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Further induction of amylase producing mutants from a highly proteolytic mutant strain of *Aspergillus flavus**

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돌연변이에의한 *Aspergillus flavus*의 아밀라아제 생성능의 개량

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ABSTRACT

A mutant strain having increased productivity of both enzymes,protease and amylase, was obtained from *A. flavus* KU 153, isolated from South Korea for its high protease production by successive ultra-violet light irradiation. Two glucoamylases from the mutant strain selected were purified from wheat branculture by successive salting out,followed by dialysis and column chromatography, and their characteristics were compared with those of the wild strain.

Glucoamylase production of the mutant selected was increased about 3.3 times compared with the wild strain, and 2.1 times compared with the parental strain,α-amylase activity of the mutant selected was about 2 times higher than that of the wild strain or the parental strain. Protease and cellulase productivities of the mutant selected were all alike compared with those of the highly proteolytic mutant, the parental strain. Therefore, it was considered that the back mutation on the protease production did not occurred in the formation process of the glucoamylase producing mutant.

Total activities of glucoamylase Ⅰ and Ⅱ from the mutant selected were 2.86 and 3.65 times higher compared with those from the wild strain, respectively. Considering the optimal pH-thermal stability and Km-Vmax value of glucoamylase Ⅰ and Ⅱ from both strains, wild and mutant, it was deduced that the characteristics of glucoamylase Ⅰ and Ⅱ from the wild strain did not altered during the mutation process.

Therefore, it was concluded that the selected mutant did not induce the formation of another glucoamylase isozyme, or the changes in the characteristics of the glucoamylase, but induce the productivity of the same glucoamylase Ⅰ and Ⅱ by the action of regulatory gene.

INTRODUCTION

Many studies concerning the induction of mutants of mold have been made to increase the enzyme productivity of the fungus. Highly proteolytic mutant srain of *Aspergillus*

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(Iguchi,1955; Metsushima and Shimada,1967; Sekine *et al.*, 1969), enhanced cellulase producing mutant strain of *Trichoderma*(Mandels et, al., 1971), and potent glucoamylase producing mutant strains of *Rhizopus* (Lin, 1972), *Aspergillus*(Meyrath *et, al.*, 1971) and *Endomxcopsis* (Afanaseva and Zeitseva, 1977) have been reported. However, most of these mutant strains were those of the enhanced producing ability of a single enzyme different from each other. If we shall develop a mutant strain having potent productivity of two or more hydrolytic enzymes, it will be possible to harvest a great deal of so many kinds of enzymes at the same time by a single culture.

In our previous paper (Lee and Koh, 1980) we selected *Aspergillus flavus* KU 153 isolated from South Korea for its high protease productivity, and obtained a UV-induced mutant strain, *A.flavus* KU 153-10, having an increased rate of about 2.7 times higher for acid protease and 1.8 times higher for neutral and alkaline proteases production, respectively, compared with those of the parental strain. In present study, attemps were made to select enhanced glucoamylase producing mutants from a powerful protease producing strain, *A. flavus* KU 153-10 by UV-light or other mutagen treatment, so that to obtain a mutant strain having increased productivity of both enzymes, protease and amylase.

The glucoamylases from the mutant strain selected were isolated, purified,and compared their characteristics with those of the wild strain, to clearify whether the mutation was caused by the variation of a single regulatory gene,or due to the formation of another glucoamylase isozymes. The results will be reported in the present paper.

MATERIALS AND METHOD

1. Experimental organism

The mutant strain, *Aspergillus flavus* KU 153-10, induced from A. *flavus* KU 153 by UV-light irradiation was used as a parental strain.

2. Selection medium

The modified clear zone production medium (Han,1977) was used for a primary screening test of mutants. The composition of the medium shows in Table 1.

Table 1. The composition of the modified clear-zone production agar medium

Starch	10g,	Citric acid	10g
$(NH_4)_2SO_4$	16g,	Acetic acid	6.9m*l*
$MgSO_4\ 7H_2O$	0.6g,	Yeast extract	2g
$FeCl_3\ 6H_2O$	0.1g,	Agar	20g
KH_2PO_4	4g,	D.W.	2,000m*l*

(pH is adjusted to 6.8 with 20% NaOH solution.)

3. Mutagen treatment

The conidia grown on a Czapek's agar slant for 10~14 days were suspended in distilled water by vibration. The suspension was filtered aseptically through glass wool. Thereafter, the conidial suspensions were agitated sufficiently to segregate clumps of conidia into individual conidium on the shake-incubator.

