KOR. JOUR. MICROBIOL. Vol. 20, No. 3, 125~133, 1982

Enhanced production of cellulase by a mutant strain of *Aspergillus phoenicis*.*

LEE, Yung Nok and Sang Kyun, KOH

(Department of Biology, Korea University)

Aspergillus phoenicis 의 한 돌연변이주에 의한 cellulase의 생성 및 그 특성

이 영 록 · 고 상 균

(고려대학교 생물학과)

ABSTRACT

Mutational experiments were performed to improve the cellulase productivity of *Aspergillus phoenicis* KU175, isolated from the southern part of Korea, as a high cellulase producer. By treatment ultra-violet light and 4-NQO (4-Nitroquinoline-N-Oxide), mutation was induced,and *A. phoenicis* KU175-115 was finally selected for its highest avicelase production.

Avicelase production of the mutant was increased about 2 times compared with those of the wild strain. However, activities of other hydrolytic enzymes, such as amylase, protease and nuclease, of the mutant strain didn't show a marked difference compared with the wild strain, except slight increase in ribonuclease activity and slight decrease in glucoamylase activity. Avicelases from the mutant strain selected were purified from wheat bran culture by successive salting out, followed by dialysis and column chromatography, and their characteristics were compared with those of the wild strain. Avicelase was separated into three peaks in the mutant strain as well as in the case of the wild strain. Avicelase II activity of the mutant strain was prominently higher than that of the wild strain, while avicelase I and III activities of those were equivalent.

The optimal pH ranges and stability of avicelase II from the mutant strain were pH4~5 and pH3.5~6.0, respectively, as well as in the case of the wild strain. The optimal temperature and thermal stability of avicelase II from the mutant strain were 40~50°C and 20~55°C, respectively. These results were same as those of the wild strain.

By the using of Eadie-Hofstee plot, K_m and V_{max} of avicelase II from the mutant and the wild strain were calculated to be 2.29mg/m*l* and 4.84μg reducing sugar as glucose per min equally, from the line fitted to the data by the least square method. Activity of avicelase II from the mutant strain was slightly activated by Mg^{++} but inhibited by Cu^{++}, Mn^{++} and Zn^{++}, as well as in the case of the wild strain.

Therefore, it was concluded that the mutant didn't induce the formation of another avice-

*This work was supported by a grant from The Korean Traders Scholarship Foundation.

lase isozyme, or the changes in the properties of avicelase, but induce the changes in the productivity of the same avicelase II by the action of regulatory gene.

INTRODUCTION

Cellulases in the nature play an essential role in the carbon cycle. Cellulases are produced by a very large number of micro-organisms. It has been known that there are multiple forms of cellulolytic enzymes in the culture filterates of various micro-organisms, such as *Trichoderma viride*(Okada *et al.*, 1968; Reese *et al.*, 1950; Toda *et al.*, 1971), *Trichoderma koningii*(Iwasaki *et al.*, 1965; Wood *et al.*, 1972), *Pseudomonas fluorescens* var. *cellulosa* (Yamane *et al.*, 1970; Yashikawa *et al.*, 1974), *Aspergillus nidulans* (Maeng *et al.*, 1980) and *Aspergillus niger* (King *et al.*,1963; Li *et al.*, 1963). Some of these cellulases have been purified and investigated extensively.

To increase the rate and yield of glucose from cellulose significant improvements have been achieved through pretreatment of cellulosic material (Knappert *et al.*, 1980) and development of better strains by mutation(Mandels *et al.*, 1971; Montenecourt *et al.*, 1980). However, there are little reports to clarify how the mutation caused.

In the present study, avicelases from the mutant strain were isolated, purified and compared their characteristics with those of the wild strain, to clarify whether the mutation was caused by the variation of a single regulatory gene,or due to the formatoin of another avicelase isozymes.

MATERIALS AND METHODS

1. Experimental organism

Aspergillus phoenicis KU175, isolated from the southern part of Korea as a high cellulase producer (Lee and Park, 1977), was used.

2. Selection medium

The clear zone production medium (Hyun *et al.*, 1978) was used for a primary screening test of mutants. The medium contains 0.5% sodium chloride, 0.2% potassium phosphate (dibasic), 0.1% ferric citrate, 0.1% sodium citrate, 1.0% ammonium sulfate, 0.1% deoxycholate, 1.0% rice straw pulp powder and 1.5% agar.

