Cellulose Hydrolysis by Digestive Enzymes of *Reticulitermes speratus*, a Native Termite from Korea[*1]

Young-Min Lee[*2], Hyun-Jung Kim[*2], Moon Jung Cho[*2], Keum Shin[*2],
Young-kyoon Kim[*2], and Yeong-Suk Kim[*2†]

ABSTRACT

This study was to investigate the enzymatic hydrolysis of cellulose using the cellulase from whole body of the native termite collected in Milyang-si, Kyungsangnamdo, Korea. In the results, optimal temperature and pH for the enzyme of native termites were 45ºC and pH 5.5 for both endo-β-1, 4-glucanase and β-glucosidase. Enzyme activity of the termite enzyme was shown 8.8×10^{-2} FPU/mℓ. And the highest glucose hydrolysis rate of cellulose by the digestive enzyme from test termites was 24.5% based on the glucan, comparing 59.7% by commercial enzyme (only celluclast 1.5 L) at 1% (w/v) substrate and 36 hours in hydrolysis time. This hydrolysis rate by the digestive enzyme from test termites was comparatively high value in 41% level of the commercial enzyme. When cellulose was hydrolyzed by the digestive enzyme of the native termite, glucose hydrolysis was almost completed in 12 hours which was the considerably reduced time for cellulose hydrolysis. It was suggested that the quiet short reaction time for cellulose hydrolysis by the enzyme from native termite could be a very high advantage for development of hydrolysis cellulase for lignocellulosic biomass.

Keywords : cellulose hydrolysis, *Reticulitermes speratus*, termite, cellulase, hydrolysis time

1. INTRODUCTION

Usually many types of termite take wooden material as a food, and most of termite digest the carbohydrate components such as cellulose and hemicelluloses in its digestive organs. The lignin component which couldn't be digested in its body would be excreted, then it would be used to build their earth tubes or habitats (Park *et al.*, 2002; Donovan *et al.*, 2001; Hogan *et al.*,

1998; Lee & Ryu, 2003). Recently there has been growing interest in development for an industrial use of the termite's symbiotic microbes and digestive enzymes. Dr. Steven Chu, the 1997 Nobel Prize Laureate in Physics, once submitted a report on a green energy-producing technology that be able to convert cellulose into ethanol using the symbiotic microbes inside a termite's body (Chu, 2005). Similarly, to develop an enzyme to degrade cellulose, there have been

studied on bacteria or flagellates, which live in the termite's gut (Bullis, 2008; Odelson & Breznak, 1985; Park & Bae, 1998; Singer, 2007). Due to the rising demand for alternative energy sources, and recent advances in biotechnology, many research has been performed to produce bioalcohol by enzymatic hydrolysis using cellulase from microorganisms. In case of bioalcohol production technology with lignocellulosic biomass, it is necessary the cellulase that are capable of decomposing lignocellulosic material in a complicate structure. The cellulase has been often developed from microorganisms like *Trichoderma sp.* and *Aspergillus sp.* (Park *et al.*, 2002; Krusa, 2005; Liang *et al.*, 2007; Moon *et al.*, 2001; Yoon & Kim, 2005; Yoon *et al.*, 2007). Some of the key challenges in bioalcohol production process include the hydrolysis enzymes development, pretreatment, hydrolysis process, and fermenting enzymes to break down the high-cost. In order to develop low-cost enzymes, the U.S. Department of Energy has invested R&D funding to Novozyme and Genencor globally renowned enzyme companies (Moon *et al.*, 2001; Azuma & Koshijima, 1984; Kim *et al.*, 1999; Lee *et al.*, 1994; Oh *et al.*, 2000).

As a new enzyme resource, animal or insects could be used. Azuma & Koshijima reported a result of hydrolytic ability of the enzymes extracted from *Coptotermes formosanus* Shiraki worker termites mixed with commercial enzyme to hydrolyze a wood biomass. Many studies related to termite enzymes are mostly the evaluations about enzyme activity of the cellulase from bacteria or protozoa living in the gut of a termite (Moon *et al.*, 2001; Azuma & Koshijima, 1984; Kim *et al.*, 1999). Despite the wide interests in ethanol production from woody biomass, cellulase development using the native termite in our country was very few. In the native termite in Korea, the characteristics of the enzyme production in each digestive organ of whole body were investigated by authors (Lee *et al.*, 2010).

A study on cellulase from bacteria living in the gut of a termite was conducted by Bae and Park (Park & Bae, 1998).

