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Characterization of β-1,4-D-Glucan Glucanohydrolase Purified from *Trichoderma koningii*

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*Trichoderma koningii*에서 분리한 β-1,4-D-Glucan Glucanohydrolase의 특성

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ABSTRACT: β-1,4-D-Glucan glucanohydrolase(EC 3.2.1.4;F-II-IV) purified from *Trichoderma koningii* was identified as a glycoprotein containing 9% carbohydrate. Isoelectric point of the enzyme was estimated to be 4.9 and molecular weight was determined to be approximately 58, 000. The porducts of *p*-nitrophenyl-cellobioside ($PNPG_2$) catalyzed by the enzyme were *p*-nitrophenol(PNP) and *p*-nitrophenyl-glucoside($PNPG_1$). The Km value for $PNPG_2$ was estimated to be 0.97 mM in case of the holoside linkage and 10.4 mM in case of the aglycon linkage and their kcat values were 1.8×10^5 min^{-1} and 7.5×10^5 min^{-1}, respectively. The product of *p*-nitrophenyl cellotriose($PNPG_3$) was only $PNPG_1$. The Km value for $PNPG_3$ was 69.5 μM and kcat was 1×10^8 min^{-1}, which implicates that the enzyme have higher affinity and higher hydrolysis rate toward $PNPG_3$ than toward $PNPG_2$. The enzyme showed its optimal activity at pH 4.0-4.5 and at 60℃. The effect of gluconolactone on the activity toward $PNPG_2$ showed competitive inhibition pattern but glucose and cellobiose did not. The enzyme contained a high content of acidic and hydroxylated amino acids in contrast to basic amino acids.

Key Words □ **β-1,4-D-Glucan Glucanohydrolase, PNP derivatives (PNP, $PNPG_1$, $PNPG_2$, $PNPG_3$).**

The cellulase system of fungi consists of β-1,4-D-glucan glucanohydrolase (endo β-1,4-glucanase, C_x, EC 3.2.1.4), β-1,4-D-glucan cellobiohydrolase (exo-β-1,4-D-glucanase, C_x, EC 3.2.1.91) and β-glucosidase (β-glucoside glucohydrolase, cellobiase, EC 3.2.1.21). Endoglucanases attack internal glycosidic bonds of cellulose chains at random, thereby producing polymer chain ends and soluble oligosaccharides. Exoglucanases cleave cellobiosyl residue from the ends of cellulose chains and oligosaccharides. β-Glucosidases catalyze hydrolysis of cellobiose and oligosaccarides to glucose(Reese *et al.*, 1950).

Having difficulties in quantitation of the products liberated from substrates(Carboxymethylcellulose(CMC), Avicel, cello-oligosaccharides), kinetic studies of endo- and exoglucanase have been limited. Recently, there has been reported the use of chromophoric disaccharide derivatives and a homologous series of 4-methylumbelliferyl glucosides of cello-oligosaccharides $(MeUmb\text{-}(Glc)^{n=2\text{-}6})$ in the studies of cellulase from *Trichoderma reesei* (Tileurgh and Pettesson, 1985). Chirico and Brown (1987) studied β-glucosidase from *Trichoderma reesei* with [1-^{3}H]cello-oligosaccharides. In *Trichoderma koningii*, two exo-, four endoglucanases and two β-glucosidases have been purified and characterized(Wood, 1968; Wood and MaCrae, 1972; 1978) but accurate kinetic parameters were not available except β-glucosidase.

In the present report, we purify and characterize the β-1,4-endoglucanase (F-II-IV) from *Trichoderma koningii* with PNP-derivatives (*p*-nitrophenyl oligosaccharides).

MATERIALS AND METHOD

Fungal strains and chemicals

Strain used was *Trichoderma koningii* ATCC 26 113 and cultured as described by Hong *et al.* (1986). All chemicals used were purchased from Sigma Chemical Co.

Enzyme assay

Activity toward CMC

The enzyme activity of column eluent was measured according to the method previously

described by Hong *et al.* (1986).