Number of conidia was counted with Haemacytometer, and the conidial suspension was diluted with distilled water to be approximately 10^5~10^6cells/m*l*.

UV-light treatment; 10m*l* of the conidial suspension in petri-dishes were exposed to ultra-violet light at the distance of 5cm, for 30sec~5min, by employing a germicidal lamp (15W, Toshiba)in the dark room to prevent from photore activation (Kelner, 1949).

4-NQO(4-Nitroquinoline-N-Oxide) treatm-

ent; the conidial suspension was treated with 3.2×10^{-7}M—3.2×10^{-3}M 4-NQO for 30min, the reaction was terminated by the $10-10^{5}$ fold dilution.

4. Measurements of survival rate

After mutagen treatment, 0.5m*l* diluted conidial suspension was poured on the Czapek's agar plates by spreading with L-shaped glass rod. After 3 days incubation at 30°C, colonies formed were counted with colony counter.

5. Induction and selection of mutants

Mutation was induced by UV-light irradiation at the does exhibiting 99.9% lethal rate (3min), or by 4-NQO treatment at the concentration of 99.1% lethal rate (3.2×10^{-5}M).

After mutagen treatment, 0.5m*l* cell suspension was poured out by spreading with glass rod on the clear zone production agar plate for a primary screening test. After 3 days incubation at 30°C, prespective colonies were selected according to the ratio of diameters of the halo and colony (HC-ratio), and transferred to Czapek's agar slant for final selection. Final selection was carried out by direct measurement of enzyme activities.

6. Isolation of enzyme solution

The mixture of 5g wheat bran and 4m*l* distilled water was autoclaved at 121°C for 30min, and cultured at 30°C for 72 hrs, after inoculation with one hook of the conidia. After cultivation, 50m*l* distilled water was added in the culture media, and then maintained at 5°C for 12 hrs. Followed by these process, centrifugation was performed at 12,000g for 20min. The filterates from the supernatant were used as a crude enzyme solution.

7. Purification of enzyme

After crude enzyme was extracted with 40% ammonium sulfate over night at 4°C, the precipitate formed was removed, by centrifugation at 7,000rpm for 20min. To the supernatant 80% ammonium sulfate was added, allowed to extract for over night at 4°C, and then it was centrifused at 7,000rpm for 20min. The precipitate was dissolved in 30m*l* of distilled water, and dialyzed against distilled water for 2 days changing distilled water several times. For further purification,the dialyzates are subjec ted to DEAE (Diethylaminoethyl) Sephadex A-50 column (2.6×3.0) chromatography equilibrated with 0.05M acetate buffer (pH 5.0). Each fraction is collected for 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.

8. Assay of enzyme activity

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 (1% mole glucose equivalent/m*l*), 1m*l* of acetate buffer (pH 5.0) 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(2-fold diluted Nelson's reagent with 1.5N H_2SO_4)was added, and the optical density was measured at 660nm. One unit is the activity which produce reducing power from starch for 10min., equivalent to 1% mole glucose.

The activity of α-amylase was assayed according to the Fuwa's blue value method with a little modification. The reaction mixture containing 1 m*l* of starch solution, 1 m*l* of starch solution, 1m*l* of acetate buffer(pH 5.0) 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 was also measured. Unit of α-amylase (DU) was expressed as enzyme activity decreasing blue value to 10%. Units were calculated by the following formula: $\frac{10(D'-D)}{D'}$(Du/m*l*)

Protease The activity of protease was assayed according to the Folin-Ciocalteu reagent method(1927). For the determination of alkaline protease activity, the reaction mixture containing 1m*l* of enzyme solution and 1.5% Hammastern milk casein in 0.1M phosphate buffer(pH 8.0) was incubated at 40°C for 20min, and reaction was halted with 2m*l* of 0.44M trichloroacetic acid(TCA).

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 (pH 3.0), or in 0.1M phosphate buffer(pH 6.0) was used as substrate solution, respectively. One unit (1PU) of protease activity is espressed the mount of enzyme which will liberate the above non-proteinous substance equivalent to 1g of tyrosine per min.