The objective of this study is to investigate the enzymatic hydrolysis of cellulose using the cellulase from whole body of the native termite in Korea, and then to find the possibility of cellulase for lignocellulosic biomass hydrolysis.

2. MATERIALS and METHODS

2.1. Test Termites

Test termites were collected in Milyang-si, Kyungsangnamdo, Korea, which were identified as *Reticulitermes speratus*, based on the termite taxonomy we observed with an image analyzer (BVS-314, SomeTech) (Park & Bae, 1997; Lee *et al.*, 2010). The adult 1000 workers were used for obtaining the digestive enzyme to determine cellulose hydrolysis.

2.2. Enzyme Preparation

The healthy 1000 adult workers were used for experiments. Whole body of test termite was homogenized, then, it was mixed in 400 $\mu\ell$ of 0.1 M sodium acetate buffer (SAB), pH 5.5 and PMSF (phenyl-methylsulfonyl fluoride) was added and ultrasonicated with a Sonic Dismem- brator. The homogenates were centrifuged at 13,000 rpm for 25 min. The supernatant was taken for saccharification evaluation of the crude enzyme from test termites and measurement of enzyme characterization. And the enzyme activities was measured after 5 times repetition of buffer changing and desalting with SAB (sodium acetate buffer, 100 mM, pH 5.5). All operations were carried out at 4ºC.

2.3. Measurement of for Enzymatic Activity

In order to measure the FPU (filter paper unit, expressed as μmol of glucose produced per minute with filter paper as a substrate) of enzymes which were used for the experiment on cellulose hydrolysis, the following process was done with filter paper (Whatman No.1) as a substrate and SAB (sodium acetate buffer, 50 mM, pH 5.5) as a buffer solution for enzyme from test termites (Adney & Baker, 2000). To begin with, the SAB and enzymes were placed in the test tube containing 50 mg of filter paper and subjected to a temperature of 50ºC for 60 minutes. The samples were then dosed with DNS reagent, boiled for 5 minutes, then the reaction was halted and the sample was allowed to cool to room temperature. The sample was then stirred carefully, and then the fibers of the filter paper were allowed to subside on the bottom, after which the values of enzyme activity, in FPU, were calculated with the absorbancy at 540 nm of its supernatant.

2.4. Measurement of Optimal Temperature and pH

Optimal temperature and pH on the enzyme from test termite (twenty adult workers) were analyzed by measuring the enzyme activity at various temperatures 20 to 70ºC in the 100 mM sodium acetate buffer at pH 5.5. The enzyme activities were measured under several different pH conditions using 100 mM citrate for pH 3.0 ~4.0, sodium acetate for pH 4.0 to 7.0 and tris buffers for pH 7.0~9.0 at 45ºC. Endo-β-1, 4-glucanase activity was assayed in 1 ㎖ reaction mixtures containing 2% (w/v) CMC (Sigma-Aldrich Fine Chemicals, Co.) in buffer solution at each pH and temperature Reducing sugars were determined by the Somogyi-Nelson method (Nel-

son, 1944). One unit (U) of activity was defined as the amount of enzyme releasing 1 nmol of reducing sugar per hour. β-glucosidase activity was assayed using p-NPG (Sigma/Aldrich) as substrate. The enzymatic reaction mixtures 1 ㎖ containing 100 $\mu\ell$ of the test solution and 10 mM p-NPG in buffer solution at each pH were incubated for 15 min at each temperature. The amount of p-nitrophenol released was measured at A_{405} in UV/Vis spectorscopy after addition of 2 M Na_2CO_3 to the reaction mixtures. One unit of p-NPG hydrolyzing activity was defined as the amount of enzyme equivalent to release 1 nmol of p-nitrophenol per hour.

2.5. Enzymatic Hydrolysis

The digestive enzyme from test termites was used to investigate the hydrolytic ability of cellulose, also, Celluclast 1.5 L and Novozyme 188 (Novozymes Biotech, Inc.) were used as the control enzyme. Natural cellulose (microcrystalline, 20~100 μm, Daejung Chemical Cat No. 2538-4405) was hydrolyzed by the digestive enzyme from test termites. The hydrolysis condition was adjusted to 45ºC and pH 5.5 for test termite enzymes, and 50ºC and pH 4.8 for commercial enzymes, then the sample was shaken at 150 rpm with a cellulose loading of 1% and 3%. To investigate the effect of hydrolysis time, the hydrolysis were conducted for 12 hours, 24 hours, 36 hours, and 48 hours in reaction time. At each hydrolysis reaction time sample was taken and placed at 100ºC water bath and boiled for 10 minutes to denature the enzyme, and then centrifuged at 10,000 × g for 5 min. The converted glucose in the supernatant was analyzed. The hydrolysis of cellulose with commercial enzymes was conducted as a control to investigate the effects of hydrolysis time.

