Enzymatic Properties of Cellobiohydrolase immobilized in Soil

Chung, Jong-Kag, Young-Ki Yang, Jin-Soo Maeng and Young-Ha Rhee

Department of Microbiology, College of Natural Sciences, Chungnam National University

토양내에 고정화되어 있는 Cellobiohydrolase의 효소학적 성질

정종각·양영기·맹진수·이영하

충남대학교 자연과학대학 미생물학과

ABSTRACT: The enzymatic properties of soil cellobiohydrolase were examined and compared with those of cellobiohydrolase-active extracts from soil in the forms of enzyme-humic complex and humic-free enzyme, and cellobiohydrolase partially purified from *Aspergillus niger*. The pH optima of soil cellobiohydrolase and cellobiohydrolase-humic complex were greater by 1.5-3.0 pH units than those of cellobiohydrolase in humic-free extract and from *A. niger*. Soil cellobiohydrolase and cellobiohydrolase-humic complex were remarkably resistant to thermal denaturation and proteolysis. These results confirm that cellobiohydrolase in soil is stable in conditions which rapidly inactivate microbial cellobiohydrolase and that its stability is due to the immobilization of this enzyme by association with humic substances. The Michaelis-Menten constants (Km) for soil, cellobiohydrolase-humic complex, humic-free extract and cellobiohydrolase from *A. niger* were 22.1 mg/ml, 11.3 mg/ml, 10.6 mg/ml and 4.5 mg/ml of Avicel, respectively.

KEY WORDS□soil cellobiohydrolase, extracted cellobiohydrolase, immobilization.

It is well known that a portion of enzymes in soil is associated with living and dead cells but the greater part of enzymes apparently occurs as extracellular forms which are largely adsorbed on organic and inorganic soil colloids. The adsorption of enzymes on soil colloids affects their function in a microenvironment which is completely different from that of free enzymes in solution (Burns, 1978 and 1982; Ross, 1983). Extraction of soil enzymes in high yields followed by investigations on their physical and chemical state have been, considering currently-available methodologies in soil enzymology, the subject of much study in recent years (Pettit *et al.*, 1976; Sarkar *et al.*, 1980; Nannipieri *et al.*, 1982A and 1982B; Bollag *et al.*, 1987).

Among the various soil enzymes cellulase has attracted considerable attentions because the decomposition of cellulosic materials and subsequent mineralization of the products have a special significance in the biological cycling of carbon. In particular, the initial phases of the decomposition processes of cellulosic materials in soil are brought about mainly by soil cellulase (Burns, 1982) and the enzymatic conversion of native cellulose to soluble carbon may make an important contribution to the total biological activity and biomass in soil (Knapp *et al.*, 1983; Sato *et al.*, 1984).

The occurrence of cellulase activity in soil profiles of various ecosystems has been reported by several investigators. In enzymatic studies, however, much work has been done with 1,4-β-D-glucan glucanohydrolase (CMCase, C_x) by using carboxymethylcellulose as a substrate (Pancholy and Rice, 1973A and 1973B; Speir and Ross, 1981; Rhee *et al.*, 1985 and 1987). Information about 1,4-β-D-glucan cellobiohydrolase (cellobiohydro-

lase, Avicelase, C_1 or exocellulase) is relatively scanty.

In the present study, cellobiohydrolase-humic complex and humic-free cellobiohydrolase were successfully extracted from a forest soil by step-wise treatment of extractants, and then some enzymatic properties of soil cellobiohydrolase, humic matter-cellobiohydrolase complex, humic matter-free extract, and partially-purified cellobiohydrolase from *Aspergillus niger* were compared to appreciate the nature and behavior of cellobiohydrolase in soil.

MATERIALS AND METHODS

Soil

A field-moist silt loam soil, with the following characteristics, was taken from the top 10 cm of a deciduous broad-leaved forest and used throughout these experiments: pH 5.4; organic matter 5.8%; total N 0.13%; moisture content 24.0%; cation exchange capacity 3.2 mequiv./100 g soil.