Cellulase:Avicelase activity was determined by Somogyi-Nelson's method. For the determination of reducing sugar, Nelson's method (1944) was used. 0.5m*l* enzyme solution diluted ten folds was added to the mixture of 1m*l* of 0.2% avicel solution and 0.5m*l* acetate buffer solution (pH 5.0). After incubation for 1hr at 50°C, 2m*l* of the low alkalinity copper reagent was added to the reaction mixture, and then heated in boiling water bath for 30min.

After cooling, 1m*l* of arsenomolybdate reagent was added, and then allowed to stand 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. Avicelase activity was defined as the amounts of glucose produced by 1m*l* enzyme solution from the avical per 1hr.

Carboxymethyl cellulase and salicinaseactivities were determined by the same method for avicelase, only except that reaction time of enzyme with substrate was for 30min at 50°C. Carboxymethyl cellulase and salicinase activities were defined as the amount of glucose produced by 1m*l* enzyme solution from the CMC and salicin per 30min, respectively.

9. Determination of protein

Protein was determined by the modified Robinson-Hogden biuret method (Herbert *et. al.* 1971). The protein profiles in column chromatography were followed by measuring the absorbances of each fractions at 280nm

RESULTS AND DISCUSSION

1. Effect of UV-light and 4-NQO on the survival rate of *A. flavus*

In the sensitivity to the ultraviolet light, there is no differences between the wild and mutant strains of *A. flavus*. As indicated in Fig. 1, lethal dose (LD_{50}) was reached at 30 second irradiation, and at a dose for 3 minute irradiation lethal rate was up to 99.9% for both strains. *A. flavus* was very sensitive to the anothther chemical mutagen, 4-NQO. It exhibited 50% lethal rate at the concentration of 2.0×10^{-7}M, and with 3.2×10^{-5}M 4-NQO, lethal rate was increased up to 99.1 % as shown in Fig. 2.

2. Selection of enhanced glucoamylase producing mutants

About 120 mutant strains, induced by ult-

raviolet light or4-NQO, were selected by using the HC-ratio as the criterion for a primary screening test. Five mutants of them with increased glucoamylase production in wheat bran were obtained by second selection. Their glucoamylase and α-amylase activities were shown in Table 2. as well as treated mutagen. *A. flavus* KU 153-1022, a UV-induced mutant, was selected as the most predominant mutant of glucoamylase production. Glucoamylase production of the selected mutant, *A. flavus* KU 153-1022, was increased 3.2 and 2.1 times compared with wild strain, KU153 and the parental strain, KU153-10, respectively. α-amylase activity of the selected mutant was increased about 2 times compared with KU153 or KU153-10(Fig. 3).

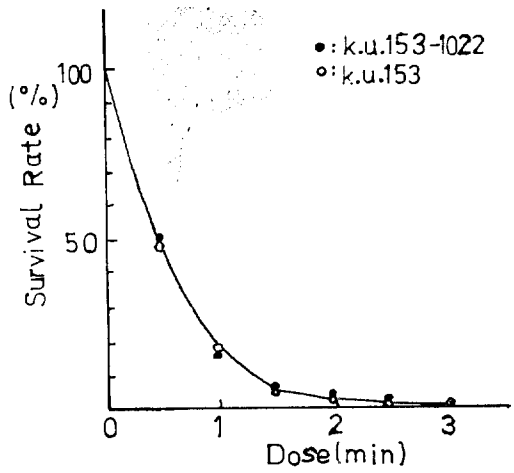

Fig.1. Survival rate of *A. flavus* KU153-10 irradiated with UV-light

3. Protease and cellulase production of the Mutant selected

Protease and cellulase production of the selected mutant strain, *A. flavus* KU153-1022, in wheat bran were shown in Table 3. Acid-, neutral- and alkaline-protease productions of the mutant strain selected were exceedingly high compared with the wild strain, and similar to compared with those of the highly proteolytic mutant strain KU153-10, the pa-

Table 2. Selected mutant strains of *Aspergillus flavus* for predominant amylase production in wheat bran

strain	mutagen	glucoamylase(GU/ml)	α-amylase(DU/ml)
KU153-109	UV	254.3	2,367
KU153-1022	UV	335.7	2,886
KU153-1072	4-NQO	248.6	2,234
KU153-1096	4-NQO	283.2	2,563
KU153-10118	UV	235.4	2,457
KU153-10*	UV	160.8	1,438
KU153**		104.5	1,395