3. Mutagen treatment

The conidia grown on a Czapek's agar slant for 7 days were suspended in distilled water. The suspension was filtered aseptically through glass wool, and it was sufficiently agitated to segregate clumps of conidia into individual conidium on the shaker-incubator. The number of conidia was counted with haemocytometer, and the conidial suspension was diluted with distilled water to be approximately 10^6cells/m*l*.

UV(Ultra-violet) light irradiation: 10m*l* of the conidial suspension in petri-dish was exposed to at the distance of 5cm by employing a germicidal lamp(15W, Toshiba) in the dark room.

4-NQO(4-Nitroquinoline-N-Oxide)treatment: The conidial suspension was treated with different concentrations of 4-NQO for 30 min.

4. Induction and selection of mutants

Mutation was induced by UV-light irradiation at the dose exhibiting 99.5% lethal rate(3min), or by 4-NQO treatment at the concentration of 95% lethal rate (3.2×10^{-5}M). After mutagen treatment, 0.5m*l* cell suspension was poured out by spreading with L-shaped glass rod on the clear zone production agar plate for a primary screening test. After 20 days incubation at 30°C, desirable mutants were selected according to the ratio of diameters halo and colony, and then were transferred to Czapek's agar slant for final selection.

Final selection was made by the treatment of cellulase activity.

5. Isolation of enzyme solution

The mixture of 5g wheat bran and 4m*l* distilled water was autoclaved at 121°C for 30 min, and was cultured at 30°C for 72 hours after inoculation with one hook of the conidia. After culture, 100m*l* distilled water was added in the culture, and then was maintained at 5°C for 24 hours. Followed by these process, which was filtered, and the filtrate was centrifuged at 10,000rpm for 20min. The supernatant were used as a crude enzyme solutin.

6. Purification of enzyme

A concentrated culture filtrate was prepared by precipitation of the filtrate of 200g wheat bran culture with $(NH_4)_2SO_4$ between the limits of 30% and 80% saturation, and it was centrifugated at 10,000rpm for 20min. The precipitate was dissolved in 0.02M acetate buffer (pH5.0), and it was dialyzed against the same buffer. The dialyzate was subjected to DEAE-Sephadex A-50 column (3.0×40cm) chromatography equilibrated with 0.02M acetate buffer (pH5.0). Each fraction was collected 5m*l*, during the elution with a linear NaCl gradient (0~0.5M) in the same buffer at the flow rate of 60m*l*/h. For futher purification, dialyzate of avicelase II fraction was applied to Sephadex G-100 column (3.0×40cm) and eluted with the same buffer at the flow rate of 40m*l*/h.

7. Assay of enzyme activity

Cellulase: Avicelase activity was determined by the method of Somogyi (1952) and Nelson (1944). 0.5m*l* of enzyme solution was added to the mixture of 1m*l* of 0.2% avicel solution and 0.5m*l* of 0.4M acetate buffer (pH5.0). After incubation for 1 hour at 50°C, 2m*l* of the low alkalinity copper reagent was added to the reaction mixture, and then was heated in boiling water bath for 30min. After cooling, 1m*l* of arsenomolybdate reagent was added, followed by standing at least 20min. When all the cuprous oxide was perfectly dissolved, the solution was diluted with 20m*l* of distilled water. The optical density was measured at 500nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1.0 μmole of reducing sugar as glucose from the substrate per min.

CMCase and salicinase were determined under the same conditions in the case of avicelase, only except that substrates were CMC and salicin, respectively, and reaction time was 30min. Unit of enzyme activity was expressed as same as avicelase.

Amylase: The activity of the glucoamylase was assayed according to the modified Somogyi-Nelson method (Yamada, 1963). The reaction mixture containing 1m*l* of starch solution (10μmole glucose equivalent/m*l*), 1m*l* of 0.4M acetate buffer (pH5.0) and 1m*l* of enzyme solution was incubated at 37°C for 10min. The reaction mixture followed by adding 3m*l* of Somogyi's reagent was boiling for 30min in water bath. After cooling, 2m*l* of Nelson-sulfuric acid reagent (two-fold diluted Nelson's reagent with 1.5N H_2SO_4) was added and the optical density was measured at 660nm. One unit (1GU) is the activity which produce reducing sugar from starch for 10min, equivalent to 1μmole glucose.

