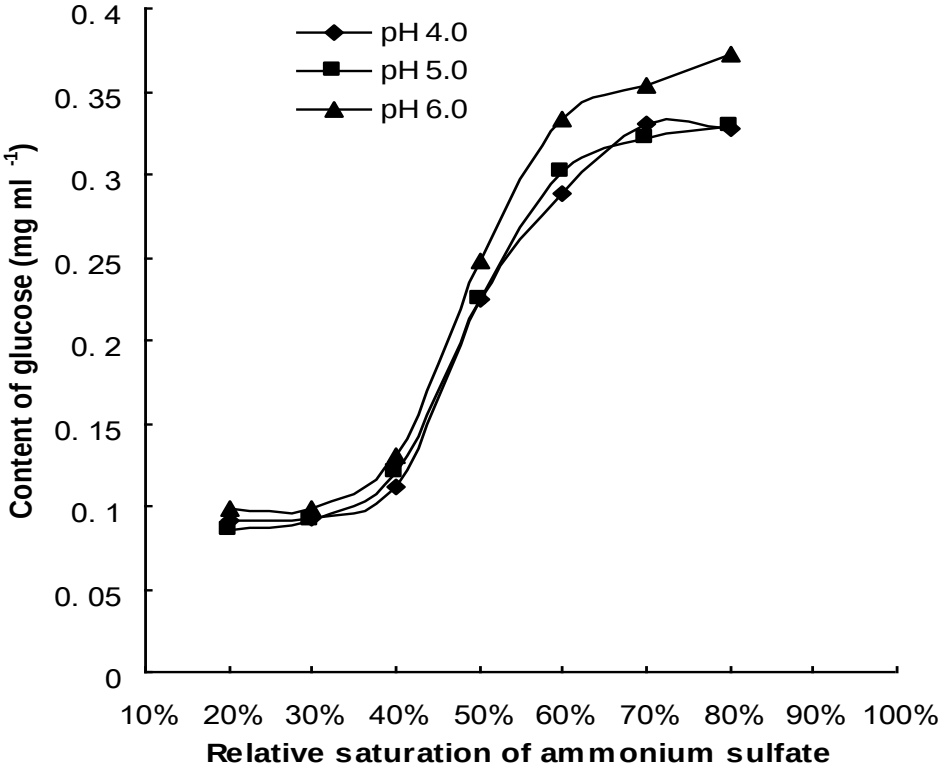

Figure 1. Effect of saturation degree of ammonium sulfate on the activity of cellulase salted out by ammonium sulfate.