* high protease producing mutant strain
** high protease producing wild strain

Table 3. Protease and cellulase activities of *A. flavus* KU153-1022

strain	protease activity(PU/ml)			cellulase activity(mg/ml)		
	acid	neutral	alkaline	Avicelase	CMCase	Salicinase
KU153-1022	56.8	84.6	78.3	4.1	3.6	7.3
KU153-10	55.0	72.8	69.4	3.6	3.5	6.8
KU153	20.6	40.4	38.3	0.9	1.1	3.7

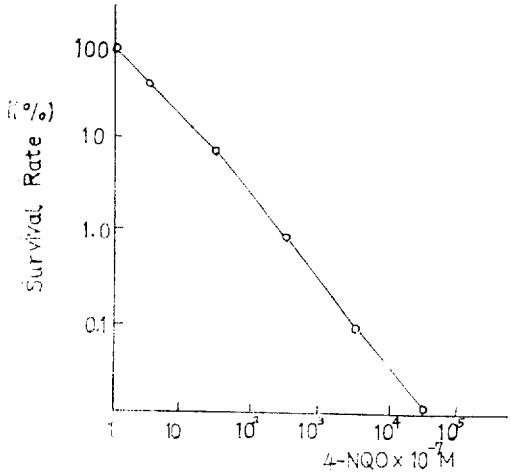

Fig. 2. Survival rate of *A. flavus* KU153-10 treated with 4-NQO

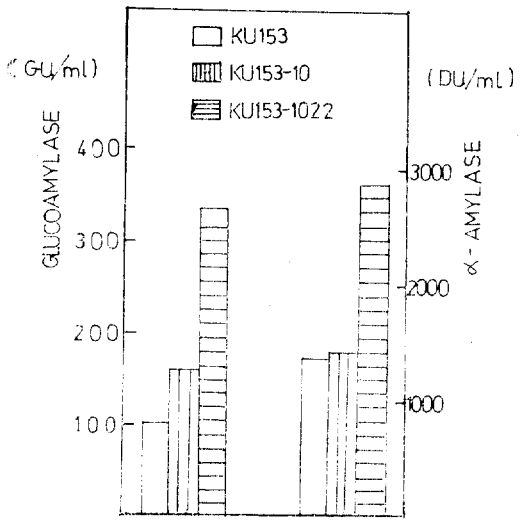

Fig. 3. Amylase activities of *A. flavus* KU153-10 and KU153-1022

rental strain. Therefore, it is considered that the back mutation on the protease production did not occurre in the formation process of glucoamylase producing mutant. On the other hand, avicelase, carboxymethyl cellulase and salicinase activities of the mutant selected were similar to those of the parental strain, KU153-10.

4. Two glucoamylase from the selected mutant

Column chromatography on DEAE-Sephadex

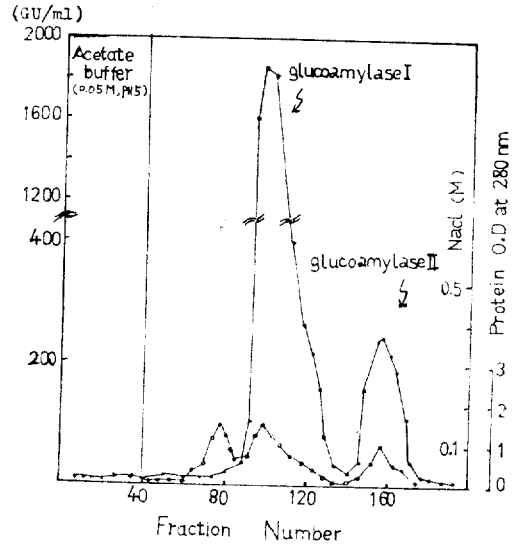

Fig. 4. Column chromatography of glucoamylase from selected mutant, *A. flavus* KU153-1022 on DEAE-Sephadex A-50

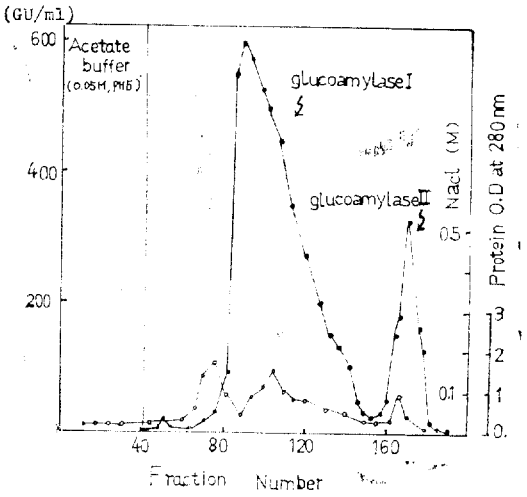

Fig. 5. Column chromatography of glucoamylase from protease producing mutant, *A. flavus* KU153-10the parental strain on DEAE-SephadexA-50

