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Properties of Glucoamylase Isozymes Produced by *Aspergillus* sp.

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Aspergillus sp.가 생산하는 Glucoamylase Isozymes의 성질

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Glucoamylase (EC 3.2.1.3) of *Aspergillus* sp. isolated from soil was partially purified by Sephacryl S-200 gel filtration and DEAE-Sephacel ion exchiange chromatography. The glucoamylase activity was separated into two isozymes after DEAE-Sephacel ion exchange chromatography. The optimum pH and temperature for both glucoamylase isozymes (GI, GII) were identical; pH 4.5 and temperature, 65°C. The molecular weights of GI and GII isozymes were estimated to be 105,000, which were measured by gel filtration on Sephacryl S-200. Both isozymes were stable at pH ranges of 2 to 7, and up to 60°C. Glycerol was effective to stabilize the both isozymes. The activation energies of GI and GII isozymes were 10.63 and 10.32 kcal/mole, respectively. The enzyme activities of both isozymes were completely inactivated by addition of 0.1% Hg^{++}. In kinetic studies, the K_m values of GI isozyme for soluble starch, dextrin, and glycogen were 0.62%, 0.32%, and 1.02%, respectively. For GII isozyme, they became 0.66%, 0.23%, and 0.14% for the substrates.

Glucoamylase (EC 3.2.1.3) is an exo-splitting enzyme which removes glucose unit consecutively from the non-reducing end of the substrate chain. Glucoamylase hydrolyzes α-1,4 glucosidic linkages but the product is β-glucose.

So far glucoamylase has been reported from bacteria(1), yeast(2,3) and fungal(4-13) sources. Glucoamylases used commercially are mainly from fungal strains. All fungal glucoamylases are reported to be glycoproteins, and the molecular weights of them range between 48,000 and 112,000 (14). Glucoamylase has been reported to have several isozymes, which can be separated by electrophoresis and chromatography. The numbers of isozymes were between two to four depending on the fungal sources(14). The properties of each isozymes were sometimes identical, or different in debranching activity or activity for raw starch digestion depending upon the fungal sources(14).

Glucoamylase has a broad application in food, fermentation, textile and pharmaceutical industry. Recently, we have isolated an *Aspergillus* strain, which has possessed high glucoamylase activity(15). This paper describes the separation and properties of two glucoamylase isozymes from the *Aspergillus* strain.

Materials and Methods

Cultivation of microorganism

Organism used was *Aspergillus* sp. which was isolated from soil (15). The medium consisted of wheat bran 200 g and water 200 m*l*, and the cultivation was carried out by addition of seed culture (inoculum size: 1%) to culture vessel (250 m*l* Erlenmeyer flask) and incubated for 7 days at 30°C.

Key words: Glucoamylase, isozyme, *Aspergillus* sp.
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Crude enzyme preparation

2 *l* of distilled water was added to wheat bran koji and the mixture was stirred with a glass rod to extract the enzyme completely. The mixture was filtered using Toyo No. 2 filter paper and the filtrate was centrifuged at 3,000 rpm for 30 minutes. The supernatant was used as a crude enzyme solution.

Paper chromatography

The product of the glucoamylase reaction from the isolated fungal strain was investigated by paper chromatography. The enzyme reaction for product identification was performed in assay mixture (1 m*l*) containing soluble starch (2%), Na-acetate buffer (0.1 M, pH 4.5) and enzyme solution (50 μl) for 15 minutes at 60 °C. Then 20 μl of reaction mixture, glucose, maltose, and soluble starch were spotted separately on Whatman No. 1 paper and they were developed in 65% n-propyl alcohol in H_2O for 4 hours by ascending method. The spots of reducing sugars were detected by dipping the paper into reducing sugar specific reagent, aniline-diphenylamine (16). It was confirmed by the paper chromatography that the product of the glucoamylase reaction was only glucose without formation of maltose and any other reducing sugars.