The activity of α-amylase was assayed according to the Fuwa's blue value method with a little modification (Yamada, 1963). The reaction mixture containing 1m*l* of the starch solution, 1m*l* of 0.4M acetate buffer (pH5.0) and 1m*l* of enzyme solution was incubated at 37°C for 10min, and the reaction was halted by 5m*l* of N/2,000 I_2-KI solution containing 0.995N HCl. The optical density of the reaction mixture was measured at 700nm(D'). The optical density of the blank test(D) in which the enzyme solution was replaced by distilled water also measured. One unit of α-amylase

(1DU) was expressed as enzyme activity decreasing blue value to 10%. Units were calculated by the following formula: 10(D-D')/D.

Protease: The activity of protease was assayed according to the Folin-Ciocalteu method (1927). For the determination of alkaline protease activity, the reaction mixture containing 1m*l* of enzyme solution and 1m*l* 1.5% Hammastern milk casein in 0.1M phosphate buffer (pH8.0) was incubated with 2m*l* of 0.44M TCA (trichloroacetic acid). The amount of TCA soluble non-proteinous material showing positive reaction to Folin reagent was determined colorimetrically at 660nm. For the assay of acid and neutral protease activities, 1.5% Hammastern milk casein in 0.1M lactate buffer (pH3.0), or in 0.1M phosphate buffer (pH6.0) was used as substrate solution, respectively. One unit (1PU) of protease activity was expressed the amount of enzyme which will liberate the above non-proteinous substance equivalent to 1μg of tyrosine per min.

Nuclease: DNase activity was determined by basis on the assay of acid soluble deoxypentose compounds released from DNA by the enzyme action (McDonald, 1964). Dexypentose in the cold 3M TCA-soluble fraction was measured by the diphenylamine method of Dische(1962). The reaction mixture consisting of 0.5m*l* of 0.1% DNA, 0.2m*l* of Tris-HCl buffer(pH9.0), 0.1m*l* of 0.1M $MgSO_4$, and 0.2m*l* of enzyme solution was incubated at 37°C. After 30min, 0.5m*l* of 3M TCA was added to the reaction mixture,and then the solution was centrifuged. To 1m*l* of supernatant was added 2m*l* of diphenylamine reagent. The reaction mixture was heated 10min at 100°C. After cooling to room temperature, readings were made at 660nm. One activity unit corresponds to an increment of 0.1 reading value in the spectrophotometer induced in 30min by one m*l* of the enzyme solution.

RNase activity was determined as described by Keto-Ikeda(1968). The reaction mixture consisted of 0.5m*l* of 0.6% RNA solution, 0.3m*l* of 0.1M acetate buffer (pH4.5), and 0.1m*l* of enzyme solution. After incubation at 37°C for 30min, the increment in absorbance at 260nm of the soup fraction was measured. One activity unit corresponds to an increment of 0.1 reading value in the spectrophotometer induced in 30min by one m*l* of the enzyme solution.

8. Determination of protein

The rotein concentration of enzyme solutions at each step of the purification were determined from the absorbance at 280nm. When necessary, protein concentration was also estimated by the Lowry *et al.*(1951) using crytalline bovine serum albumin as a standard, the absorbance at 550nm being measured.

RESULTS AND DISCUSION

1. Selection of enhanced cellulase producing mutants

About 200 mutant strains, induced by UV-light or 4-NQO, were selected by using the halo and colony ratio as the criterion for a primary screening test. Four mutants among them with the increased cellulase production in wheat bran culture were obtained by second selection. Their cellulase activities were shown in Table 1.

Table 1. Selected mutant strains of *Aspergillus phoenicis* for predominent cellulase production in wheat bran.

Strain	Mutagen	Cellulase (10^{-2}unit/m*l*)		
		Avicelase	CMCase	Salicnase
KU 175*		1.08	1.85	3.08
KU 175-37	UV	1.69	2.16	2.27
KU 175-67	4-NQO	1.54	2.16	1.23
KU 175-70	4-NQO	2.16	2.46	2.77
KU 175-115	UV	2.31	1.85	2.16

*wild strain

A. phoenicis KU175-115, a UV-induced mutant, was selected as the most predominent mutant of avicelase production. Avicelase production of the selected mutant, *A. phoenicis* KU175-115, was increased about 2 times compared with the wild strain.