A-50 of glucoamylase from selected mutant strain, KU153-1022, was shown in Fig. 4, and those from the parental strain, KU153-10 and the wild strain, KU153 were shown in Fig. 5 and Fig. 6, respectively. Two glucoamylase were isolated for each strains. They

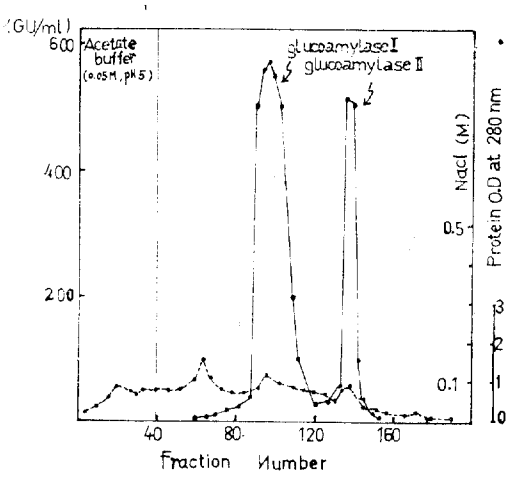

Fig. 6. Column chromatography of glucoamylase from wild strain, *A. flavus* KU153 on DEAE-Sephadex A-50

were named glucoamylase I and glucoamylase II according to elution rate,respectively. Peak of glucoamylase I from selected mutant strain was very high compared with that from the parental strain or the wild strain. Specific activities of purified two glucoamylase I and II from the mutant selected were estimated to be about 10.7 and 8.4 times higher compared with those of crude enzyme, respectively. Total activities of glucoamylase I and II from the selected mutant strain were increased about 2.86 and 3.65 times compared with those from the wild strain, respectively(Table 4).

5. Effect of pH on the activity and stability of glucoamylase

The optimal pH ranges of the glucoamylase I and glucoamylase II from the mutant strain selected were pH 5-7 equally. These optimal pH ranges were very similar to those from the wild strain exhibiting pH 4~6.5 and pH 4~7, respectively, as shown in Fig. 7 and Fig.8. On the other hand,pH stability of glucoamylase I and glucoamylase II from mutant strain were pH 4~8 and pH 4~7, respectively. These were quite same value, compared with those from the wild strain, as shown in the Fig. 9 and Fig. 10.

6. Effect of temperature on the activity and stability of glucoamylase

The optimal temperature ranges for both glucoamylase activities from the mutant selected were 40~50°C, and both enzymes were

Table 4. Purification and overall recovery of glucoamylase from *A. flavus* KU 153-1022

Fraction	Vol. (ml)	Total Activity value (units)	Activity increase relative to wild strain (times)	Total Protein(mg)	Specific Activity (units/mg)
Crude enzyme					
KU 153	900	133,825.0		5,635.5	23.7
KU 153-1022	900	407,833.0		10,458.5	39.0
Dialysis					
KU 153	30	125,449.3		878.5	142.8
KU 153-1022	35	365,506.0		1,439.3	253.9
DEAE-Sephadex					
KU 153					
glucoamylase I	135	70,139.5	1	338.8	207.0
glucoamylase II	55	21,648.0	1	107.2	201.9
KU153-1022					
glucoamylase I	230	200,748.6	2.86	479.1	419.0
glucoamylase II	130	78,990.5	3.65	240.8	328.0

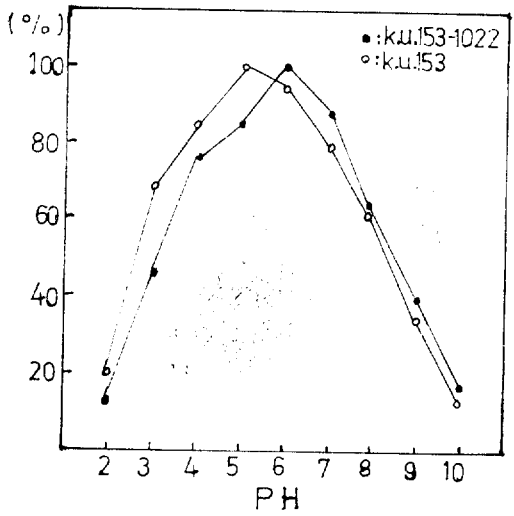

Fig. 7. Effect of pH on the activity of glucoamylase I from the selected mutant strain, *A. flavus* 153-1022