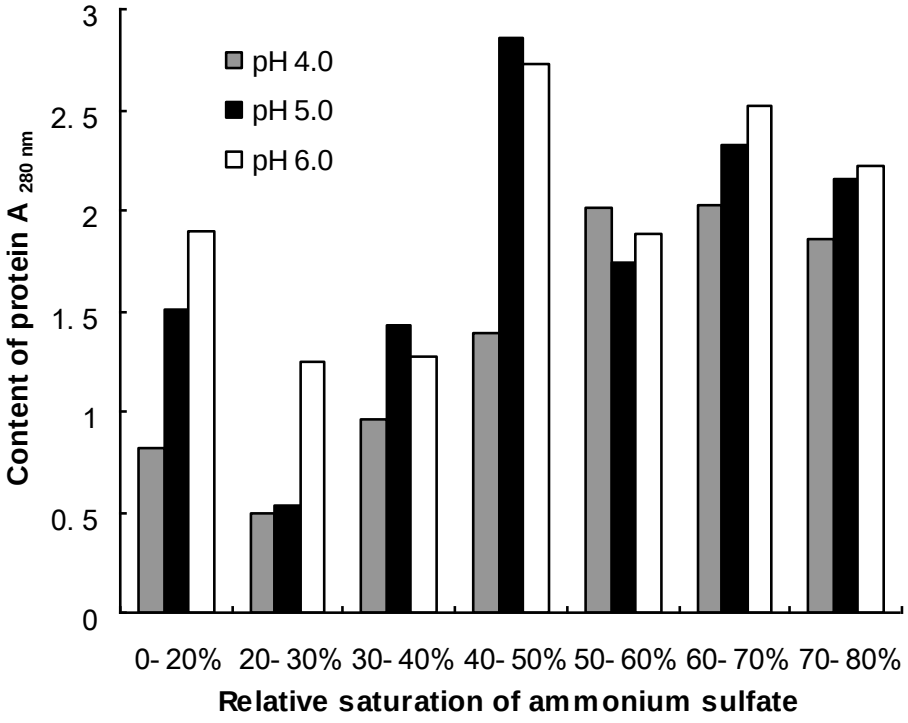

Figure 2. Effect of saturation degree of ammonium sulfate on the content of protein salted out by ammonium sulfate.

Table 1. Isolation and purification of the β-endoglucanase.

Purification stage	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification - fold	Recovery (%)
Crude cellulase	571.4	3869.6	6.7	1	100
Ammonium sulphate	195.2	1805.4	9.2	1.4	46.9
Sephadex G100	57.8	1238.2	21.4	3.2	32.2
DEAE FF	5.4	288.6	53.4	8.1	7.5

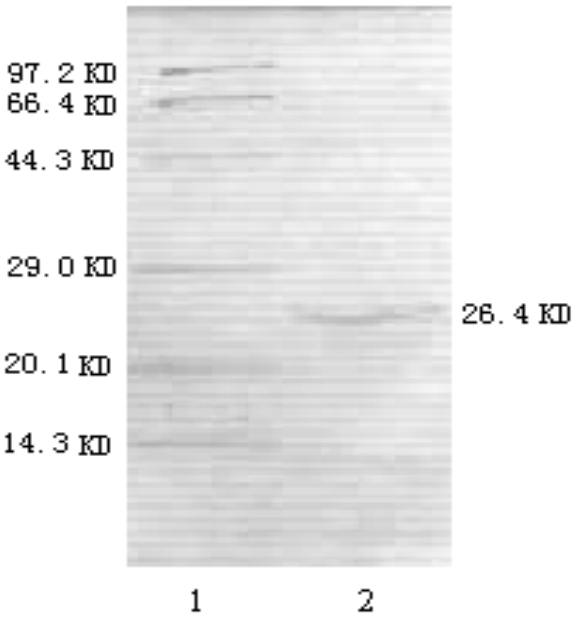

Figure 3. SDS-PAGE analysis of the purification and molecular weight of β-endoglucanase. Lane 1, protein markers; lane 2, the purified β-endoglucanase.

purification reached to the electrophoretically pure samples.

Confirmation of the side-chain radical modified

The modification of pure cellulase by excess methanol was studied to determine the functional groups of cellulase. Information about the side-chain radical of modified and unmodified cellulase was confirmed by analysis of infrared spectra as shown in Figures 4 and 5. The absorption peak at 1653 cm^{-1} in Figure 5 clearly shows the existence of C = O, which had a little blue shift compared to C=O at the peak of 1625 cm^{-1} in Figure 4. In Figure 5, strong absorption peak at 1020 cm^{-1} within the fingerprint region was caused by the ester C-O stretching vibration. It is considered that the absorption peaks at 2840 and 2950 cm^{-1}, along with the double peaks near 1430 cm^{-1} in Figure 5 corresponded to CH_3 ligand. In Figure 4, the broad and intense band at 3700~3000 cm^{-1} covered over the OH ligand. Therefore, these analyses of infrared spectra confirmed that the carboxyl in the side-chain radical of cellulase was modified by methanol (Zhu, 2000) (Figure 6).

Effect of methanol on the activity of cellulase

Modification of cellulase with different concentrations of methanol is shown in Figure 7. The relative enzyme activity of cellulase modified by methanol decreased almost linearly with the increasing addition of methanol concentration. Curves plotted with relative enzyme activity against reaction time are shown in Figure 8. It can be seen that the relative activity of cellulase modified by a certain concentration of methanol decreased with the increase of reaction time, while the relative activity of cellulase at a certain reaction time decreased with the increase of methanol concentrations. These results show that the activity of cellulase was linear with modification-degree by methanol, which preliminary conformed that the modified side-chain radical by methanol lies at the active site of cellulase or on essential groups (Tao et al., 1995).