Extraction procedure

To extract cellobiohydrolase-humic complex, field-moist soil was suspended in five times its volume of 140 mM sodium pyrophosphate, pH 7.0, and gently agitated at 25 °C for 24h in a shaking water bath (60 strokes/min). The suspension was centrifuged and the clear supernatant filtered through a glass filter (porosity 40-60 μm). The filtrate was then stored at 4 °C and used in this study as cellobiohydrolase-humic complex or sodium pyrophosphate(SP) extract.

The SP extract was partially purified by precipitation with ammonium sulfate at a final concentration of 90% saturation. The precipitated proteins were collected by centrifugation at 12,000 g for 10 min and were dissolved in a minimal volume of 10 mM phosphate buffer, pH 7.0. To this enzyme solution 50ml of protamine sulfate (Sigma, grade III) was added and followed by dialysis against 10 mM phosphate buffer, pH 7.0 for 24h. After removal of brown-colored precipitates formed by centrifugation at 26,000 g for 20 min, the supernatant which had no brown color was used as protamine-treated (PT) extract.
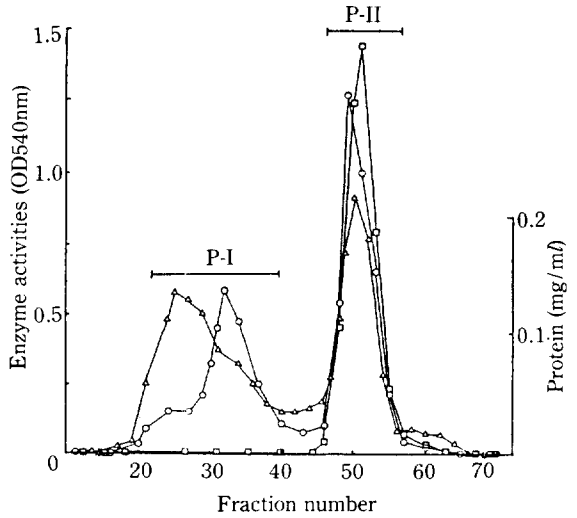

Fig. 1. *Sephadex G-150 column chromatography of the crude cellulase from A. niger.*
⊐—⊐ : cellobiohydrolase, ○—○ : CMCase,
△—△ : protein

Preparation of cellobiohydrolase from *A. niger*

A commercial cellulase powder produced by *A. niger* (Fluka) was used as a starting material for the separation of cellobiohydrolase fraction. A sample (20 ml) of the crude enzyme was applied on a Sephadex G-150 column (2.2 × 80 cm) previously equilibrated with 50 mM phosphate buffer, pH 6.0. The protein was eluted with the same buffer at a flow rate of 22 ml/h with a fraction size of 4.4 ml, and separated into two major peaks of enzyme with different cellulolytic activity (Fig. 1). The second peak (P-II) which was rich in cellobiohydrolase activity was pooled and concentrated 4-fold by ultrafiltration through a PM 10 diaflo membrane (Amicon Corp.). For the further purification of cellobiohydrolase, the concentrated P-II fraction was put on a column of DEAE-Sephadex A-50 (3.0 × 40 cm) previously equilibrated with 20 mM phosphate buffer, pH 6.0. The column was eluted using a linear gradient of NaCl (0-1.5 M) in the same buffer at a flow rate of 32 ml/h with a fraction size of 6.4 ml. P-II fraction was separated by this ion-exchange chromatography into two cellobiohydrolase peaks (Fig. 2). The second peak (P-II-II, fractions; 40-45), which eluted at 0.5 M NaCl solution, has much cellobiohydrolase activity and was used throughout this experiments.
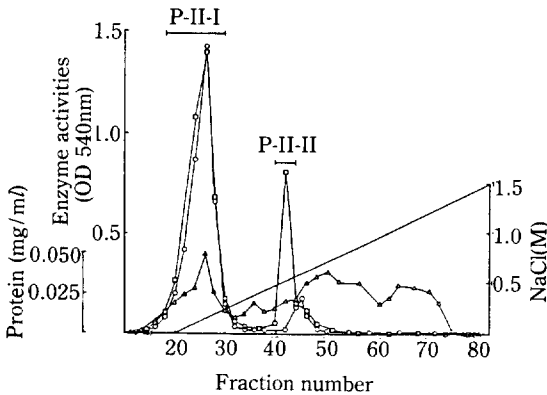
Fig. 2. *Ion-exchange chromatography of P-II fraction on DEAE-Sephadex A-50.*