Activity toward PNP derivatives

Assay with spectrophotometer

The activity of purified enzyme toward *p*-nitrophenyl cellobioside ($PNPG_2$) was assayed by measuring the amount of *p*-nitrophenol(PNP) liberated from the $PNPG_2$. The reaction mixture was composed of 40 μl of 2 mM $PNPG_2$ solution in 0.1 M acetate buffer pH 5.0 and 10 μl of enzyme solution. After incubation at 40℃ for 5 min, 100 μl of 1 M sodium carbonate solution was added to the mixture. The mixture was then diluted with 500 μl of distilled water and the absorbance at 420 nm was measured. One unit of enzyme activity was defined as the amount of enzyme per producing 1 μmol min^{-1} of PNP under the standard condition of the assay.

Assay with HPLC

After the reaction mixture was incubated at the same condition described above, samples were withdrawn and analyzed with HPLC system (Model 600 A, Waters Associates Co.) and Partisil PXS 10/25 column. The eluent was 22%(v/v) water in acetonitrile at the flow rate of 1.0 m*l* min^{-1}. The PNP derivatives were detected by Waters Model 440 absorbance detector at 254 nm. One unit of enzyme activity was defined as the amount of enzyme per producing 1 μmol min^{-1} of PNP, $PNPG_1$ under the standard condition of the assay.

Biosynthesis of the PNP derivatives and purification

The reaction mixture was composed of 20 mM PNPG and purified 0.5 μM low molecular weight endoglucanase(F-IV-I; Hong *et al.*, 1986). After incubation at 40℃ for 60 min, the enzyme was filtered off with Centricon and the reaction products were analyzed and purified with HPLC system(Model 600 A, Waters Associates Co.) and μ-Porasil column. The sample was eluted with linear gradient of 10%(v/v) methanol in chloroform and 2%(v/v) water in methanol. The flow rate 1.0 m*l* min^{-1}.

Determination of protein concentration

Protein concentration was determined by method of Lowry *et al.*, (1951) and Bradfford (1976), with bovine serum albumin(Sigma) as standard.

Enzyme purification

The crude enzyme preparation was fractionated by gel filtration on Bio-Gel P-150 100-200 mesh (Bio-Rad Laboratories, Richmond, CA, USA) as described by Hong *et al.* (1986). The endoglucanase fraction(F-II, 150 m*l*) from the Bio-Gel P-150 column was concentrated ten-fold by ultrafiltration through a Diaflo membrane, type PM 10(Amicon, Lexington, MA. USA). The ultrafiltrate was diluted fifty-fold with 0.02 M phosphate buffer, pH 6.9 and concentrated again. Further fractionation of enzyme from the concentrated solution was performed on a DEAE-Sephadex A-50(2.2×50 cm) column equilibrated with 0.02 M phosphate buffer, pH 6.9. The column was eluted with 300 m*l* of the same buffer, thereafter a linear NaCl concentration gradient(0-0.5 M) was applied at the flow rate of 20 m*l* $hour^{-1}$. The fraction volume was 4 m*l*. The endoglucanase fraction(F-II-IV, 120 m*l*) was concentrated to 15 m*l* by ultrafiltration. The buffer of the enzyme solution was exchanged for 0.025 M histidine-HCl buffer, pH 5.5 by repeated dilution and ultrafiltration. The concentrated solution was loaded on Polybuffer exchanger PBE 94(Pharmacia Co.) chromatofocusing column(0.9×35 cm) equilibrated with 0.025 M histidine-HCl buffer, pH 5.5. The column was eluted with 250 m*l* of ten-fold diluted Polybuffer 74(Pharmacia Fine Chemicals)-HCl buffer, pH 3.5. The flow rate was 9 m*l* hr^{-1} and the fraction volume was 2 m*l*.

Determination of carbohydrate in enzyme

The content of carbohydrate in the purified enzyme was analyzed quantitatively by the method of Dubois *et al.* (1956), using 5% phenol solution and sulfuric acid.

SDS-linear polyacrylamide gradient gel electrophoresis

The SDS-linear polyacrylamide gradient gel electrophoresis was performed by the modified method of Lambin, (1978). After electrophoresis, the fixation and staining and destaining were performed by the method of Neuhoff *et al.* (1988).

Analytical isoelectric focusing

Analytical isoelectric focusing was performed on a T=5%, C=3% gel slap containing 6.25% Pharmalyte(Pharmacia) in the pH range 4-6.5, as described by Hong *et al.* (1986).