Enzyme assays

Since the glucoamylase produced only glucose from soluble starch, glucoamylase activity was determined by measuring the amount of glucose released from soluble starch by dinitrosalicyclic acid (17). The enzyme reaction was performed with 0.5 m*l* of 40% soluble starch, 0.45 m*l* of 0.2 M Na-acetate buffer (pH 4.5) and 50 μl of enzyme solution at 60 °C for 15 minutes. One unit of glucoamylase activity was defined as the amount of enzyme to produced one μ mole of glucose per minute under the above conditions.

The glucoamylase activity was also measured by using color formation of *p*-nitrophenol from *p*-nitrophenyl α-D-glucopyranoside (18). The reaction mixture consisted of 0.1 m*l* of 0.3% *p*-nitrophenyl α-D-glucopyranoside, 0.8 m*l* of 0.2 M Na-acetate buffer (pH 4.5) and 0.1 m*l* of the enzyme. After incubation for 30 minutes at 60 °C, the reaction was stopped by addition of 1 m*l* of 1% Na-carbonate solution. The released *p*-nitrophenol was measured at 420 nm. One unit of the enzyme activity was defined as the amount of enzyme that release 1 μmole of *p*-nitrophenol from *p*-nitrophenyl α-D-glucopyranoside per minute under the specified condition.

Determination of protein concentration

The protein concentration was measured by the method of Bradford with bovine serum albumin as a standard (19).

Results and Discussion

Separation of glucoamylase isozymes

20 m*l* of crude enzyme was loaded to Sephacryl S-200 column (2.5 × 38 cm), which was equilibrated with equilibration buffer (50 mM Na-phosphate, pH 7.0). Elution was carried out with the buffer and the elute was collected in 7.5 m*l* fraction (Fig. 1) The active fractions (38 m*l*) after gel filtration were collected, and then loaded to DEAE-Sephacel ion exchange chromatography column (2.5 × 15 cm), which was preequilibrated with the equilibration buffer. The column was washed with the same buffer and eluted with 600 m*l* of linear gradient of 0-0.5 M NaCl in the buffer. Each fraction of 7.5 m*l* was collected (Fig. 2). As shown in Fig. 2, the glucoamylase activity was separated into 2 isozyme (GI, GII) after DEAE-Sephacel ion exchange chromatography. Lineback *et al.* (4) reported two types of glucoamylase are purified from *Aspergillus niger* possessing different pI. Razzaque *et al.* (6) also revealed the existence of three forms of gluco-

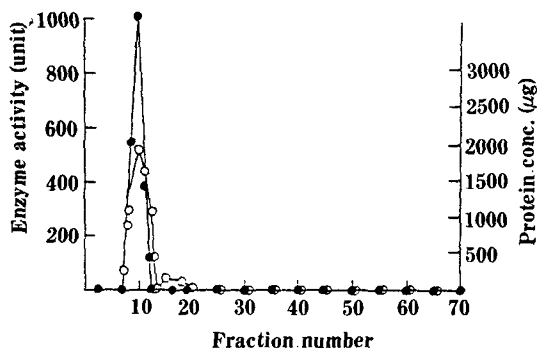

Fig. 1. Elution profile of the enzyme on Sephacryl S-200.

20 m*l* of crude extract of the enzyme was applied to Sephacryl S-200 (2.5 × 38 cm) column previously equilibrated with 50 mM sodium phosphate buffer (pH 7.0). Elution was performed with the same buffer and fractions of 7.5 m*l* were collected.
protein conc. (○-○), enzyme activity (●-●).

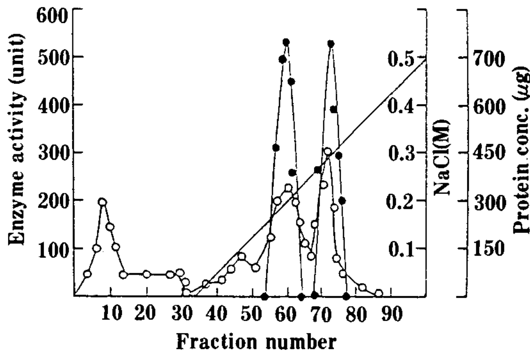

Fig. 2. Elution profile of the enzyme on DEAE-Sephacel column.