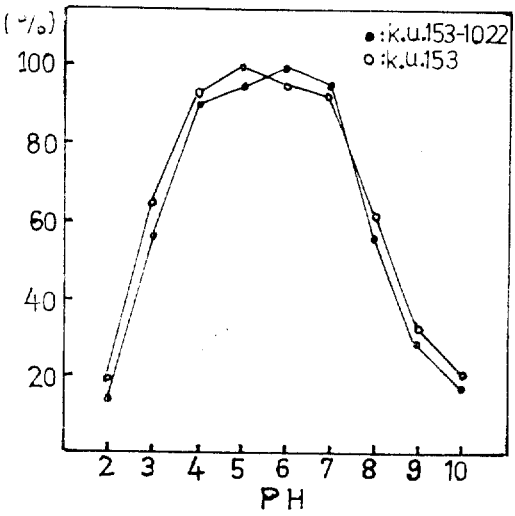

Fig. 8. Effect of pH on the activity of glucoamylase Ⅱ from the selected mutant strain, *A. flavus* KU 153-1022

stable at the ranges of 20~50°C, but their activities were abruptly decreased at 60°C. As shown in the Fig. 11~14, these optimal temperature ranges and thermal stability of the two glucoamylases from the selected mutant strain were not altered with those from the wild strain.

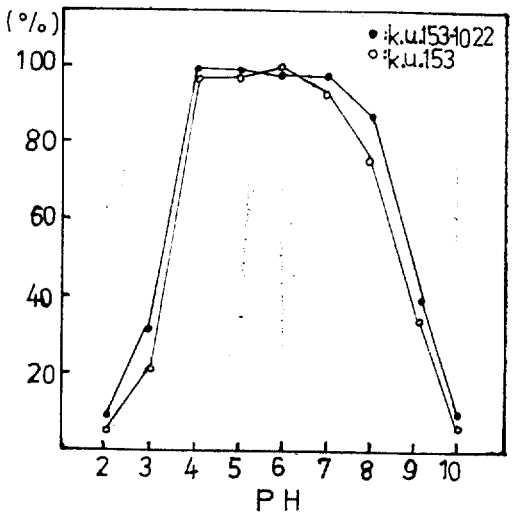

Fig. 9. pH stability of glucoamylase, I, from the selected mutant, *A. flavus* KU 153-1022.

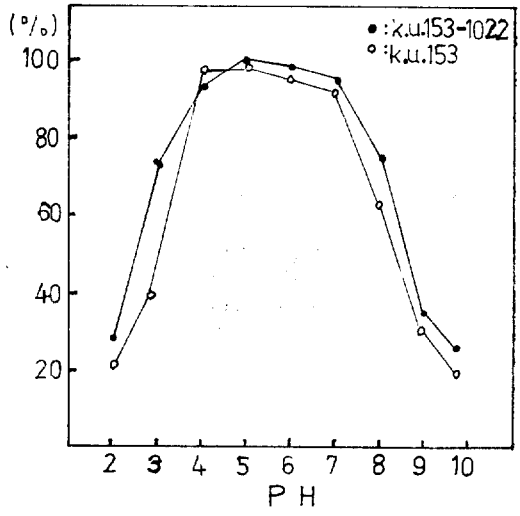

Fig. 10. pH stability of glucoamylase Ⅱ, from the selected mutant, *A. flavus* KU 153-1022

7. Vmax and Km value of the two glucoamlases

According to Lineweaver and Burk plot of the influence of substrate concentration upon the reaction velocity of the two glucoamylases from the mutant selected, Km values of glucoamylase I and Ⅱ were calculated to be 2.38mg/m*l* and 1.89mg/m*l*, and