Amino acid composition

The amino acid composition was determined by the method of Bidlingmeyer *et al.* (1984). About 15 μg of enzyme were hydrolyzed with 6 N HCl at 105℃ for 24 hr in sealed evacuated tubes. The hydrolysate was dried with redrying agent(Waters Associate Co.) and derivatized with phenylisothiocynate(PITC) at room temperature for 20 min. The derivatized amino acids were analyzed with HPLC system and a Pico-Tag column(Waters Associate Co.). The samples were eluted with linear gradient of solvent A (2% sodium acetate, 0.05% triethylamine, and 6% acetonitrile) and solvent B(60% acetonitrle). The flow rate was 1.0 m*l* min^{-1}.

RESULT AND DISCUSSION

Enzyme purification and purity

The endoglucanase fraction(F-II, III) was effectively separated from other components by gel filtration chromatography on Bio-Gel P-150 column as described Hong *et al.* (1986). Further purification of F-II was achieved by ion exchange chromatography on the DEAE-Sephadex A-50 column. As showon in Fig. 1, the endoglucanase

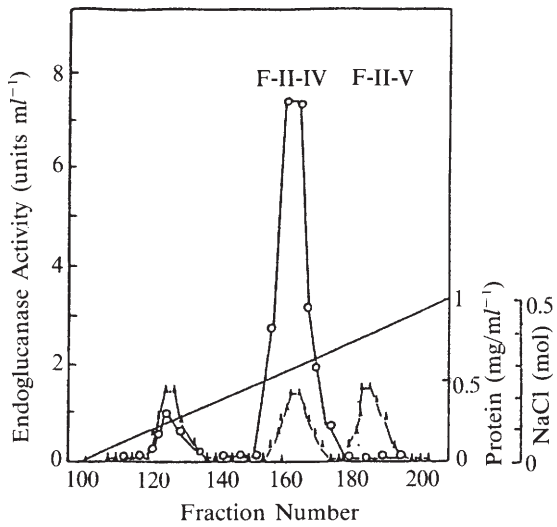

Fig. 1. *Ion exchange chromatograph of F-II on DEAE-Sephadex A-50.*
Column dimension: 2.5×50 cm. Fraction volume: 4 m*l*. Observed values: ▲, protein; ○, endoglucanase activity.

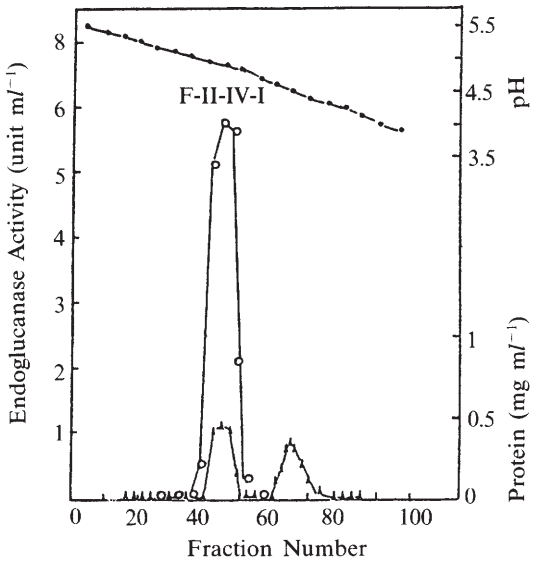

Fig. 2. *Chromatofocusing of F-II-IV on polybuffer exchanger PBE 94.*
Column dimension: 0.9×35 cm Fraction volume 2 m*l*. Observed values: ▲, protein; ○, endoglucanase activity.

fraction(F-II-IV) was separated from other protein fraction. Fig. 2 shows the result of chromatofocusing of F-II-IV-I fraction on the polybuffer exchanger PBE 94 column. The purity of the

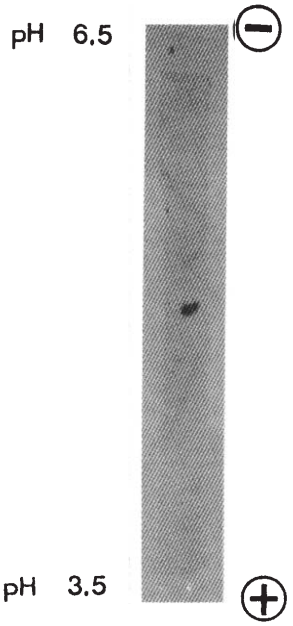

Fig. 3. *Analytical isoelectric focusing of F-II-IV using Pharmalyte (pH 4-6.5). T=5%; C=3%.*