2. Other enzyme activities of the mutant

Protease, amylase and nuclease activities of the mutant strain in wheat bran culture were shown Table 2. RNase activity of the mutant strain was slightly increased, while glucoamylase activity was slightly decreased, compared with those of the wild strain.

Table 2. Amylase, protease and nuclease activities of *A. phoenicis* KU 175-115.

Strain	Amylase activity		Protease activity (PU/m*l*)			Nuclease activity (unit/m*l*)	
	Glucoamylase (GU/m*l*)	α-amylase (DU/m*l*)	acid	neutral	alkaline	DNase	RNase
KU 175-115	4.31	73.0	4.24	1.41	1.41	2.4	7.0
KU 175	5.70	73.0	4.24	1.41	1.82	2.3	5.5

3. Chromatography on DEAE-Sephadex A-50

Column chromatography on DEAE-Sephadex A-50 of avicelase from the mutant and wild strain was shown in Fig.1. Avicelase was separated into three peaks, I (fraction No's. 11 ~21), II (fraction No's. 31~45) and III(fraction No's. 79~86) in the mutant as well as the wild strain. Avicelase II activity of the mutant strain was prominently higher than that of the wild strain, while avicelase I and III activities of those equivalent.

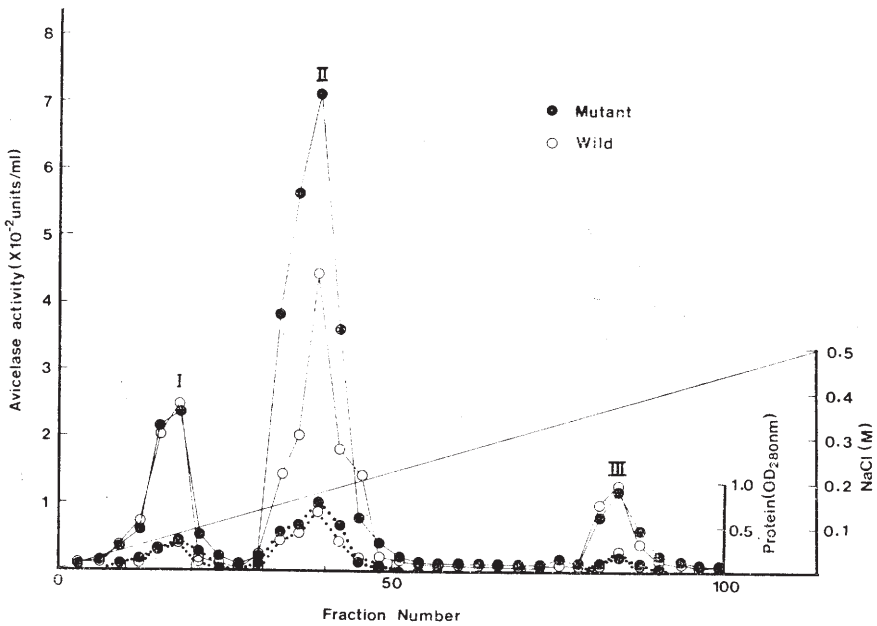

Fig. 1. Fractionation of avicelase by ion-exchange chromatography on DEAE-Sephadex A-50. (—— : avicelase activity, ····· : protein)

4. Column chromatography of avicelase II on Sephadex G-100

Avicelase II was rechromatographed on Sephadex G-100 as shown in Fig.2, and Table 3 is shown the purification procedures. Avicelase II from the mutant and wild strain was 6.5 fold and 8.7 fold purification respectively, compared with those of crude enzymes.

Table 3. Purification and specific activity of avicelase from *A. phoenicis* KU 175-115.

Fraction	Volume(ml)	Total activity (unit)	Total protein (mg)	Specific activity (10^{-2}unit/mg)
Crude enzyme				
KU 175-115	750	15.34	142.3	11
KU 175	740	7.80	140.6	6
Dialysis				
KU 175-115	40	9.54	20.1	31
KU 175	40	4.10	15.4	27
DEAE-Sephadex A-50(Peak II)				
KU 175-115	75	3.24	5.3	61
KU 175	70	1.92	4.3	45
Sephadex G-100				
KU 175-115	100	1.92	2.7	71
KU 175	100	1.04	2.0	52

