

## Purification and Characteristics of Glucoamylase in *Aspergillus oryzae* NR 3-6 Isolated from Traditional Korean *Nuruk*

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The purification system of glucoamylase (glucan 1,4- $\alpha$ -glucosidase, EC 3. 2. 1. 3), some characteristics of the purified enzyme and hydrolysis rate of various raw starch were investigated through several experiments. The enzyme was produced on a solid, uncooked wheat bran medium of *Aspergillus oryzae* NR 3-6 isolated from traditional Korean *Nuruk*. The enzyme was homogeneously purified 6.8-fold with an overall yield of 28.3% by the criteria of disc- and SDS-polyacrylamide gel electrophoresis. The molecular weight was estimated to be 48 kDa by SDS-PAGE. The optimum temperature and pH were 55°C and 4.0, respectively. The enzyme was stable at a pH range of 3.0~10.0 and below 45°C. Enzyme activity was inhibited about 27% by 1 mM Hg<sup>2+</sup>. The hydrolysis rate of raw wheat starch was shown to be 17.5-fold faster than the hydrolysis rate of soluble starch. The purified enzyme was identified as glucoamylase because the product of soluble starch by the purified enzyme was mainly glucose by thin layer chromatography.

**Key words:** *Aspergillus oryzae* NR 3-6, glucoamylase, *Nuruk*, raw starch

*Nuruk* has been used to brew folkcraft spirits in Korea. General shapes of *Nuruk* are mostly round with a diameter of about 16 to 17 cm, a height of 4 to 5 cm, and a weight of 600 to 700 g (7). *Nuruk* consists of unboiled raw barley and various grains. They are ground to paste and moistened, then naturally inoculated by airborne microorganisms. Therefore, many kinds of microorganisms such as fungi, yeast, and some bacteria grow in *Nuruk*. In *Nuruk*, most fungi and raw grains produce amylase which represent the saccharifying activity of *Nuruk*. The alcohol fermentability is mainly due to yeast and some species of fungi. Dominant species of *Nuruk* fungi are *Aspergillus*, *Rhizopus* and *Absidia*, and *Mucor* was found less frequently. *Aspergillus* sp. and *Rhizopus* sp. are known to play important roles in saccharification of *Nuruk* (20). Most fungi isolated from traditional Korean *Nuruk* showed better productivity of the saccharogenic and dextrinogenic enzymes in raw wheat bran medium than in cooked wheat bran medium. The productive ability of acid and flavor production are good in the raw wheat bran medium, and aflatoxins and citrinine are not produced under the same culture conditions. The majority of *Aspergillus* sp. have

high cell growth and amylase activity in the microflora of *Nuruk* (8).

Glucoamylase is a saccharogenic enzyme among various enzymes in *Nuruk*. It is considered to be an important enzyme in the formation of good taste and ethylalcohol of folkcraft Korean spirits. Glucoamylase (glucan 1,4- $\alpha$ -glucosidase, EC 3. 2. 1. 3) is an exo-acting carbohydrase which liberates glucose units from the non-reducing end of starch. Cooked starch is easily hydrolyzed by amylase, but uncooked starch is not. The enzymatic hydrolysis of raw starch has become the subject of much attention in recent years from the viewpoint of energy and effective utilization of nature resources. Starch has been gelatinized at first by cooking in the course of conventional enzymatic hydrolysis. In order to reduce the cost of cooking, several studies were done on the enzymatic hydrolysis of raw starch. However, these studies were not only technically, but also economically difficult. The production of an active enzyme depends on the selection of a suitable mould for the purpose. Furthermore, it is also necessary to screen useful fungi for manufacturing *Nuruk*. Although the enzyme is well known to be produced by the genera of *Aspergillus* (3, 11, 13), *Penicillium* (15, 16), *Rhizopus* (10), *Mucor* (17) and *Monascus* (18). This paper deals with the purification and some enzymatic characteristics of glucoamylase from *Aspergillus*

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*oryzae* NR 3-6 isolated from the traditional Korean *Nuruk*.

## Materials and Methods

### Materials

Molecular weight standards for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were purchased from Pharmacia Co., Sweden. DEAE-cellulose, Sephadex G-100, Phenyl Sepharose, glucose oxidase (from *Aspergillus niger*), and peroxidase (type 1, from horseradish) were obtained from Sigma Co., U.S.A.

### Microorganism

*A. oryzae* NR 3-6 from our laboratory, previously reported (9), was used.

### Medium and cultivation

The medium composition and culture conditions were the same as those for *A. oryzae* NR 3-6 reported previously (19). An uncooked medium was sterilized at room temperature by ethylene oxide gas (Ethylene oxide sterilizer, IKI-M, Japan).