Active fractions from gel giltration (38 m*l*) were pooled and applied to DEAE-Sephacel (2.5 x 15 cm) previously equilibrated with 50 mM Na-phosphate buffer, pH 7.0. Elution was performed by 0-0.5 M NaCl linear gradient, and fractions of 7.5 m*l* were collected.
protein conc. (○-○), enzyme activity (●-●), NaCl conc. (- - -)

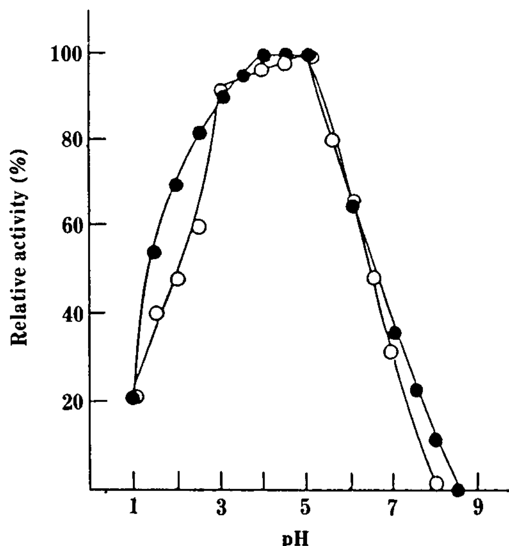

Fig. 3. Effect of pH on activity of glucoamylase isozymes.

The enzyme activities were measured using standard assay conditions with variation of pH. The pH used was explained in the text.
GI (●-●), GII (○-○).

amylase from *Aspergillus niger*. Takahashi (7) suggested that *Rhizopus* sp. have three isozymes of glucoamylase. Yamasaki *et al.* (20) proposed that *Mucor* sp. contain two glucoamylase isozymes. The fact that the *Aspergillus* sp. glucoamylase has two types of isozymes was similar to Lineback's results.

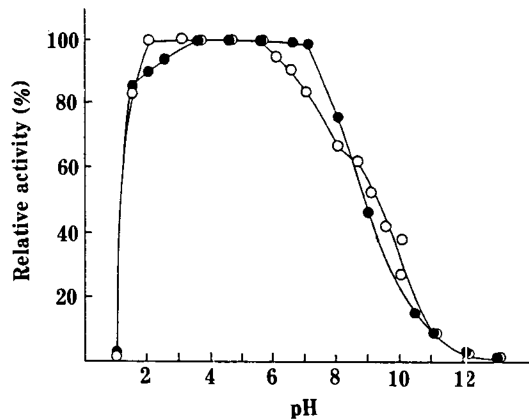

Fig. 4. Effect of pH on stability of glucoamylase isozymes.

Both isozymes were preincubated for 30 minutes at various pH (37 °C), and the pH was adjusted to pH 4.5 after preincubation. The residual activities were assayed under the standard assay conditions.
The pH used was explained in the text. GI (●-●), GII (○-○).

Effect of pH on activity and stability

The effect of pH on activity of the partially purified glucoamylase was examined over pH 1 to 12 (Fig. 3). The optimal pH was measured using soluble starch as a substrate. The buffers (0.2 M) used were as follow; pH 1.0-1.5, KCl/HCl; pH 2.0-2.5 glycine; pH 2.5-3.5, citric acid; pH 4.0-5.5, Na-acetate; pH 6.0-7.5, K-phosphate; pH 8.0-8.5, Tris/HCl; pH 9.0-10.0, boric acid; pH 10.5, carbonic acid; pH 11.0-11.5, Na-phosphate; pH 12.0-13.0 KCl/NaOH. Both isozymes (GI, GII) showed the identical pH optimum, pH 4.5. The acidic pH optimum was similar to results obtained from glucoamylases produced by *Aspergillus shirousamii* (10) and *Amylomyces rouxii* (21). The pH stability of the glucoamylase was measured by standard assay conditions after preincubation of the enzyme at given pH values at 37 °C for 30 minutes. Both glucoamylase isozymes were stable between pH 2 to 7, and the stability of the both isozymes was decreased sharply at acidic pH (Fig. 4).