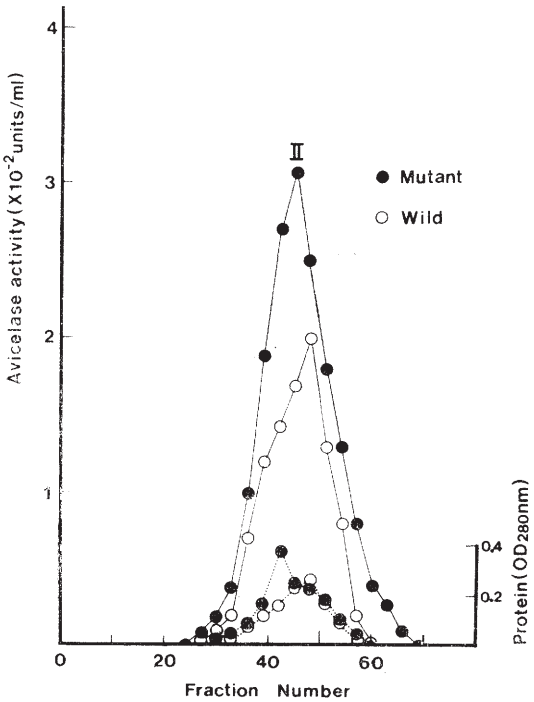

Fig. 2. Gel-filtration of avicelase II on a Sephadex G-100.
(—— : avicelase activity, ····· : protein)

5. Effect of pH and temperature on the activity and stability of avicelase II

The optimal pH ranges and stability of avicelase II from the mutant strain were pH4~5 and pH3.5~6.0, respectively, as well as in the case of the wild strain, as shown in Fig. 3 and Fig.4.

The optimal temperature and thermal stability of avicelase II from the mutant strain were 40~50°C and 20~55°C, respectively. These results were same as those of the wild strain as shown in Fig.5 and Fig.6.

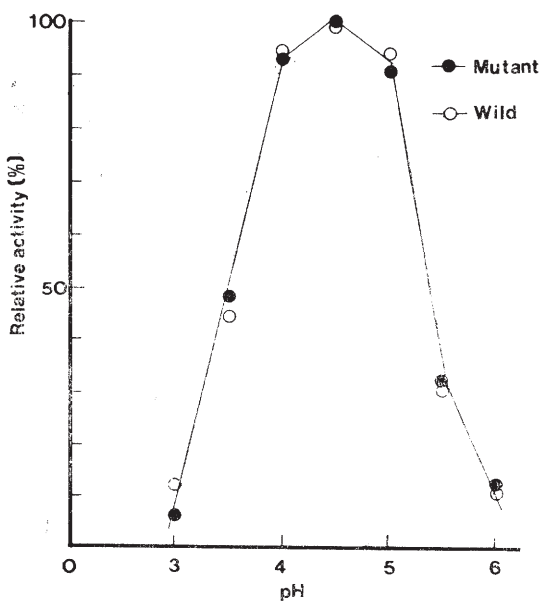

Fig. 3. Effect of pH on the avicelase II activity.

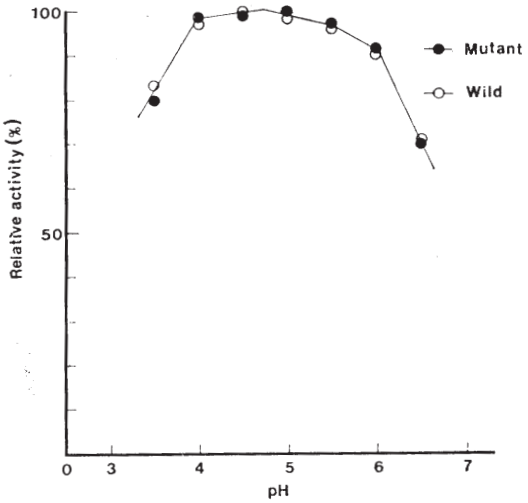

Fig. 4. pH-stability curve of avicelase II.

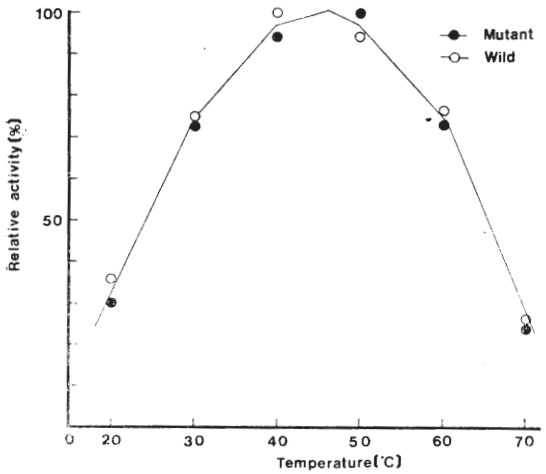

Fig. 5. Effect of temperature on the avicelase II activity.