### Preparation of crude extract

The solid culture was soaked in 0.5% NaCl solution at 4°C overnight and then the suspension was filtered with a steel net. The materials were removed by centrifugation at  $13,000 \times g$  for 10 min. The resulting supernatant was used as the crude enzyme preparation.

### Assay of glucoamylase activity

Glucoamylase activity was assayed by the release of glucose from boiled soluble starch by the method previously reported (19). Determination of glucose concentration was measured by ultraviolet absorption at 505 nm by the method of Allain *et al.* (1). One unit of the enzyme activity was defined as the amount of glucoamylase liberating 1 mg of glucose per h at 40°C.

### Protein determination

Protein concentration was determined by the method of Bradford (2) using a protein assay kit (Bio-Rad Co., U.S.A.) with bovine serum albumin as a standard protein or by measuring the absorbance at 280 nm.

### Electrophoresis

Polyacrylamide gel electrophoresis was performed by a modified version of Davis' method (5). Stacking and running gels were polymerized with 7.5% (w/v)

stab gel. After running, the gel was stained with 1% Amido black 10 B (E. Merck, Darmstadt, Germany), electrophoretically destained, and stored in 7% acetic acid. SDS-polyacrylamide gel electrophoresis was performed according to Weber and Osborn (14) on a 7.5% (w/v) gel with the normal amount of cross-linker at 5 mA per gel. The gel was stained with Coomassie brilliant blue R-250 (Sigma Co., U.S.A.), electrophoretically destained, and stored in 7% acetic acid.

### Molecular weight

The molecular weight of the enzyme was estimated by polyacrylamide gel electrophoresis in the presence of 0.1% SDS according to Weber and Osborn (14). The standard proteins used were rabbit muscle phosphorylase b (94 kDa), transferrin (76 kDa), bovine serum albumin (67 kDa), glutamate dehydrogenase (53 kDa), and ovalbumin (43 kDa).

### Analysis of hydrolyzed products

The reaction mixture was centrifuged, and the amount of reducing sugar in the supernatant was determined as glucose by thin layer chromatography (TLC). 1  $\mu$ l of the supernatant was developed on a TLC plate (Silica gel 60 aluminium plate, E. Merck, Darmstadt, Germany) with a solvent (acetone : H<sub>2</sub>O = 9 : 1) at room temperature for 30 min. The TLC plate was dried and spots were detected by spraying 10% potassium permanganate.

### Raw starch digestion

The reaction mixture consisted of 0.2 g of various raw, wheat, corn, potato, rice and soluble starches, in 10 ml of 0.1 M acetate buffer (pH 5.0) containing the enzyme. The mixture was incubated at 40°C for 5 h and stirred occasionally. Reducing sugar formed in 1 ml of the reaction mixture was determined by the method of Allain *et al.* (1) and the degree of hydrolysis was calculated.

## Results and Discussion

### Purification of glucoamylase

The glucoamylase from *A. oryzae* NR 3-6 was thermostable, and so all operations were done at temperatures below 10°C unless otherwise noted. Purification of glucoamylase was accomplished as follows.

#### Step 1: Evaporation

The crude enzyme solution (600 ml) was evaporated with a rotary evaporator at 30°C. The evaporated crude enzyme solution (30 ml) was dialyzed

**Table 1.** Summary of purification steps of glucoamylase from *Aspergillus oryzae* NR 3-6

Purification step	Total protein (mg)	Total activity (U) <sup>a</sup>	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	244	16,173	66.2	100.0	1.0
Evaporation	102.3	14,555	142.3	90.0	2.2
DEAE-cellulose chromatography	34.1	5,269	154.6	32.6	2.3
Sephadex G-100 gel filtration	26.1	4,991	191.5	30.9	2.9
Phenyl Sepharose chromatography	10.2	4,573	450.2	28.3	6.8

<sup>a</sup> One unit (U) of enzyme activity is defined as the amount of glucoamylase liberating 1 mg of glucose per h at 40°C.

overnight at 4°C against 50 volumes of 0.05 acetate buffer (pH 5.0) for 24 h. The precipitate formed was removed by centrifugation at  $13,000 \times g$  for 10 min.

#### Step 2: DEAE-cellulose column chromatography

The concentrate was applied to a DEAE-cellulose column ( $3.5 \times 15$  cm) equilibrated with 0.05 M acetate buffer (pH 5.0). The column was washed thoroughly with the same buffer. After removing much of the inactive protein, the enzyme was eluted in a linear gradient with the same buffer containing 0 to 0.5 M NaCl. The active fractions were pooled and concentrated by an Amicon ultrafiltration kit (model 8050, Grace Company, U.S.A.). The concentrated active fraction was dialyzed overnight at 4°C against 4 changes of 10 volumes of the same buffer.