Generally speaking, when the activity of a post-modification enzyme did not show significant changes, the radical of modification is definitely not in the active site. But, if a post-modification enzyme lost its activity to a great extent, there may be two possibilities: one is at the active site of cellulase, and the other is on essential groups. So, further experiments need to be done to determine whether the modification occurs at the active site of the enzyme or essential groups.

Protective action of substrate

Effect of substrate on cellulase modification is shown in Figure 9. As concentration of CMC increased, the relative activity of cellulase was increased correspondingly. The relative activity was 95% when the concentration of CMC was 0.5 g/ml; while it dropped to 68% when the concentration decreased to 0.1 g/ml. The phenomena indicate that the substrate had the function of protection on cellulase, and the protective action increased with the increase of the concentration of substrate.

From the results, the inactivation of cellulase modified by methanol can be reduced by increasing the concentration of substrate. Hence, it can be assumed that the carboxyl modified by methanol is not only a structural

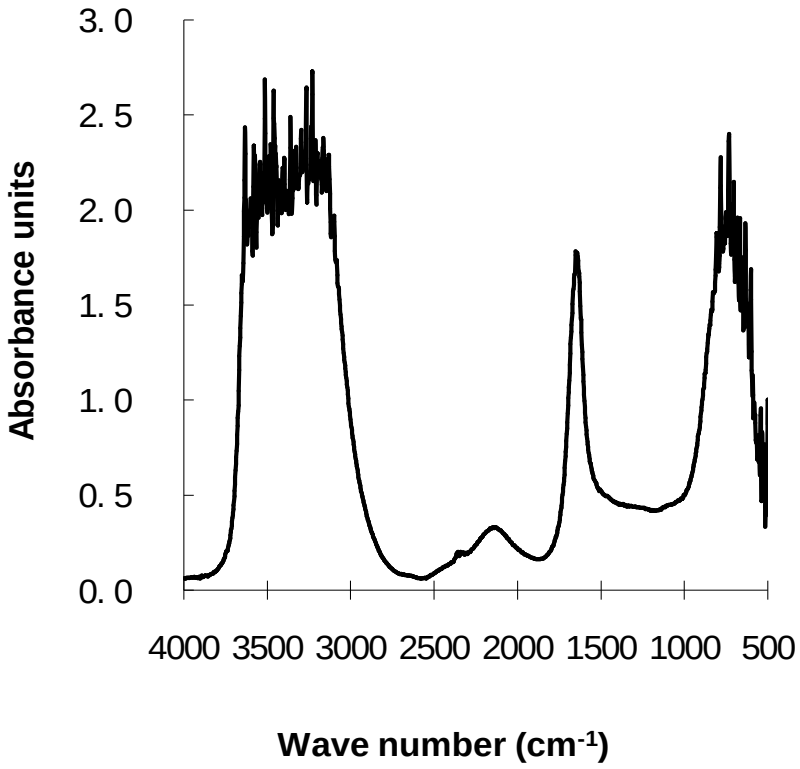

Figure 4. FTIR spectrum of cellulase unmodified.