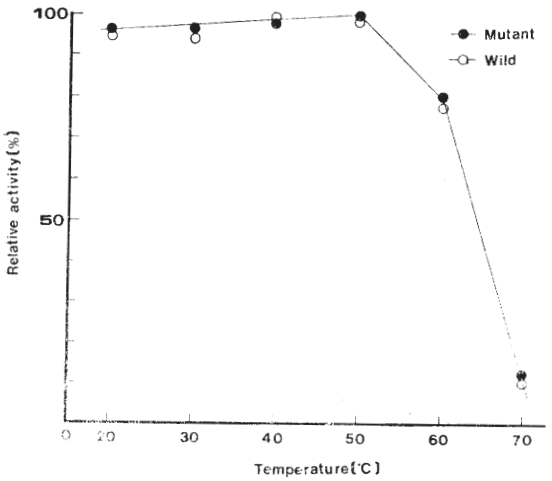

Fig. 6. Thermal-stability curve of avicelase II.

6. K_m and V_{max} value of avicelase II

According to Eadie-Hofstee plot of influence of substrate concentration upon the reaction velocity of avicelase II from the mutant and wild strain, K_m and V_{max} were calculated to be 0.229% (2.29mg/ml) of avicel and 2.686×10^{-2} units (4.84μg reducing sugar as glucose per min) from the line fitted to the data by the least square method, as shown Fig.7. The K_m value was similar to that of *Trichoderma-koningii* (Hong *et al.*, 1976) but this result is far from that of Halliwell *et al.*, (1973), 0.05%

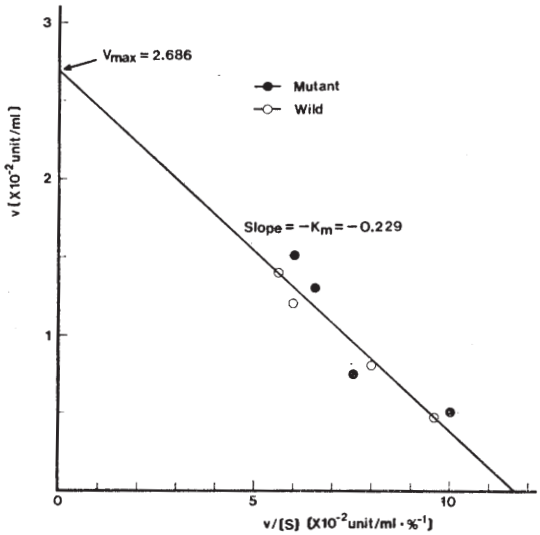

Fig. 7. Eadie-Hofstee plot of the action of avicelase II. The line fitted by the least square method to the points.

7. Effect of metal ions on avicelase II activity

In the effect of metal ions, avicelase II from the mutant strain slightly activated by Mg^{++} but inhibited by Cu^{++}, Mn^{++} and Zn^{++}, as well as in the case of the wild strain, as

Table 4. Effects of metal ions on the activity of avicelase II.

Treatment	Relative activity (%)	
(2×10^{-3}M)	KU 175-115	KU 175
$CuSO_4$	80.2	81.4
$MgCl_2$	107.6	103.6
$MnSO_4$	90.6	90.3
$ZnSO_4$	78.3	76.7

shown in Table 4. Considering the optimal pH-temperature ranges, pH-termal stability, K_m-V_{max}, and effect of metal ions of avicelase II from the mutant and wild strain, it was concluded that the mutant did not induced the formation of another avicelase isozyme, or the changes in the properties of avicelase, but induce the increased productivity of the same avicelase II by the action of regulatory gene.