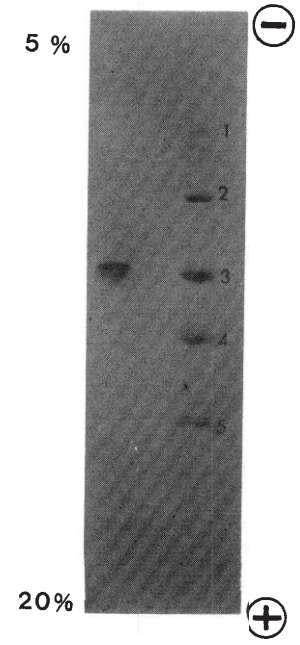

Fig. 4. *Molecular weight estimation of F-II-IV by SDS-linear polyacrylamide gradient gel electrophoresis.*
T=5−20% C=2.7%. Molecular weight markers; 1, α-2 macroglobulin(170,000); 2, phosphorylase(97,000); 3, glutamate dehydrogenase(55.400); 4, lactate dehydrogenase(36,500); 5, trypsin inhibitor(20,100).

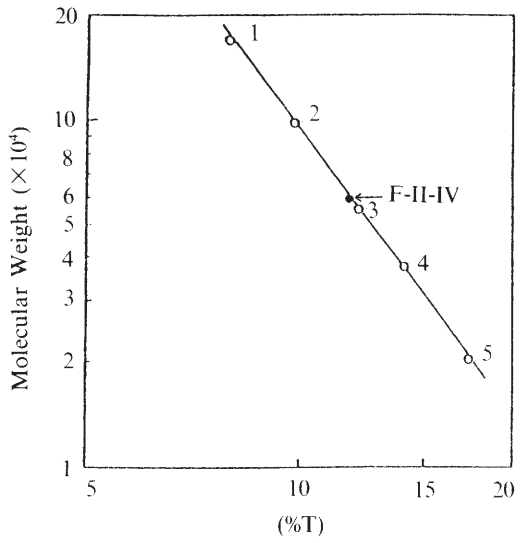

Fig. 5. *Molecular weight estimation of F-II-IV by SDS-linear polyacrylamide gradient gel electrophoresis.*
T=5−20% C=2.7%. Molecular weight markers; 1, α-2 macroglobulin(170,000); 2, phosphorylase(97,000); 3, glutamate dehydrogenase(55,400); 4, lactate dehydrogenase (36,500); 5, trypsin inhibitor(20,100).

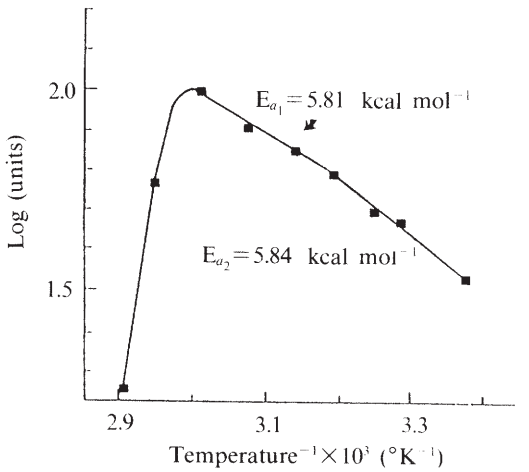

Fig. 6. *Effect of temperature on the activity of F-II-IV.*

enzyme was detected by electrophoretic method. Isoelectric focusing and SDS-linear polyacrylamide gradient gel electrophoresis showed a single band(Fig. 3, 4), which indicates the hommogeneity of the enzyme.

Isoelectric point and Molecular weight

The isoelectric point of the enzyme was determined to be 4.9 by analytical isoelectric focusing(Fig. 3). This value is a slightly higher than