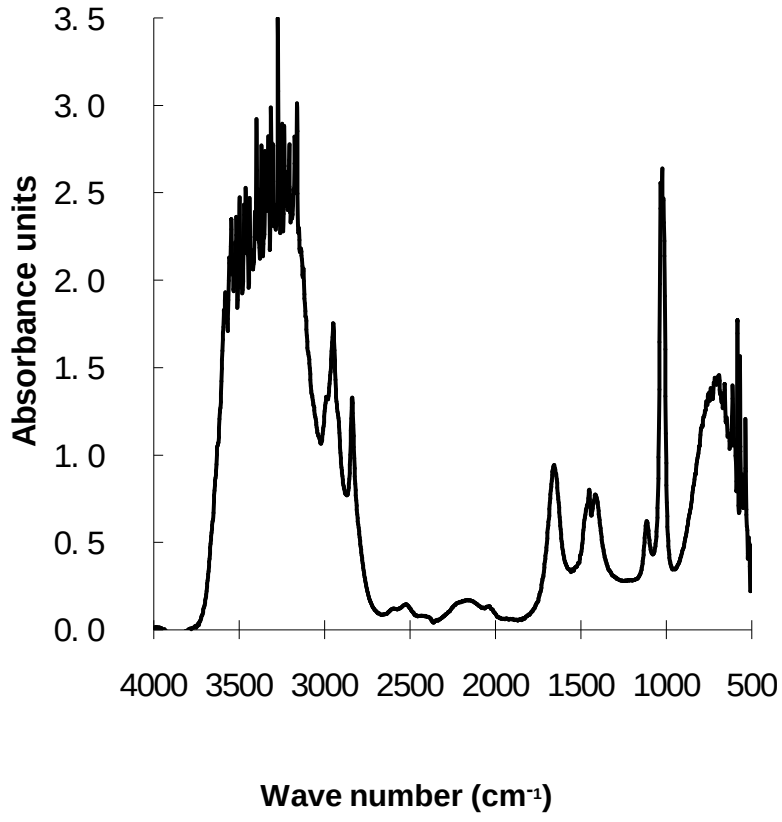

Figure 5. FTIR spectrum of cellulase modified by excess methanol.

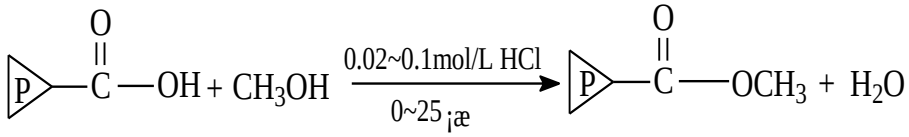

Figure 6. The carboxyl in the side-chain radical of cellulase modified by methanol.

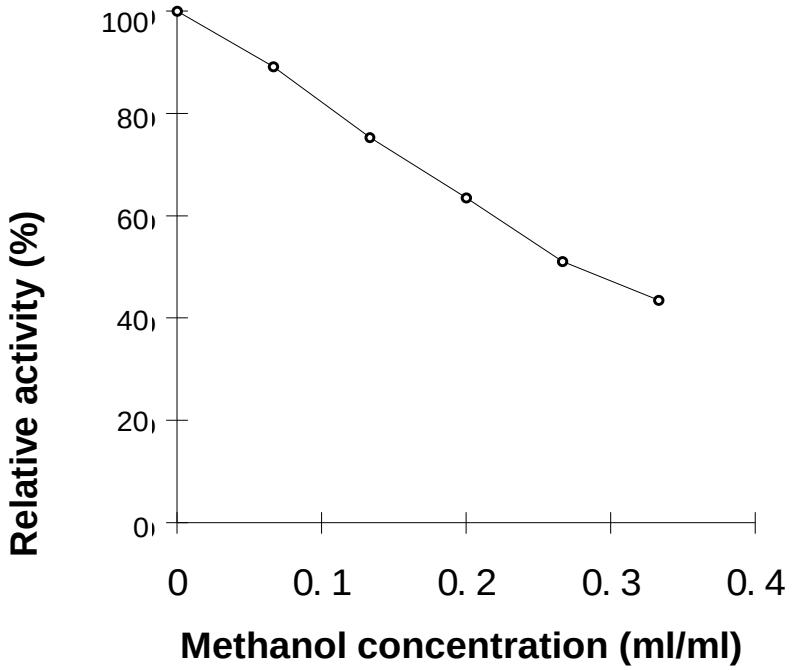

Figure 7. Modification of cellulase with different concentrations of methanol. The activity of cellulase modified by deionized water as control was defined as 100%.