적 요

셀룰라아제의 생성능이 비교적 우수한 야생균주 *A. phoenicis*에 자외선 또는 4-NQO(4-Nitroquinoline-N-Oxide)를 처리하여 다수의 돌연변이를 유발시키고 셀룰라아제의 생성능이 증가된 한 돌연변이 주를 선별하였다. 이 변이주의 다른 생리적, 형태적 특징을 조사하여 표현형에 있어서의 특징을 밝히고 이 균주가 생성한 셀룰라아제를 분리정제하고 그 효소학적 성질을 조사하여 모균주의 그것과 비교하므로써 이 돌연변이 주가 조절 유전자의 변화에 기인하는 것인지 또는 다른 isozyme의 생성을 유발한 것인지의 여부를 밝히고저 하였다.

돌연변이를 유발시킨 포자 현탁액은 펄프가루를 탄소원으로 하는 deoxycholate agar 배지에 접종하여 30°C에서 20일간 배양한 후 clear zone method로 200여 균주를 일차적으로 선별하였다. 일차적으로 선별한 돌연변이 균주들은 그들의 셀룰라아제 활성을 avicel, CMC 또는 salicin 등을 각각 기질로 하여 Somogyi-Nelson의 방법으로 측정비교하여 이들 효소의 생성능이 비교적 우수한 4균주를 이차선별하고, 그중 avicelase 활성이 가장 높은 우량균주, KU 175-115를 최종 선별하였다.

이 돌연변이 균주는 천연상태의 셀룰로오즈를 일차적으로 공격하는 C_1-효소에 해당하는 avicelase 활성이 모균주(야생균주)에 비해 2배 가량 높았다. 이 돌연변이 균주의 아밀라아제, 푸로테아제 및 뉴클라아제 등 셀룰라아제 이외의 다른 가수분해효소의 활성은 야생균주의 그것과 큰 차이가 없었으나 glucoamylase 활성은 약간 감소하였고 ribonuclease 활성은 약간 증가하였다.

이 돌연변이주가 생성한 셀룰라아제를 황산암모니움에 대한 염석과 투석 그리고 DEAE-Sephadex A-50 및 Sephadex G-100 Column Chromatography 등으로 분리정제하여 온도 및 pH에 대한 안정도 및 최적 활성, V_{max} 및 K_m값, 그리고 활성에 미치는 Cu^{++}, Mg^{++}, Mn. , Zn^{++} 등 여러가지 금속이온의 영향 등을 조사하여 모균주의 그것과 비교하였다.

DEAE-Sephadex A-50 Column Chromatography에서 3개의 avicelase fraction이 분리되었는데, 그중 돌연변이 균주의 avicelase Ⅱ fraction의 전체활성은 야생균주의 그것에 비해 현저히 높았다. 그러나 avicelase Ⅰ 및 avicelase Ⅲ fraction의 전체활성은 야생균주나 돌연변이 주가 큰 차이를 나타내지 아니하였다. Sephadex G-100 Column으로 더욱 정제한 avicelase Ⅱ의 최적 및 안정도 pH영역은 돌연변이주나 야생균주에서 다같이 pH 4~5 및 3.5~6.0이었고 최적 및 안정 영역도 다같이 40~50°C 및 20~55°C였다. avicelase Ⅱ의 기질농도에 따른 반응속도로부터 산출한 Eadie-Hofstee plot에서 K_m 및 V_{max}의 값은 돌연변이주나 야생균주에서 다같이 각각 2.29mg/ml 및 4.84μg/min을 나타내었다. 또한 이들 avicelase Ⅱ의 활성에 미치는 Cu^{++}, Mn^{++}, Zn^{++} 등 금속이온의 영향도 돌연변이 주에서나 야생균주에서 비슷한 경향을 나타내었다. 따라서 이 돌연변이주는 다른 avicelase isozyme의 생성이나 avicelase의 성질의 변화를 유발한 것이 아니라 조절 유전자의 변화로 같은 avicelase Ⅱ의 생성능이 증가된 돌연변이주인 것으로 생각되었다.

REFERENCES

1. Dische, Z., 1962. Color reactions of pentoses.In Methods in Carbohydrate Chemistry. Vol. 1,484~488. Academic Press Inc., New York.
2. Folin, O. and V. Ciocalteu, 1927. Tyrosine and tryptophan determinations in proteins. *J. Biol. Chem.* **73**, 627~650.
3. Halliwell, G. and M. Griffin, 1973. The nature and mode of action of the cellulolytic component of *Trichoderma koningii* on native cellulose. *Biochem. J.* **135**, 587~594.
4. Hofstee, B.H.J., 1952. On the evaluation of the constants V_m and K_m in enzyme reactions. *Science* **116**, 329~331.