□—□ : cellobiohydrolase, ○—○ : CMCase,
△—△ : protein

Enzyme assays

Soil cellobiohydrolase activity was measured by reducing sugars liberated from Avicel (FMC Co.). Field-moist soil (5 g) was placed in a 100 m*l* flask and 0.6 m*l* of toluene added (Rhee *et al.*, 1987). After mixing thoroughly for 15 min, 10 m*l* of 100 mM phosphate buffer (pH 7.0) and 10 m*l* of 0.5% Avicel were also added. The reaction mixture was then held in a shaking water bath at 50 °C for 15h. Then 50 m*l* distilled water was added, the suspension was filtered and distilled water added to increase the volume of filtrate to 100 m*l*. The reducing sugar content of the filtrate was then determined.

The cellobiohydrolase activity of the extracted enzyme preparations was measured in a reaction mixture of 0.4 m*l* of 0.5% Avicel, 0.2 m*l* of 100 mM phosphate buffer (pH 6.0), and 0.4 m*l* of enzyme solution. After incubation at 40 °C for 15h, the concentration of reducing sugar was determined. Cellobiohydrolase fractionated by chromatography was assayed by the same method but with a 1h incubation. CMCase activity was measured as described in a previous paper (Rhee *et al.*, 1987). In all cases the concentration of reducing sugar was determined colorimetrically by the Nelson-Somogyi method (Somogyi, 1952).

Measurement of protein

Protein concentration was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma) as standard.

Stability of cellobiohydrolase

(a) Thermal: All forms of enzyme preparation were maintained at 60 °C and 80 °C for up to 5h in the presence of buffer solution but in the absence of substrate. After pre-incubation, the residual enzyme activities were measured under their standard assay conditions.

(b) Proteolysis: Enzyme reaction mixtures were mixed with 1.0 mg/m*l* and 1.5 mg/m*l* of proteinase K(Sigma, type XI) and incubated under their standard assay conditions. Controls without proteinase K were also incubated. After incubation, remaining enzyme activities were measured and compared with the controls.

Kinetics

The cellobiohydrolase activities were determined at substrate concentrations ranging from 1.0 to 30 mg/m*l*. The Michaelis-Menten constant(Km) values were derived by using the Lineweaver-Burk plot and determined by computed least-squares of linear plots.

RESULTS AND DISCUSSION

1. Effect of pH

The hydrogen-ion concentration in the reaction system may affect the ionization groups of enzyme or influence the ionization state of substrate, or both; thus a comparison of the pH-activity profile of an immobilized enzyme and that of a free enzyme can show the effect of the microenvironment surrounding enzymatic protein on the hydrogen-ion concentration (Zaborsky, 1973).