Effect of temperature

The effect of temperature on activities of both isozymes was investigated at various temperatures ranged from 5 °C to 80 °C (Fig. 5). Both isozymes,

GI and GII, exhibited the maximal activities at 65 °C when they were assayed using soluble starch as a substrate for 15 minutes. Above that temperature, the enzyme activities decreased, indicating the inactivation of the both isozymes. To estimate activation energies of both isozymes, Fig. 5 was replotted as log velocities *versus* reciprocal absolute temperature. From the slopes obtained, the activation energies were calculated by Arrhenius equation (22). The activation energies of GI and GII isozymes were measured to be 10.62 kcal/mole for GI, and 10.23 kcal/mole for GII.

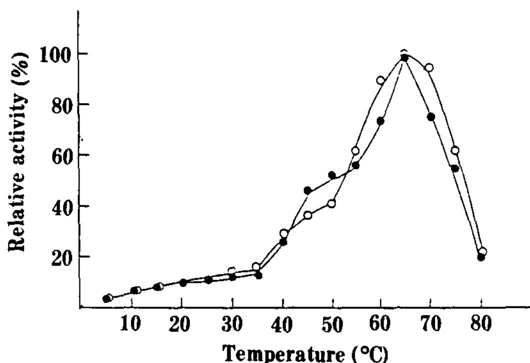

Fig. 5. Effect of temperature on activity of glucoamylase isozymes.
The reaction was carried out for 15 minutes at various temperature in standard assay conditions.
GI (●-●), GII (○-○).

Fig. 6 exhibited the inactivation of both isozymes at various temperatures ranged from 55 °C to 75 °C in 0.2M Na-acetate buffer (pH 4.5). Both isozymes were stable below 50 °C, and GII isozyme is slightly more thermostable than GI at 60 °C. Both isozymes lost most of their activities in 15 minutes at 65 °C, but they are most active for hydrolysis of soluble starch at that temperature as shown in Fig. 5. The enzyme reaction was performed in assay mixture containing 2% soluble starch, and glucose was produced during the reaction. Therefore, it seems likely that the substrate (soluble starch) or product (glucose) increases the stability of both isozymes from thermal inactivation at 65 °C.

Glycerol has been used as a stabilizing agents for many enzymes. Therefore, glycerol was used to increase the thermostability of the both isozymes (Fig. 7). The half life of the both isozymes were about 3 minutes without glycerol, but it was increased to about 12 minutes with 50% glycerol in

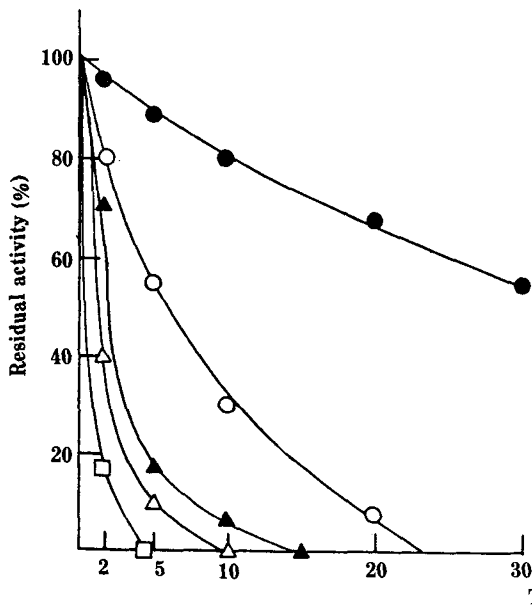

Fig. 6. Thermostability of glucoamylase isozymes at various temperature.
Enzyme solutions were heated at various temperature (55-75 °C) for 30 minutes. After heating, the remaining activities were determined immediately under the standard assay conditions. GI (left), GII (right).
55 °C(●-●), 60 °C(○-○), 65 °C(▲-▲), 70 °C(△-△), and 75 °C (□-□).