5. Hong, S.W., K.H. Min and Y.H. Rhee, 1976. Partial purification and some properties of cellulase components from *Trichoderma koningii*. *Kor. Jour. Microbiol.* **14**, 84~94.
6. Hyun, H.H., H.S. Baik, I.B. Lee and S.Y. Lee, 1980. Plating technique for cellulase mutants of *Trichoderma viride*. *Korean J. Appl. Microbiol. Bioeng.* **6**, 129~133.
7. Iwasaki, T.R. Ikeda, K. Hayashi and M. Funatsu, 1965. Biochemical studies on cellulase. II. Properties of two types of cellulase from *Trichoderma koningii*. *J. Biochem.* **57**, 478~487.
8. Kato, M. and Y. Ikeda, 1968. On the deoxyribonucleases, K1 and K2, isolated from mycelia of *Aspergillus oryzae*. I. Isolation and purification of DNase K1 and K2. *J. Biochem.* **64**, 321~328.
9. King, K.W.and R.M. Smibert, 1963. Distinctive properties of glucosidases and related enzymes derived from a commercial *Aspergillus niger* cellulase. *Appl. Microbiol.* **11**, 315~319.
10. Knappert, D., H. Grethlein and A. Converse, 1980. Partial acid hydrolysis of cellulosic materials as a pretreatment for enzymatic hydrolysis. *Biotech. Bioeng.* **22**, 1459~1463.
11. Lee, Y.N. and Y.K. Park, 1977. Cellulase activities of Aspergilli distributed in South Korea. II. Avicelase, CMCase and salicinase activities of the strains surveyed in taxonomical viewpoint. *Kor. Jour. Microbiol.* **15**, 113~121.
12. Li, L.H. and K.W. King, 1963. Fractionation of β-glucosidases and related extracellular enzymes from *Aspergillus niger*, *Appl. Microbiol.* **11**, 320~325.
13, 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.
14. Maeng, P.J., S.W. Hong and Y.C. Hah, 1980. Purification and properties of carboxylmethylcellulases from *Aspergillus nidulans* FGSG 159. *Kor. Jour. Microbiol.* **18**, 133~147.
15. Mandels, M., J. Weber and R. Parizek, 1971. Enhanced cellulase production by a mutant of *Trichoderma viride*. *Appl. Microbiol.* **21**, 152~154.
16. McDonald, M.R., 1964. Deoxyribonucleases. In Methods in Enzymology. Vol. II, 437~447. Academic Press Inc., New York.
17. Montenecourt, B.S. and D.E. Eveleigh, 1978. Hypercellulolytic mutants and their role in saccharification. In Second Annual Fuels from Biomass Symposium, pp. 613~625.
18. Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **153**, 375~380.
19. Okada, G.K. Nisizawa and H. Suzuki, 1968. Cellulase components from *Trichderma viride*. *J. Biochem.* **63**, 591~607.
20. Reese, E.T., R.G.H. Sui and H.S. Levinson, 1950. The biological degredation of soluble cellulose derivatives and its relationship to the mechanism of cellulase hydrolysis. *J. Bacteriol.* **59**, 485~497.
21. Somogyi, M., 1952. Notes on sugar determinations. *J. Biol. Chem.* **195**, 19~23.
22. Toda, S. H. Suzuki, and K. Nisizawa, 1971. Some enzymatic properties and substrate specificities of *Trichoderma* cellulases with special reference to their activity toward xylan. *J. Ferment. Technol.* **49**, 499~521.
23. Wood, T.M. and S.I. McCrae, 1972. The purification and properties of the C_1 component of *Trichoderma koningii* cellulase. *Biochem. J.***128**, 1183~1192.
24. Yamada, N., 1963. Studies on acid-resistant amylase(II). An improved method of amylase determination. *Agr. Chem. Soc. of Japan.* **37**, 633-636.
25. Yamane, K., H. Suzuki and K. Nisizawa, 1970. Purification and properties of extracellular and cell-bound cellulase components of *Pseudomonas fluorescens* var. *cellulosa*. *J. Biochem.* **67**, 19~35.
26. Yashikawa, T., H. Suzuki and K. Nisizawa, 1974. Biogenesis of multiple cellulase components of *Pseudomanas fluorescens* var. *cellulosa*. I. Effects of culture conditions on the multiplicity of cellulase. *J. Biochem.* **75**, 531~540.