The pH dependences of cellobiohydrolase activities of soil, SP extract, PT extract, and *A. niger* were examined using universal buffer (Perrin and Dempsey, 1974) to cover the pH range 2-10. The variations of activity with pH were shown in Fig. 3. The soil cellobiohydrolase showed the highest activity at pH 6.5 and became vulnerable to inactivation at pH values below 5.5. The activity of SP extract was maximum at pH 5.5 and more than 70% of maximal activity were observed over the range 4.0-6.5. PT extract exhibited the highest cellobiohydrolase activity at pH 5.0. For the
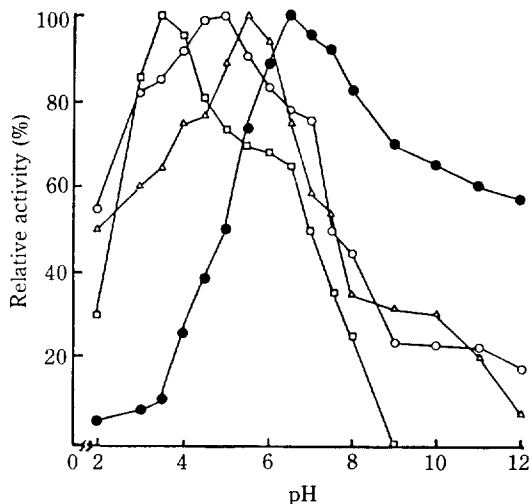
Fig. 3. Effect of pH on the cellobiohydrolase in soil, SP
extract, PT extract, and from A. niger.
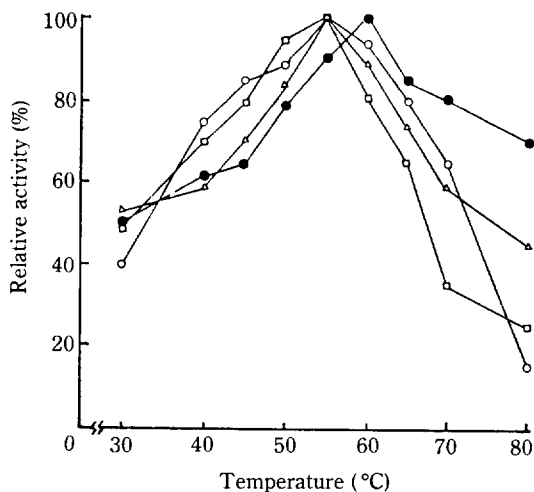●—● : soil, △—△ : SP extract, ○—○ : PT
extract, □—□ : A. niger

Fig. 4. Effect of incubation temperature on the cello-
biohydrolase in soil, SP extract, PT extract, and
A. niger.
●—● : soil, △—△ : SP extract, ○—○ : PT
extract, □—□ : A. niger.

cellobiohydrolase from A. niger, the optimum pH
was 3.5 and was lower by 1.5-3.0 pH units than
those of other forms of cellobiohydrolase.

The pH optima of enzymes in soil are about 2
pH units higher than the same enzymes in solution
because of the influence of the polyanionic humic
moiety on the concentration of hydrogen-ions in
the microenvironment of the active sites of the
bound enzymes (Frankenberger and Tabatabai,
1980; Nannipieri et al., 1982A). It is likely,
therefore, the shift of pH optima of cellobiohydro-
lase in soil and SP extract to larger values is due to
the immobilization of these enzymes by associa-
tion with humic matrix.

2. Effect of incubation temperature

Cellobiohydrolase in SP extract, PT extract,
and from A. niger showed their highest activities
at 55 °C, while at 60 °C in that of soil cellobio-
hydrolase (Fig. 4). And comparable differences
between the enzyme sources were observed in
their sensitivities to inactivation temperature. The
soil cellobiohydrolase was relatively insensitive to
the change of temperature above 65 °C, and in-
cubation mixture at 80 °C showed 70% of max-
imum activity. Inactivation of 56% of cellobiohy-
drolase activity at 80 °C appeared in SP extract,
while the PT extract and cellobiohydrolase from

A. niger lost respectively 75% and 86% of their
maximum activity at the same incubation tempe-
rature. These results are consistent with the fin-
dings of Browman and Tabatabai (1978) who
found that the temperature needed to inactivate
the phosphodiesterase in soil was about 10 °C hig-
her than the temperature needed to inactivate the
same enzyme in the absence of soil.

3. Thermal stability

Generally immobilization of enzymes enhances
their thermal stabilities.Resistance of immobilized
enzymes to thermal denaturation may be related
to the stabilization of the tertiary structure of pro-
teins (Zaborsky, 1973).