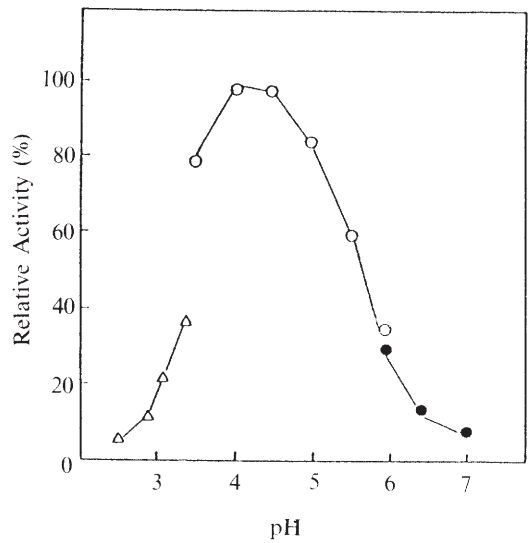

Fig. 7. *Effect of pH on the activity of F-II-IV.*
△, 25 mM citrate buffer; ○, 25 mM acetate buffer; ●, 25 mM phosphate buffer.

that of F-IV-I(4.8) from the same strain estimated by Hong *et al.* (1986). The molecular weight of the purified enzyme was estimated by SDS-linear polyacrylamide gradient gel electrophoresis with standard proteins(Fig. 4). A linear relationship was obtained when the relative acrylamide concentration(T %) located in the standard proteins was plotted against the logarithmic values of the molecular weight. The molecular weight of the enzyme was estimated to be about 58,000(Fig. 5).

The carbohydrate content

The carbohydrate content of the enzymes was estimated to be about 9% of molecular weight, using glucose as a standard carbohydrate.

Effect of temperature and pH on the enzyme activity

The reaction mixture was incubated at various temperature and enzyme activity toward $PNPG_2$ was assayed with spectrophotometer. As shown in Fig. 6, the optimum temperature for the enzyme activity was 60 ℃. The Arrheius plot indicates an activation energy of 5.84 kcal mol^{-1} from 23 to 45 ℃ and 5.8 kcal mol^{-1} from 45 to 65 ℃. Ea_1 value is almost the same as that of low molecular weight endoglucanase from same strain(F-IV-I; Hong *et al.* 1986) but Ea_2 value is lower than that of F-IV-I(12.1 kcal mol^{-1}). The dependence of enzyme activity on pH was investigated by measuring the PNP formed at various pH values. The reaction mixture was incubated at 40 ℃ for 5 min. As shown in Fig. 7, the optimum pH for the enzyme activity wa 4.0-4.5.

Kinetic study

PNP derivatives($PNPG_{1,2,3,4}$) formed from PNPG and cellotetraose with the purified low molecular

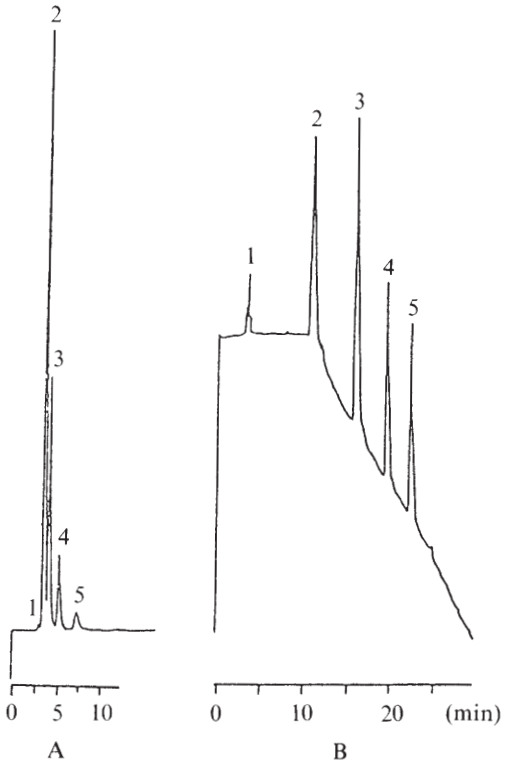

Fig. 8. *Separation of PNP-derivatives using HPLC.*
A; Separation of reaction products using Partisil 10 PAC column. Mobile phase; acetonitrile/water (78:22). Flow rate; 1.0 ml/min. Reaction mixture; PNPG (30 mM)+cellotetraose (20 mM)+F-IV-I(4×10^{-4} mM).
B: Separation of reaction products using μ-Porasil column. Initial condition, chroloform/methanol (90:10); eluent, methanol/water (98:2). 1, PNP; 2, $PNPG_1$; 3, $PNPG_2$; 4, $PNPG_3$; 5 $PNPG_4$.