#### Step 3: Sephadex G-100 column chromatography

The dialyzed enzyme solution was then applied to a Sephadex G-100 column ( $1.5 \times 15$  cm) equilibrated with 0.05 M acetate buffer (pH 5.0). The active fractions were combined and concentrated by an Amicon ultrafiltration kit.

#### Step 4: Phenyl Sepharose column chromatography

Glucoamylase was finally purified by Phenyl Sepharose column ( $1.8 \times 6$  cm) chromatography. The concentrated enzyme solution was introduced into the column equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) containing 4 M NaCl. The column was washed thoroughly with the same buffer. Elution was done in a linear gradient with the same buffer containing 4 to 2 M NaCl, conversely, at a flow rate of 15 ml per h.

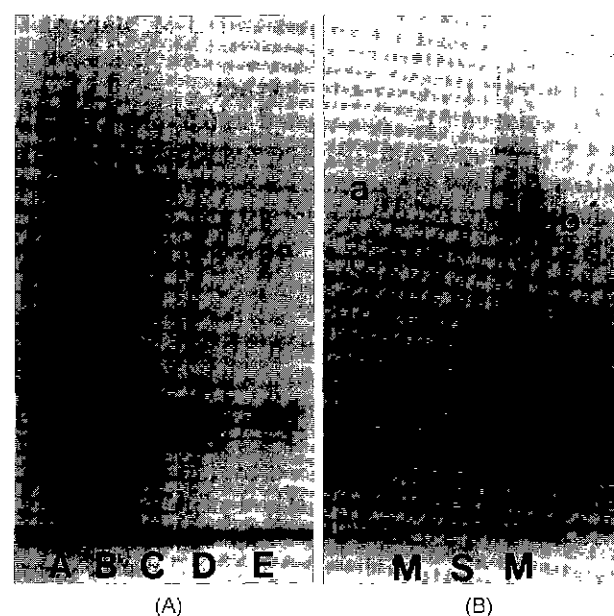
The final step yielded a single and symmetrical protein peak, and enzyme activity was entirely associated with this protein peak. The purification procedure is summarized in Table 1. By these procedures glucoamylase from *A. oryzae* NR 3-6 was purified about 6.8-fold with a 28.3% yield.

### Homogeneity and molecular weight

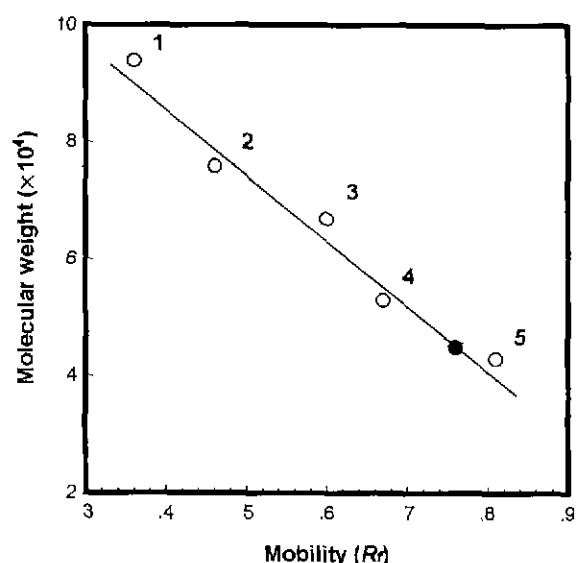
The homogeneity of the purified glucoamylase

was investigated by disc- and SDS-polyacrylamide gel electrophoresis. The final preparation showed a single band in the polyacrylamide gel both in the absence and presence of SDS (Fig. 1).

The molecular weight of the enzyme was estimated to be 48 kDa by polyacrylamide gel electrophoresis in the presence of SDS, as shown in Fig. 2. This value is lower than those of *A. niger* (56 kDa) (3), *A. usamii* AM 2185 (67 kDa) (4), *A. oryzae* (68.4 kDa) (11), *A. saitoi* (90 kDa) (13) and *A. awamori* (90 kDa) (6).