The cellobiohydrolase activities of soil, SP ex-
tract, PT extract, and A. niger following incuba-
tion for up to 5h at 60 °C and 80 °C are shown in
Fig. 5. PT extract and cellobiohydrolase from A.
niger were unstable at high temperature and inac-
tivated completely by heating at 80 °C for 3.5h. By
contrast, soil and SP extract revealed their greater
resistance to thermal denaturation and approxi-
mately 53% and 30% of the initial activities, res-
pectively, were retained after preincubation at
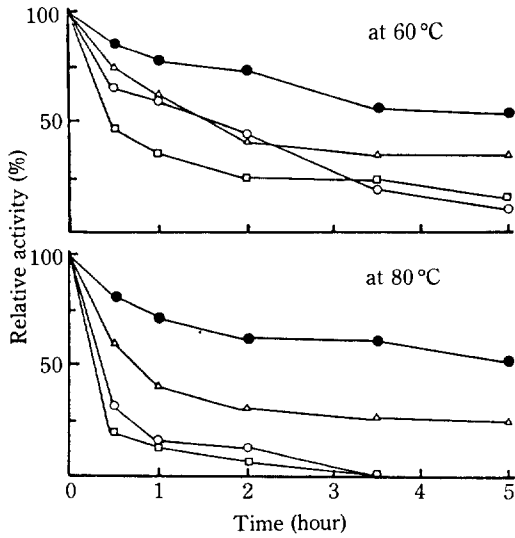80 °C for 5h.

4. Stability to proteolysis

Fig. 5. *Stability of cellobiohydrolase activities at 60°C and 80°C.*
●——● : soil, △——△ : SP extract, ○——○ : PT extract, □——□ : *A. niger*

Table 1. *Effect of proteinase K on cellobiohydrolase activity.*

Cellobiohydrolase	Concentration of proteinase K (mg/ml)	Relative activity* (%)
Soil	None	100
	1.0	98
	1.5	97
SP extract	None	100
	1.0	93
	1.5	90
PT extract	None	100
	1.0	73
	1.5	70
A. niger	None	100
	1.0	80
	1.5	73

*The relative activity is expressed in terms of percentage to the cellobiohydrolase activity in the absence of proteinase K.

The fungal proteolytic-enzyme, proteinase K is very active in the breakdown of carboxymethyl-cellulase (Rhee, 1985) and hence has been used to test the resistance of cellobiohydrolase to proteolysis. In Table 1, the observed activities of cellobiohydrolase are expressed as a percentage of the control. The cellobiohydrolase activities of PT extract and *A. niger* were significantly inactivated by the action of proteinase K. The activities of soil and SP extract, however, were fairly resistant to proteolysis retaining respectively 97% and 90% of their original activities in the presence of 1.5 mg/ml of proteinase K. These results indicate that soil cellobiohydrolase is remarkably stable in conditions which rapidly inactivate free cellobiohydrolase (cellobiohydrolase in PT extract and from *A. niger*) and these stabilities can be maintained to some extent in the cellobiohydrolase-humic complex (SP extract).

Soil is an inhospitable environment for extra-cellular enzymes in that non-biological denaturation and degradation by proteolytic microorganisms all conspire to take their toll of enzymes (Burns, 1982). Thus the marked stabilities of soil cellobiohydrolase and SP extract to thermal denaturation and proteolysis demonstrate the ecolo-

gical importance of immobilized cellobiohydrolase in soil ecosystems. In this context, it is desirable to envisage humic matter as a complex and recalcitrant backbone supporting enzyme molecules and enabling enzymes to become resistant to denaturation and proteolysis.
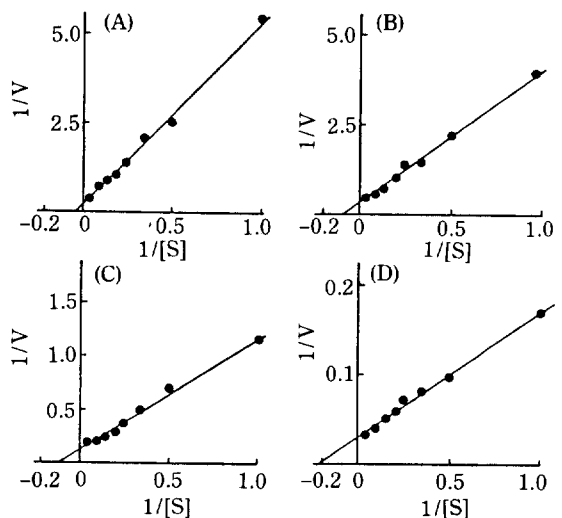