2.6. Sugar Analysis

After filtering the sample obtained from the hydrolysis process through a sterilized filter (0.20 μm), the sugar analysis was performed using the HPLC system (Younglin Acme 9000 Vacuum Degasser, Avme 9000 RI detector and Column Oven) and Aminex HPX-87P column from BioRad. The solvent used was DI water, the injection amount of a sample was 10 $\mu\ell$ per sample, the velocity was 0.6 ㎖/min, and the column oven was measured at 85ºC.

3. RESULTS and DISCUSSION

3.1. Optimal Condition of the Digestive Enzyme of Test Termites

To measure the optimal temperature for the enzyme of test termites, enzymatic activity was stimulated in intervals of 5ºC from 20ºC up to 70ºC, and the results are shown in Fig. 1 and 2. As shown in figures, endo-β-1,4-glucanase reacted most actively at 45ºC, and its activity was shown a tendency to increase gradually from 20ºC to 45ºC but to decrease at above 45ºC. Also, β-glucosidase was shown the maximum activity at the temperature of 45ºC as well as endo-β-1,4-glucanase, however, it was shown a drastic decline in activity at above 55ºC. Azuma *et al.* (1984) reported that the optimal temperature for carboxymethylcellulase of *Coptotermes formosanus*, a Japanese termite, was at 50ºC, while it was the maximum activity at 45ºC in β-glucosidase (Azuma *et al.*, 1984). Sun (2002) reviewed that the cellulase-catalyzed hydrolysis of lignocellulosic biomass is usually carried out at 40~50ºC, and which greatly reduces the sugar degradation at high temperature. Also, Ballesteros *et al.* (2004) reported that the compromise of enzymatic hydrolysis temperature (40~50ºC) and fermentation temperature (in case
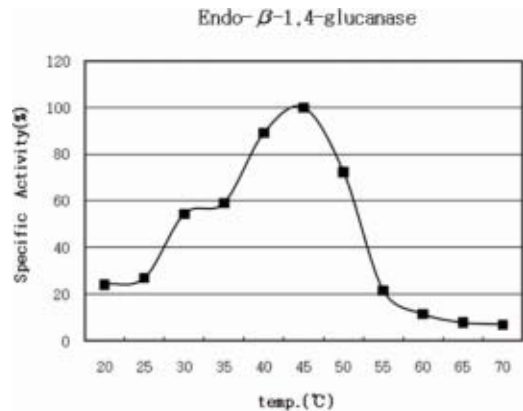

Fig. 1. Effect of temperature on the endo-β-1,4-glucanase activity of the test termite.
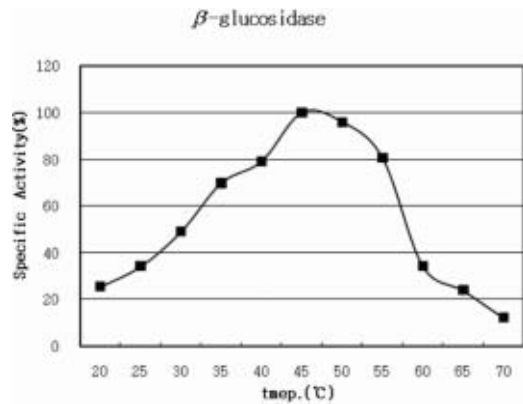

Fig. 2. Effect of temperature on the β-1,4-glucosidase activity of the test termite.