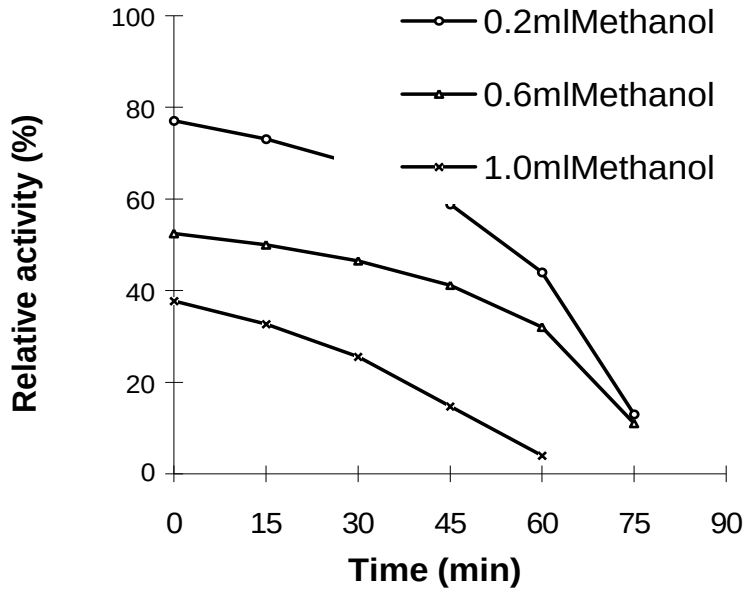

Figure 8. The relative activity of cellulase modified by methanol at different times. The activity of cellulase modified by deionized water as control was defined as 100%.

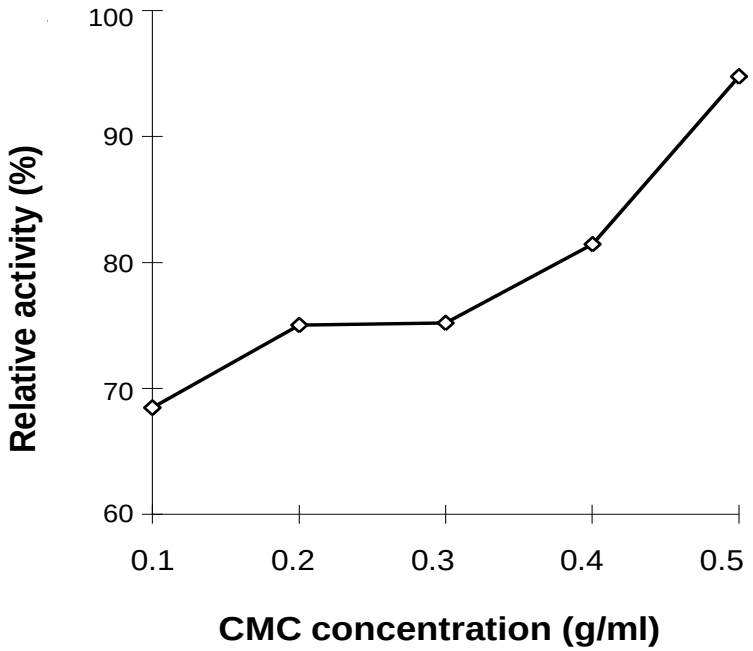

Figure 9. Effect of the concentration of substrate on the relative activity of cellulase modified by methanol. The activity of cellulase using deionized water as control instead of methanol and CMC-Na was defined as 100%.

radical but also lies at the active site (Tao et al., 1995). Methanol molecules approaching the radical at the active site of enzyme were obstructed effectively by competitive binding or dimensional block because of the increase of medial complex of enzyme-substrate, which can reduce the contact probability between the modifier and radical, and accordingly decreases the modified inactivation. Then, these confirmations show that the substrate puts up a protective action on cellulose.

Inhibiting action of glucose

Effect of glucose on cellulase modification is shown in Figure 10. The relative activity of cellulase increased when the concentration of glucose changed from 0 to 0.2 g/ml, and then tended to show steady-state over the concentration of 0.2 g/ml. The result show that glucose had an inhibiting action to cellulase modification by methanol.

Glucose is one of the inhibitor of cellulase, and the action of inhibition was produced by competing for the active site of cellulase jointly with the substrate. The inactivation degree of cellulase modified by methanol could be decreased by glucose, and it showed that the modification occurred in the active site of cellulase.