Fig. 6. *Lineweaver-Burk plots for cellobiohydrolase activities of soil(A), SP extract(B), PT extract(C), and A. niger(D).*

5. Kinetics

The Michaelis-Menten constant (Km) is a characteristic parameter of each enzyme under definite assay conditions. It is related to a number of reaction rate constants and measures the affinity of the enzyme for the substrate. Determination of Km values following the same procedure employed for a single enzyme in homogenous experimental conditions has been widely used in soil enzymology. However, some soil enzymes which did not follow Michaelis-Menten kinetics were reported (Irving and Cosgrove, 1976; McLaren, 1978; Nor, 1982) and such a deviation was explained as being to the heterogeneity of the soil system.

Fig. 6 shows the Lineweaver-Burk plots applied to the cellobiohydrolase activity values of four preparations obtained as a function of concentration of substrate (Avicel). All reactions catalyzed by different forms of cellobiohydrolase followed Michaelis-Menten kinetics. The correlation coefficients for the Lineweaver-Burk plots were high and they were in the range of 0.98-0.99. The Km values obtained for four enzyme preparations were: soil 22.1 mg/ml, SP extract 11.3 mg/ml, PT extract 10.6 mg/ml and cellobiohydrolase from *A. niger* 4.5 mg/ml. The data confirm that the association of cellobiohydrolase with soil colloids gives rise to increase in the Km value. These results indicate that the affinity of free cellobiohydrolase to its substrate is greater than that of same enzyme in an adsorbed state, and are in line of the results of Chhonkar and Rao (1982) who found high Km values of C_1-cellulase in the presence of soils rich in kaolinite as compared to pure system (in absence of soils).

적　요

미생물의 대사활동에 의하여 토양내에 기생성되어 있는 abiontic soil cellobiohydrolase의 생태학적 특성을 이해하기 위하여 이 효소와 토양으로부터 분리한 서로 다른 성상의 cellobiohydrolase 추출물, *Aspergillus niger*로부터 부분정제된 cellobiohydrolase의 효소학적 성질을 비교검토하였다.

Soil cellobiohydrolase 및 cellobiohydrolase-부식교질 복합체의 최적 pH는 부식교질이 제거된 추출물이나 *A. niger*의 cellobiohydrolase의 최적 pH에 비하여 1.5-3.0 pH units가 높았으며 이들의 활성이 억제될 수 있는 열처리 및 단백질가수분해효소에 대하여서도 매우 높은 안정성을 나타내었다. Soil cellobiohydrolase와 cellobiohydrolase-부식교질 복합체의 이와같은 특성은 토양미생물로부터 생성된 cellobiohydrolase가 부식교질에 의하여 고정화됨으로써 토양생태계내에서 안정된 효소활성을 유지할 수 있음을 보여 주었다. 반면에 soil cellobiohydrolase와 cellobiohydrolase-부식교질 복합체의 기질 Avicel에 대한 Km 값은 각기 22.1mg/ml와 11.3mg/ml로서 4.5mg/ml의 Km 값을 보인 *A. niger*의 cellobiohydrolase에 비하여 현저하게 낮은 기질친화력을 보였다.