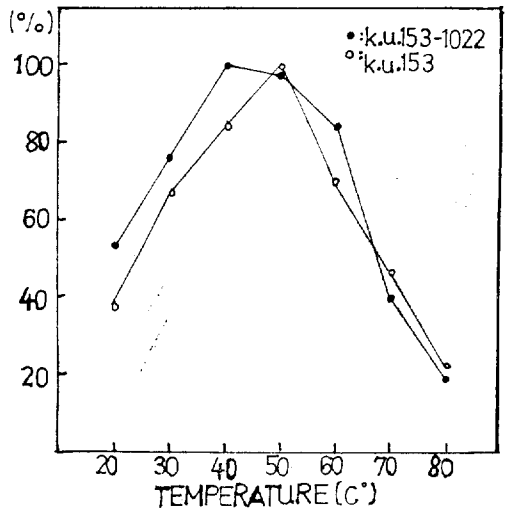

Fig. 11. Effect of temperature on the activity of glucoamylase I from the mutant strain, *A. flavus* KU 153-1022

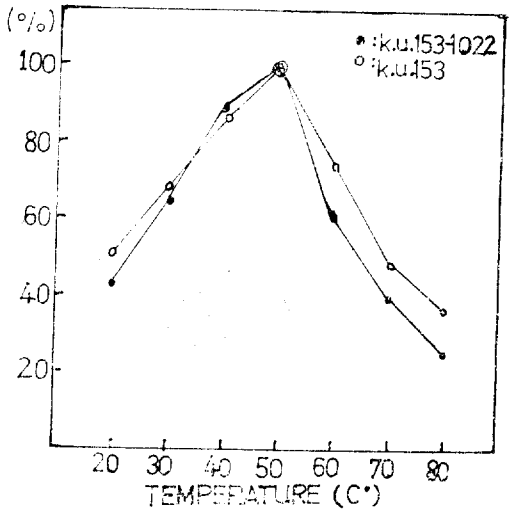

Fig. 12. Effect of temperature on the activity of glucoamylase Ⅱ from the mutant strain, *A.flavus* KU 153-1022

Vmax values $1.25\times10^{-2}\mu$mol/mg/min and $2.0\times10^{-2}\mu$mol/mg/min, respectively(Fig. 15, 16). The Km and Vmax values of glucoamylase I and Ⅱ from the mutant strain, KU 153~1022, were quite same value with those from the wild strain KU 153.

Considering the optimal pH-temperature

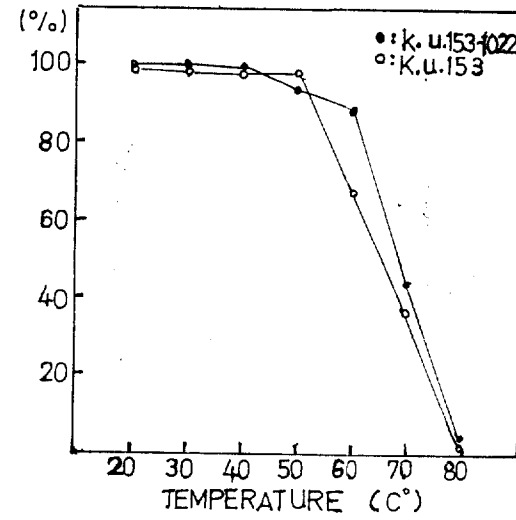

Fig.13. Thermal stability of glucoamylase I from the mutant strain, *A. flavus* KU 153-1022

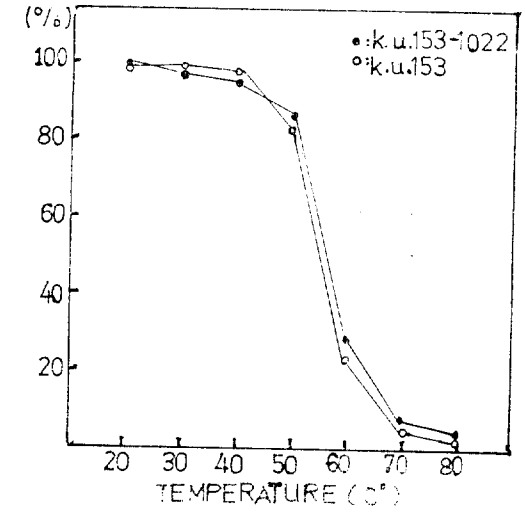

Fig.14. Thermal stability of glucoamylase Ⅱ from the mutant strain, *A. flavus* KU 153-1022

ranges, pH-thermal stability, and Km-Vmax values of the two glucoamylases, from the both strains, wild and mutant, it is deduced that the characteristics of both glucoamylase I and Ⅱ were not altered in the process of the mutation. Therefore, it is concluded that the mutation didn't induce the changes in the characteristics of the glucoamylase, or the formation of the another glucoamylase