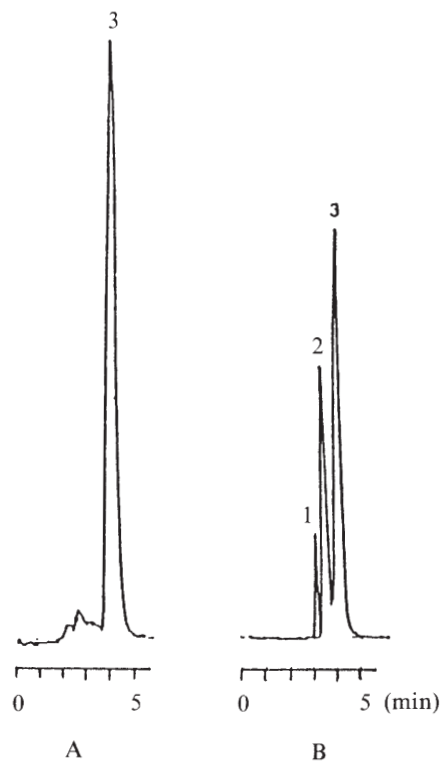

Fig. 9. *Analysis of reaction products from enzymatic hydrolysis of $PNPG_2$ using HPLC.*
A; substrate ($PNPG_2$), B; enzymatic $PNPG_2$ hydrolysis products, 1, PNP; 2, $PNPG_1$; 3, $PNPG_2$.

weight endoglucanase(F-IV-I; Hong *et al.*, 1986) were separated and purified(Fig. 8). The reaction products liberated from $PNPG_2$ by the enzyme were PNP and $PNPG_1$(Fig. 9). This enzyme hydrolyzes the holoside as well as the aglycon linkage of $PNPG_2$. These reaction products are same as that by endoglucanase from *Irpex lasteus* (Nisizawa, 1973). The reaction product by the enzyme toward $PNPG_3$ was only $PNPG_1$(Fig. 10). The Km and V_{max} values of the enzyme were determined by using Lineweaver-Burk plot(not shown) and listed in Table 1. Km values for the holoside linkage and the aglycan linkage of $PNPG_2$ were 0.97 and 10.1 mM, kcat were 1.8×10^5 and 7.5×10^5 min^{-1}, respectively. These result showed that the enzyme had higher affinity toward holoside linkage than aglycon linkage but hydrolyzed $PNPG_2$ preferentially at the aglycon linkage. The Km value for $PNPG_3$ was 69.5 μM and kcat was 1×10^8 min^{-1}. Comparing the Km value and kcat for substrates, it appears that this enzyme have higher affinity and higher turnover number toward $PNPG_3$ than toward $PNPG_2$. From the results mentioned above, it is thought that the higher enzymatic activity toward $PNPG_3$ than $PNPG_2$ comes from the addition of glucosyl moiety to $PNPG_2$, through higher binding feasibility. Considering the results that the only product($PNPG_1$) was liberated from $PNPG_3$ and two products(PNP, $PNPG_1$) from $PNPG_2$, this enzyme is thought to have each binding site that recognize at least cellobiosyl moiety. This supposition is supported by additional result that the activity toward $PNPG_2$ was increased in the presence of cellobiose and dependent on its concentration(1-10 mM: not shown). In conclusive remark, these results could mean that there are at least three glucosyl binding site on one side of the catalytic site and three on the other similar to lysozyme.

When the effect of the gluconolactone on the activity of the enzyme toward $PNPG_2$ was

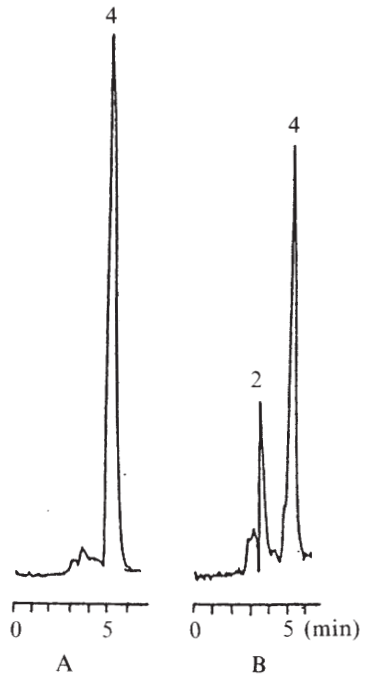

Fig. 10. *Analysis of reaction products from enzymatic hydrolysis of $PNPG_3$ using HPLC.*
A; substrate ($PNPG_3$), B; enzymatic $PNPG_3$ hydrolysis products, 2, $PNPG_1$; 4, $PNPG_3$.