Analysis of time process of methanol modification

The relative activity of cellulase modified by methanol at different reaction times is shown in Figure11; the relative activity decreased with the increase of times. From the time process curve, when the relative activity dropped to half, the corresponding time was at 44.6 min. That is to say, $t_{1/2}$, the time required for 50% decrease in activity was 44.6 min. A linear equation was fitted by the curve plotted with logarithmic of activity against times (data not shown), which illustrated that the inactivation reaction can be assumed as first-order kinetics (Liu et al., 2002). The inactivation rate constant K_i was the slope of the straight; 0.01794.

According to the obtained result, time process curves were plotted, respectively from different reaction mixtures with ratios of methanol to cellulase solution of 0.2:0.5, 0.35:0.5, 0.5:0.5, 0.65:0.5 and 0.80:0.5 (v/v). The results show that logarithm of all the relative activity of cellulase had a linear relationship against time (data not shown). Then, values of $t_{1/2}$ were calculated from equations of fitting straight lines, respectively. Based on the equation deduced by Keech and Farrant (1968) and Liang et al. (2001): $\lg(1/t_{1/2}) = \lg k' + n\lg I$ (k', apparent first-order inactivation rate constant; I, modifier's concentration, n, apparent molecule number of inhibiter needed to form the enzyme-inhibiter complex), the standard curve plotted with logarithmic of $1/t_{1/2}$ against logarithmic of concentration of methanol was then drawn as shown in Figure 12. It can be seen that the standard curve was nearly a straight line, which can be deduced that the inactivation process followed first order reaction.

Based on the fitting straight line in Figure 12, n and k' were calculated as n=1.01 and $k'=6.01\times10^{-2}$. The results

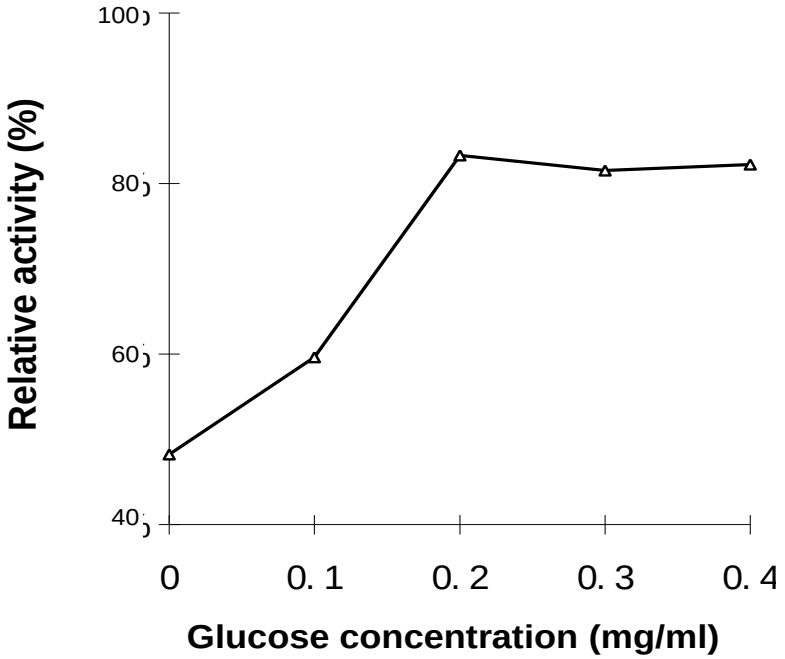

Figure 10. Effect of the concentration of glucose on the relative activity of cellulase modified by methanol. The activity of cellulase using deionized water as control instead of methanol and glucose was defined as 100%.