REFERENCES

1. Bollag, J.M., C.M. Chen, J.M. Sharkar and M.J. Loll, 1987. Extraction and purification of a peroxidase from soil. *Soil Biol. & Biochem.* **19**, 61-67.
2. Browman, M.G. and M.A. Tabatabai, 1978. Phosphodiesterase activity of soils. *J. Soil Sci. Soc. Am.* **42**, 284-290.
3. Burns, R.G., 1978. Enzyme activity in soil: Some theoretical and practical considerations. In *Soil Enzymes* (ed. by R.G. Burns), Academic Press, pp 295-340.
4. Burns, R.G., 1982. Enzyme activity in soil: Location and possible role in microbial ecology. *Soil Biol. & Biochem.* **14**, 423-427.
5. Chhonkar, P.K. and Y.S.S. Rao, 1982. A kinetic study of cellulases in soils. *J. Indian Soc. Soil Sci.* **30**, 398-400.
6. Frankenberger, W.T. Jr. and M.A. Tabatabai, 1980. Amidase activity in soil: Method of assay. *Soil Sci. Am. J.* **44**, 282-287.
7. Irving, G.G.J. and D.J. Cosgrove, 1976. The kinetics of soil acid phosphatase. *Soil Biol. & Biochem.* **8**, 335-340.
8. Knapp, E.B., L.F. Elliott and G.S. Campbell,

1983. Microbial respiration and growth during the decomposition of wheat straw. *Soil Biol. & Biochem.* **15**, 319-323.

9. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

10. McLaren, A.D., 1978. Kinetics and consecutive reactions of soil enzymes. In *Soil Enzymes* (ed. by R.G. Burns), Academic Press, pp. 97-116.

11. Nannipieri, P., B. Ceccanti, C. Conti and D. Bianchi, 1982A. Hydrolases extracted from soil: Their properties and activities. *Soil Biol. & Biochem.* **14**, 257-263.

12. Nannipieri, P., B. Ceccanti, S. Cervelli and C. Conti, 1982B. Hydrolases extracted from soil: Kinetic parameters of several enzymes catalysing the same reaction. *Soil Biol. & Biochem.* **14**, 429-432.

13. Nor, Y.M., 1982. Soil urease activity and kinetics. *Soil Biol. & Biochem.* **14**, 63-65.

14. Pancholy, S.K. and E.L. Rice, 1973A. Soil enzymes in relation to old field succession. *Soil Sci. Soc. Proc.* **37**, 47-50.

15. Pancholy, S.K. and E.L. Rice, 1973 B. Carbohydrolase in soil as affected by successional stages of revegetation. *Soil Sci. Soc. Am. Proc.* **37**, 227-229.

16. Perrin, D.D. and B. Dempsey, 1974. *Buffers for pH and metal ion control.* Chapman and Hall, p 156.

17. Pettit, N.M., A.R.J. Smith, R.B. Freeman and R.G. Burns, 1976. Soil urease: Activity, stability and kinetic properties. *Soil Biol. &*

Biochem. **8**, 479-484.

18. Rhee, Y.H., 1985. Characteristics and Distribution of Abiontic Carboxymethylcellulase in Forest Soils. Ph. D. thesis. Seoul National University

19. Rhee, Y.H., Y.C. Hah and S.W. Hong, 1985. Distribution of abiontic carboxymethylcellulase in relation to microbial growth and activity in forest soils. *Kor. J. Microbiol.* **23**, 147-156.

20. Rhee, Y.H., Y.C. Hah and S.W. Hong, 1987. Relative contributions of fungi and bacteria to soil carboxymethylcellulase activity. *Soil Biol. & Biochem.* **19**, 479-481.

21. Ross, D.J., 1983. Invertase and amylase activities as influenced by clay minerals, soil-clay fractions and topsoils under grassland. *Soil Biol. & Biochem.* **15**, 287-293.

22. Sarkar, J.M., L. Batistic and J. Mayudon, 1980. Les hydrolase du sol et leur association avec les hydrates de carbone. *Soil Biol. & Biochem.* **12**, 325-328.

23. Sato, K., J. Antheunisse and E.G. Mulder, 1984. A possible effect of cellulase-decomposition on soil bacterial flora. *J. Gen. Appl. Microbiol.* **30**, 1-14.

24. Somogyi, M., 1952. Notes on sugar determination. *J. Biol. Chem.* **195**, 19-23.

25. Speir, T.W. and D.J. Ross, 1981. A comparison of the effects of air-drying and acetone dehydration on soil enzyme activities. *Soil Biol. & Biochem.* **13**, 225-229.

26. Zaborsky, O.R., 1973. *Immobilized Enzymes.* . CRC Press, Cleveland, Ohio