Table 1. *Km and kcat of F-II-IV on $PNPG_2$, $PNPG_3$*

Substrate	Product	Km	kcat (min^{-1})
$PNPG_2$	PNP	0.97 mM	1.8×10^5
	$PNPG_1$	10.10 mM	7.5×10^5
$PNPG_3$	$PNPG_1$	49.50 μM	1.0×10^8

investigated, the gluconolactone was proved to competitive inhibitior. This inhibition constant for gluconolactone was estimated to be about 11.6 μM by using Lineweaver-Burk plot(not shown). But glucose and cellobiose did not show inhibition.

Amino acid composition

The result of the amino acid analysis of the enzyme is summarized in Table 2. The enzyme had a high content of acidic and hydroxylated amino acids in contrast to low content of basic amino acids. The fact is consistent with the isoelectric point and water soluble property of the enzyme. When amino acid composition was expressed in mol percent of constituent amino acids, this enzyme is very similar to endoglucanases(EGI, EGII) from *Trichoderma reesei* and exoglucanase(F-II-V) from *Trichoderma koningii.*

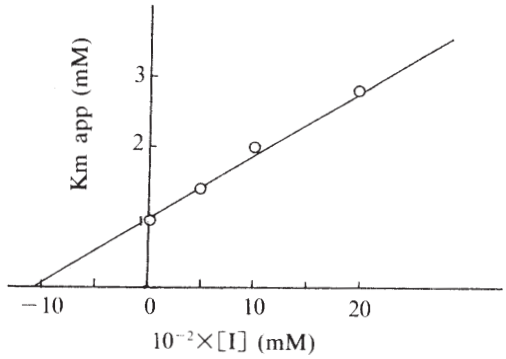

Fig. 11. *Determination of Ki value of gluconolactone.*

Table 2. *Amino acid composition of F-II-IV.*

Amino acid	F-II-VI residue per molecular (mol%)
Asp+Asn	65 (13.5)
Glu+Gln	39 (8.1)
Ser	64 (13.4)
Gly	65 (13.5)
His	7 (1.5)
Arg	11 (2.3)
Thr	48 (10.0)
Ala	31 (6.4)
Pro	48 (10.0)
Tyr	28 (5.8)
Val	17 (4.5)
Met	9 (1.5)
Cys	5 (1.0)
Ile	9 (1.9)
Leu	25 (5.2)
Phe	9 (1.9)
Lys	10 (2.1)
Trp	n.d.
Total	481 (100)

The values are based on a molecular weight of 52,000 assuming that only analyzed amino acids are present.

적 요

*Trichoderma koningii*에서 순수 분리된 β-1,4-D-glucan glucanohydrolase (EC 3.2.1.4; endoglucanase; F-II-IV)는 9%의 탄수화물 포함한 당단백질로 분자량은 58,000이고 등전점은 4.9이였다. 기질 *p*-nitrophenyl cellobioside($PNPG_2$)의 분해산물은 *p*-nitrophenol(PNP)와 *p*-nitrophenyl glucoside($PNPG_1$)으로, 각 분해산물(holoside, aglycon linkage)에 대한

Km 값은 0.97과 10.4 mM이고 kcat 값은 1.8×10^5 min^{-1}와 7.5×10^5 min^{-1}로 나타났다. *p*-Nitrophenyl cellotraose(PNPG$_3$)을 기질로 사용하였을 경우 $PNPG_1$만이 생성되고 Km 값이 69.5 μM, kcat 값은 1×10^8 min^{-1}으로 측정된 점으로 보아 이 효소는 $PNPG_2$ 보다 $PNPG_3$에 대한 기질 친화도가 훨씬 높고 잘 분해하는 것으로 생각된다. 이 효소의 최적 반응 온도는 약 60℃이고 최적 pH는 4.0-4.5이였다. Glucose나 cellobiose는 이 효소에 대하여 억제 효과를 나타내지 않는 반면 gluconolactone은 강력한 경쟁 억제 효과를 보였다. 효소의 아미노산 조성을 분석한 결과, 산성 및 glycine이 포함된 친수성 아미노산이 높은 비율로 존재한 반면 염기성 아미노산은 낮은 비율로 존재하였다.

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