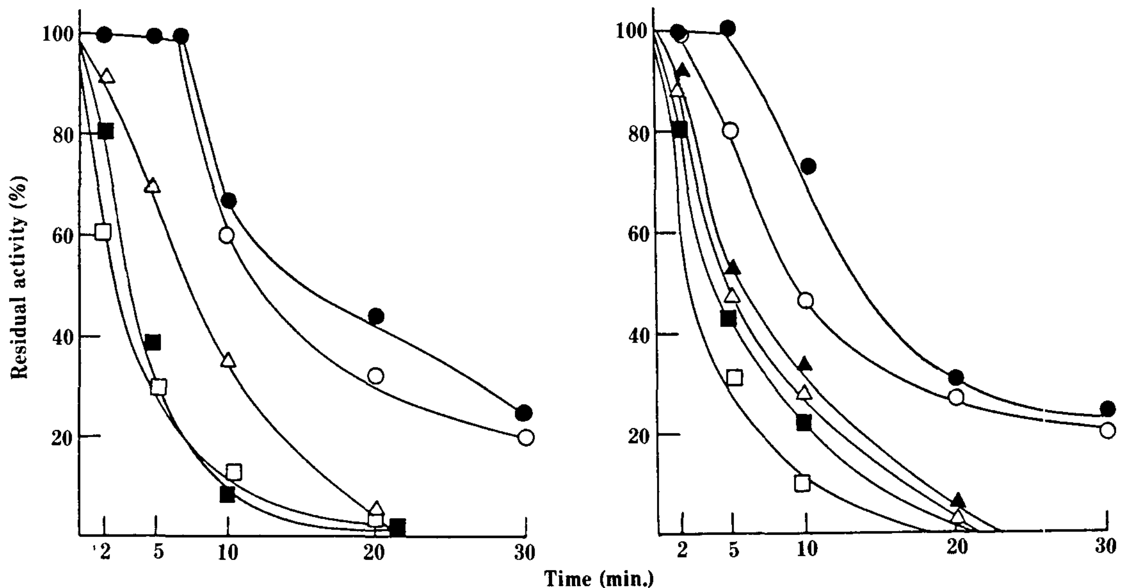

Fig. 7. Effect of glycerol on the thermostability of the glucoamylase isozymes.
The enzyme solutions containing various concentrations of glycerol were treated for 30 minutes at 60 °C. The residual activity was measured using standard assay conditions. GI (left), GII (right).
control(□-□), 10%(■-■), 20%(△-△), 30%(▲-▲), 40%(○-○), and 50%(●-●).

Table 1. Effect of metal ions on activities of glucoamylase isozymes.

Metal ions	Relative activity (%)	
	GI	GII
None	100	100
$MgSO_4$	100	87
$CaCl_2$	108	101
$ZnCl_2$	80	78
$Pb(CH_3COO)_2$	18	17
$CoCl_2$	66	52
$HgCl_2$	0	0
$Ba(OH)_2$	40	36
Na_2WO_4	86	56
EDTA	80	72

The concentrations used were 0.1%.

the enzyme solutions. Glycerol was known to form strong hydrogen bonds with water, effectively slowing down the motion of water molecules, so reducing water activity. Therefore, the effect of glycerol seems to be due to the fact that the enzyme preferentially binds water, and the structure so formed is less able to unfold against the structured glycerol solvent than it would be water alone (23).

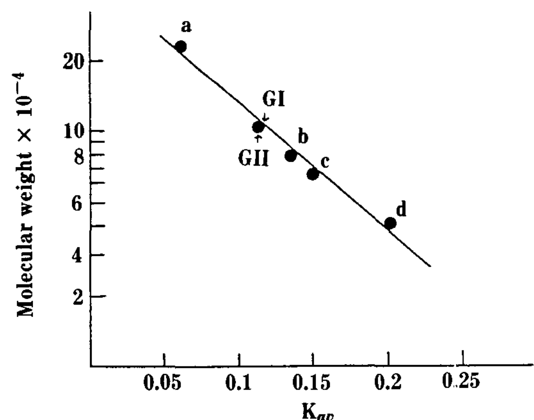

Fig. 8. Estimation of molecular weights of glucoamylase isozymes on Sephacryl S-200 column (1.5×84 cm).
Fractions of 0.85 m*l* were collected at a flow rate of 6m*l*/hr by using 0.1M Na-phosphate buffer, pH 7.0. Standard proteins used were catalase(a), conalbumin(b), bovine serum albumin(c), and ovalbumin(d).