of utilizing the thermotolerant yeasts like *Kluyveromyces marxianus* which can be cultured at 42~43ºC) makes it feasible for the simultaneous saccharification and fermentation (SSF), which will decrease end product inhibition and ethanol production cost. Therefore, it was discussed that *Reticulitermes speratus*, a native termite in Korea, with optimal temperature (45ºC) for it's endo-β-1,4-glucanase and β-glucosidase would be comparatively feasible in application for cellulose hydrolysis in fuel ethanol production.
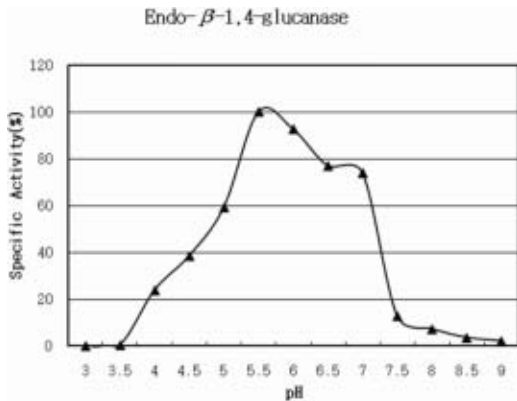
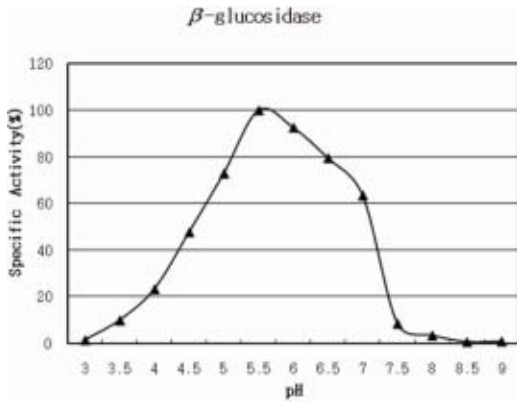Fig. 3. Effect of pH on the endo-β-1,4-glucanase activity of the test termite.

Fig. 4. Effect of pH on the β-1,4-glucosidase activity of the test termite.
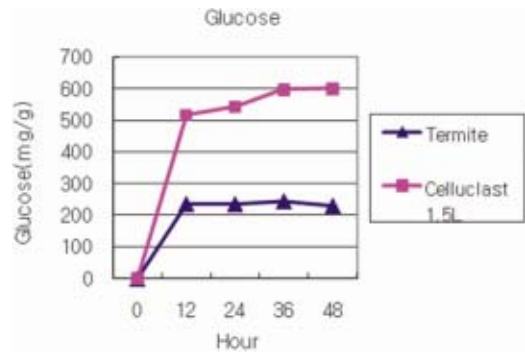

Fig. 5. Change of hydrolysis rate of glucose with reaction time by the enzyme from test termites (Substrate concentration : Cellulose 1%).

Fig. 6. Change of hydrolysis rate of glucose with reaction time by the enzyme from test termites (Substrate concentration : Cellulose 3%).

The optimal pH of endo-β-1,4-glucanase and β-glucosidase is shown in Fig. 3 and Fig. 4. Endo-β-1,4-glucanase and β-glucosidase were the maximum enzyme activity at pH 5.5. Optimal condition pH 5.5 of *Reticulitermes speratus*, a native termite, was different from that of *Coptotermes formosanus, a Japanese termite by* Azuma *et al.* and Yamaoka and Nagatani, while it was the same tendency to it by Yokoe (Azuma *et al.*, 1984; Yokoe, 1964; Yamaoka & Nagatani, 1975). Based on the results and some literatures it was found that optimal pH level might be different

in the same termite species.

3.2. Enzymatic Hydrolysis with the Digestive Enzyme of the Native Termite

The results of the cellulose hydrolysis by the digestive enzyme of the native termite in Korea, are shown in Table 1 and Figs. 5～6. To investigate the level of hydrolytic ability of termite enzyme two kinds of commercial enzyme were included to cellulose hydrolysis experiment. Table 1 showed cellulose hydrolysis results by the enzymes at each conditions. Enzyme activity

Table 1. Results of cellulose hydrolysis by the digestive enzyme of the test termites

enzymes	temp. (ºC)	pH	Time (hr)	Substrate conc. (w/v)	sugar (mg/g)		Hydrolysis rate (%)	
					Glucose	Xylose	Glucose	Xylose
Enzyme from termite[1]	45	5.5	12	1%	236.0	7.2	23.6	0.7
				3%	87.0	7.4	8.7	0.7
			24	1%	235.9	7.5	23.5	0.8
				3%	89.1	7.9	8.9	0.8
			36	1%	244.9	7.8	24.5	0.8
				3%	91.5	8.1	9.2	0.8
			48	1%	230.6	7.2	23.1	0.7
				3%	96.6	8.6	9.7	0.9
Commercial enzyme[2]	50	4.8	12	1%	516.9	-	51.7	-
				3%	436.0	-	43.6	-
			24	1%	543.0	-	54.3	-
				3%	491.7	-	49.2	-
			36	1%	596.6	-	59.7	-
				3%	547.3	-	54.7	-
			48	1%	600.3	-	60.0	-
				3%	563.1	-	56.3	-
Commercial enzyme[3]	50	4.8	12	1%	684.3	32.5	68.4	3.3
				3%	606.0	32.9	60.6	3.3
			24	1%	778.1	36.0	77.8	3.6
				3%	663.4	35.4	66.3	3.5
			36	1%	765.6	35.7	76.6	3.6
				3%	779.5	40.9	78.0	4.1
			48	1%	833.4	38.5	83.3	3.9
				3%	794.8	41.4	79.5	4.1