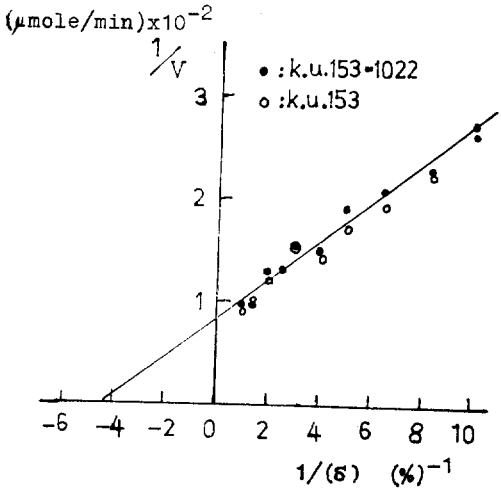

Fig. 15. Lineweaver and Burk plot of the action of glucoamylase I. Km value was caculated to be 2.38mg/m*l* and Vmax to be 1.25×10^{-2}μmole/mg/min.

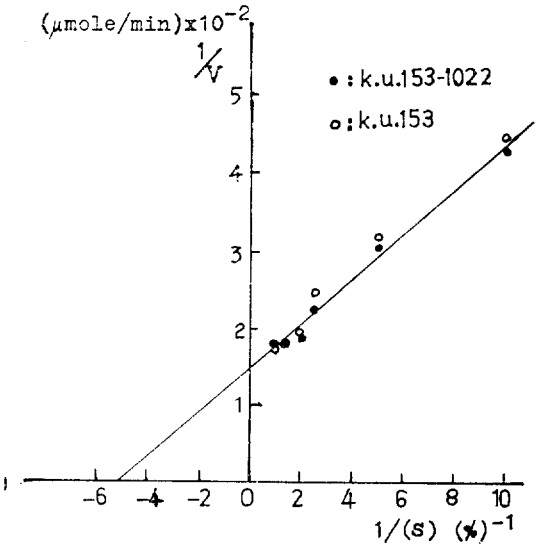

Fig. 16. Lineweaver and Burk plot of the action of glucoamylase II. Km value was calculated to be 1.89mg/m*l* and Vmax to be 2.0×10^{-2}μmole/mg/m*l*.

isozyme, but induced the variation in the pro ductivities of the same character's glucoamylase owing to the variation of the regulatory genes, so that promoted the production of glucoamylase I and II.

적 요

Protease의 생성능이 우수한 야생균주 *A. flavus* ku 153에 자외선을 조사하여 보다 강력한 protease 생성능을 가지는 돌연변이주, *A. flavus* KU 153-10을 얻고 이에 다시 자외선을 조사하여 glucoamylase의 생성능이 강화된 돌연변이주 *A. flavus* KU 153-1022를 선별하였다.

이 돌연변이주의 glucoamylase 생성능은 야생균주에 비해서는 약 3.3배, 모균주에 비해서는 2.1배 가량 증가하였고, α-amylase 활성은 야생균주나 모균주에 비해 각각 약 2배 가량 증가하였다. 선별된 이 돌연변이주의 acid-, neutral- 및 alkaline-protease 활성은 야생균주에 비해 월등히 크며, 모균주의 활성을 그대로 보존하고 있었다. 따라서 이들 protease의 생성능에 대한 back mutation을 유발하지는 아니하였고, avicelase, carboxymethy cellulase, 및 salicinase 활성도 모균주의 그것과 비슷한 값을 나타내었다.

선별된 돌연변이주가 생성한 순화된 glucoamylase I 및 II의 total activity는 야생균주의 그것에 비해 각각 2.86 및 3.65배나 증가하였다. 선별된 돌연변이주와 야생균주가 생성한 순화된 glucoamylase I 및 II의 최적 pH 및 최적 온도 영역, pH 및 열에 대한 안정도, 그리고 Km 및 Vmax 값 등을 비교할 때, 야생균주의 glucoamylase I 및 II의 특성은 돌연변이 과정에서 전혀 변화되지 아니하였음을 알 수 있었다.

따라서 선별된 이 돌연변이주는 glucoamylase의 특성의 변화나 다른 glucoamylase isozyme의 형성을 유발한 것이 아니라 조절유전자의 변화로 같은 성질의 glucoamylase의 생성능의 변화를 유발하여 glucoamylase I 및 II의 생성능이 강화된 것으로 생각되었다.

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