Effect of metal ions

The effects of various metal ions on the glycoamylase activities for GI and GII were determined by adding 0.1% of metal ions to the standard assay mixture. The enzyme activities were completely inactivated by addition of Hg^{++}. As ex-

hibited in Table 1, some metals showed negative effect on the glucoamylase activities for both isozymes, while no metal ions tested demonstrated the increasing effect on the enzyme activities for both isozymes.

Estimation of molecular weight

The molecular weights of both isozymes, GI and GII, were measured using gel filtration on Sephacryl S-200 (1.5×84cm). Both GI and GII isozymes were eluted at same fraction during gel filtration step, and therefore the molecular weights of them were estimated to be identical, 105,000 as shown in Fig. 8. Most fungal glucoamylases have been reported to have a molecular weight of between 48,000 and 112,000 (14). Therefore, the molecular weight of glucoamylase from this fungal strain was similar to those of other fungal origin.

Kinetic Properties

The Michaelis-Menten constants of glucoamylase isozymes for soluble starch, dextrin, and glycogen were measured at pH 4.5, and 60 °C. From the double reciprocal plots for the substrates, the K_m values of GI and GII isozymes for the substrates were measured and shown in Table 2. The differences between GI and GII isozymes were clearly demonstrated in K_m values for glycogen. GII isozyme exhibited lower K_m value for glycogen than that of GI, indicating higher affinity to glycogen. Both isozymes prefer dextrin or glycogen to soluble starch as a substrate, and the rate of glucose formation is higher with dextrin or glycogen than soluble starch as a substrate (Table 3). The GI isozyme has no ability for utilizing amylose or amylopectin, while GII isozyme possesses a poor capacity to decompose them.

Table 2. Michaelis-Menten constants of glucoamylase isozymes for various substrates.

Substrate	K_m value (%)	
	GI	GII
Soluble starch	0.62	0.66
Dextrin	0.32	0.23
Glycogen	1.02	0.14
p-Nitrophenyl α-D-glucopyranoside*	4.54	3.33

The temperature and pH used were 60 °C and pH 4.5.
*: mM

Table 3. Activities of glucoamylase isozymes with various substrates.

Substrate	Glucoamylase Activity (Unit/ml)	
	G(I)	G(II)
Soluble starch	24.0	24.0
Dextrin	67.5	52.5
Glycogen	54.6	54.0
Amylose	—	0.06
Amylopectin	—	0.09

The temperature and pH used were 60 °C and pH 4.5. The concentrations employed were 2% for all substrates.

요 약

Aspergillus sp.로부터 추출한 glucoamylase를 Sephacryl S-200과 DEAE-Sephacel 이온교환 칼럼을 통과시킨 후, 2개의 isozyme으로 분리하였다. 분리된 glucoamylase isozyme(GI, GII)의 효소학적 특성을 각각 조사하였다. GI 및 GII isozyme의 활성 최적 pH 및 온도는 pH 4.5 및 65℃였다. 분리된 효소는 pH 3-7 사이에서 안정했으며, 또한 55℃ 이하에서 안정하였다. Hg^{++}는 효소 활성을 완전히 저해하였으나, 글리세롤은 효소의 안정성을 크게 증가시켰다. 효소반응의 활성화에너지는 GI의 경우 10.62 kcal/mole이었으며, GII의 경우에는 10.23 kcal/mole이었다. GI isozyme의 기질에 대한 K_m값은, 0.62%(soluble starch), 0.32%(dextrin), 1.02%(glycogen)이었으며, GII isozyme의 경우에는, 0.66%(soluble starch) 0.23%(dextrin), 그리고 0.14%(glycogen)이었다.

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