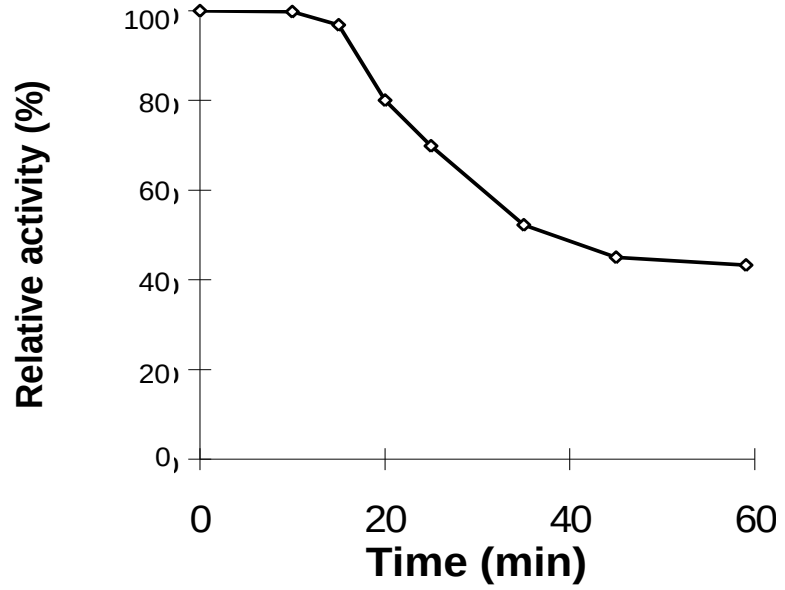

Figure 11. The relative activity of cellulase modified by methanol at different reaction times. The activity of cellulase modified by deionized water as control was defined as 100%.

showed that inactivation of cellulase was caused by the carboxyl reacting with one methanol molecule, which can be concluded that there was one carboxyl which lies at the active site of cellulase during the processing of inactivation of cellulase.

Conclusions

In this study, crude cellulase from *T. viride* could be purified and concentrated by salting out with ammonium sulfate at the optimum conditions of saturation range of

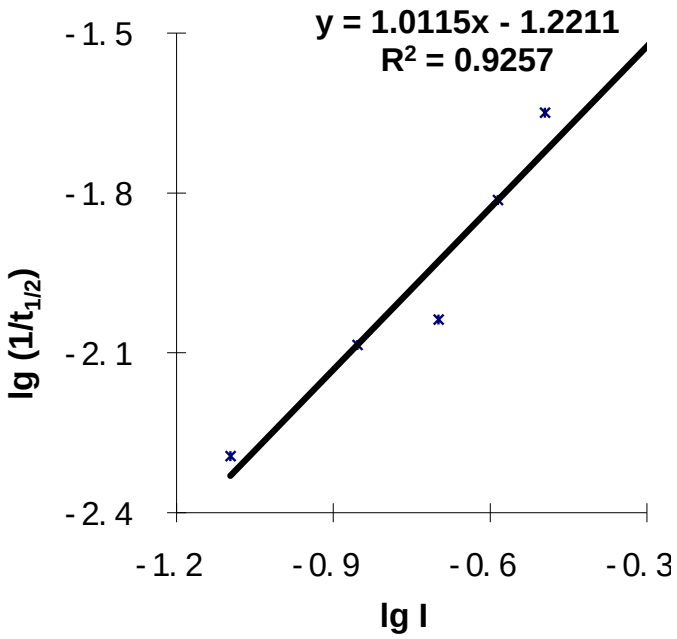

Figure 12. The standard curve plotted with logarithmic of $1/t_{1/2}$ against logarithmic of concentration of methanol. $t_{1/2}$ and I are the time required for 50% decrease in activity and the concentration of methanol, respectively.

40 to 60%, and a pH of 6.0. Analyses of infrared spectra of cellulase unmodified or modified demonstrated that the carboxyl in the side-chain radical of cellulase was modified by methanol. Effect of methanol on cellulase activity showed that the modified side-chain radical lies at the active site of cellulase or on the essential groups. CMC-Na which protected the cellulase from inactivation by methanol suggest that carboxyl modified by methanol is not only a structural radical but also lies at the active site. Effect of glucose on modification showed that the modification occurred in the active site of cellulase. The kinetic analysis confirmed that one carboxyl was an essential group for cellulase activity.

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