[1] 8.8×10^{-2} FPU/mL
[2] Celluclast 1.5 ℓ (60 FPU/㎖),
[3] Celluclast 1.5 ℓ (60 FPU/㎖) + Novozyme 188 (3:1)

of termite enzyme on the basis of filterpaper unit was shown 8.8×10^{-2} FPU/㎖. The highest hydrolysis rate of cellulose by the digestive enzyme from the termites was 24.5% based on the glucan, comparing 59.7% by commercial enzyme (only celluclast 1.5 L, 60 FPU/㎖) at 1% (w/v) substrate and 36 hours in hydrolysis time. The hydrolysis rate by the digestive enzyme from test termites was comparatively high value in 41% level of the test commercial enzyme, despite of the very lower enzyme activity than commercial enzyme. Also the hydrolysis rate of this study was evaluated as the considerably higher value compared with 4.5% to 9.3% hydrolysis rate of wood pulp by enzymes of *C. formosanus*, a Japanese termite, as reported by

Young-Min Lee, Hyun-Jung Kim, Moon Jung Cho, Keum Shin, Young-kyoon Kim, and Yeong-Suk Kim

Azuma & Koshijima (Azuma & Koshijima, 1984). Based on this results and literature reviews, it was realized that the feasibility of the native termite enzyme for development of hydrolysis enzyme with lignocellulosic biomass would be high.

To find the effect of the substrate concentration on the the substrate was added at 1% and 3% (w/v). Glucose hydrolysis rates by all test enzymes were considerably low when the substrate concentration was increased. It was discussed that this might be caused by insufficient enzyme for substrate or enzyme inhibitions of the resultant accumulation of cellobiose or glucose. And to determine the reduction effect of reaction time for hydrolysis by termite enzyme the cellulose was hydrolyzed for 12, 24, 36, 48 hours, and the change of glucose hydrolysis rate with increasing of reaction time was measured. Figs. 5 ~ 6 showed the changes of glucose hydrolysis rate by increasing of hydrolysis time. The results showed the differences of hydrolysis rate change between the termite enzyme and commercial enzymes. When cellulose was hydrolyzed by the digestive enzyme of the native termite, hydrolysis rate was rarely increased even though the hydrolysis time was extended to more than 12 hours. However, the cellulose hydrolysis rate was increased with increasing of hydrolysis time in the results of commercial enzymes used, showing need the more hydrolysis time. Generally, disadvantage of most of enzymatic hydrolysis is to have a long reaction time for completion of hydrolysis, mostly it need about 72 hours for enzymatic hydrolysis with commercial enzyme or cellulase from fungi. However it was known that cellulose components would be almost degested within 12 hours after feeding by the enzyme of digestive organs of termite body. Accordingly it would be very high feasible for commercialization compared to the enzymes from fungi.

4. CONCLUSION

This study was to investigate the enzymatic hydrolysis of cellulose using the cellulase from whole body of the native termite collected in Milyang-si, Kyungsangnamdo, Korea. In the results, optimal temperature and pH for the enzyme of native termites were 45ºC and pH 5.5 for both endo-β-1,4-glucanase and β-glucosidase, it was discussed that *Reticulitermes speratus*, a native termite in Korea with optimal temperature (45ºC) for enzyme activities would be more feasible in application for cellulose hydrolysis. Also, based on the results and literatures it was suggested that optimal pH level might be different in the same termite species. Enzyme activity of termite enzyme on the basis of filterpaper unit was shown 8.8 × 10^{-2} FPU/㎖. The highest hydrolysis rate of cellulose by the digestive enzyme from test termites was 24.5% based on the glucan, comparing 59.7% by commercial enzyme (only celluclast 1.5 L) at 1% (w/v) substrate and 36 hours in hydrolysis time. This hydrolysis rate by the digestive enzyme from test termites was comparatively high value in 41% level of the commercial enzyme and was the considerably higher value compared with enzyme of *C. formosanus*, a Japanese termite. When cellulose was hydrolyzed by the digestive enzyme of the native termite, glucose hydrolysis was almost completed in 12 hours. 12 hours in enzymatic hydrolysis time is the considerably reduced time for cellulose hydrolysis, comparing 72 hours in commercial cellulase based on the fungi. It was suggested that the quiet short reaction time for cellulose hydrolysis by the enzyme from native termite could be a very high advantage for development of hydrolysis cellulase for lignocellulosic biomass.

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