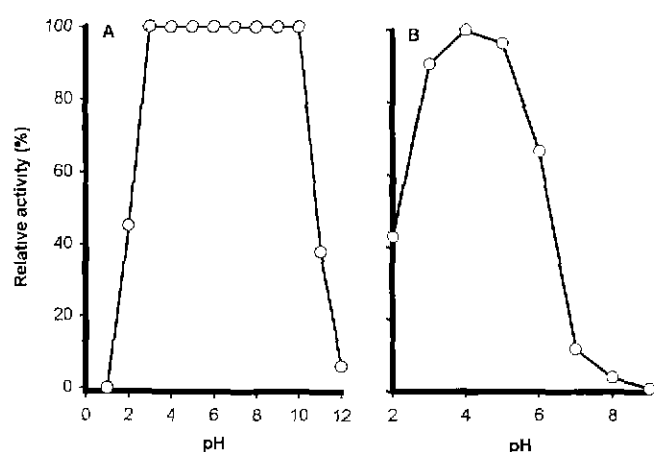
**Fig. 1.** Polyacrylamide gel electrophoresis of the purified glucoamylase in the absence (A) or presence (B) of sodium dodecyl sulfate. (A) Polyacrylamide gel electrophoresis of the glucoamylase in the absence of sodium dodecyl sulfate was carried out at various purification steps. Lane A, crude enzyme; lane B, evaporation; lane C, DEAE-cellulose column chromatography; lane D, Sephadex G-100 column chromatography; lane E, Phenyl Sepharose column chromatography. (B) Polyacrylamide gel electrophoresis of the purified glucoamylase in the presence of sodium dodecyl sulfate. S, purified glucoamylase; M, standard protein, a,  $\beta$ -galactosidase (116 kDa); b, phosphorylase b (94 kDa); c, transferrin (76 kDa); d, bovine serum albumin (67 kDa); e, glutamate dehydrogenase (53 kDa); f, ovalbumin (43 kDa).



**Fig. 2.** Determination of molecular weight of the purified glucoamylase by SDS-polyacrylamide gel electrophoresis. Molecular weights of standard proteins were as follows 1, phosphorylase b (94 kDa); 2, transferrin (76 kDa); 3, bovine serum albumin (67 kDa); 4, Glutamate dehydrogenase (53 kDa); 5, ovalbumin (43 kDa). ○: standard protein, ●: purified glucoamylase.

### Effect of pH and temperature on enzyme stability and activity

In order to examine the effect of pH on enzyme stability, the enzyme were kept in 0.1 M buffers of pH 0.5 to 12.0 at 4°C for 24 h. The residual activity was assayed under standard conditions. As shown in Fig. 3A, enzyme activity was approximately 55% and 40% at pH 2.0 and pH 11.0 at 4°C for 24 h, respectively, and was stable at a pH range of 3.0 to 10.0. The above results suggest that glucoamylase of *A. oryzae* NR 3-6 is very acidic and alkaline stable compared with those of *A. niger* (3), *A. oryzae* (11) and *Rhizopus oryzae* (10). The optimum pH for the enzyme activity was found to be 4.0, as shown in Fig. 3B. Moreover, as shown in Fig. 4B, the optimum temperature for the glucoamylase was found to be between 50 to 55°C, and enzyme activity decreased by half at 58°C for 20 min. After heating at 65°C for 20 min even at the optimal stable pH, more than 90% of the activity disappeared, as shown in Fig. 4A. Glucoamylase from *A. oryzae* NR 3-6 was very thermo-unstable at temperatures higher than 60°C, unlike that from *A. niger* (3) and *A. oryzae* (11), which were relatively stable at room temperature. Optimum temperatures of glucoamylase from *A. oryzae* (11), *A. usamii* (4) and *A. niger* (3) were found to be 56, 60, and 65°C, respectively. Optimum temperature of the *A. oryzae* NR 3-6 enzyme was lower than those of other *Aspergillus* sp. enzymes (3, 4).

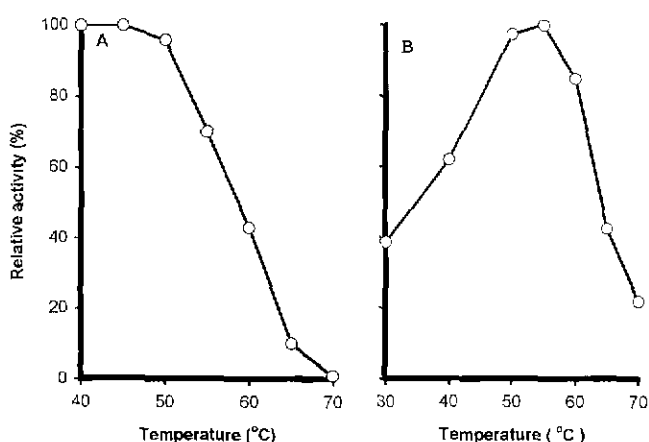


**Fig. 3.** Effect of pH on stability (A) and activity (B) of the purified glucoamylase. (A) Enzyme solutions were kept in 0.1 M buffer of pH 0.5 to 12.0 at 4°C for 24 h. The residual activity was assayed under standard conditions using glycine-HCl buffer (pH 0.5 to 3.0), acetate buffer (pH 4.0 to 5.0), citrate phosphate buffer (pH 6.0), Tris-HCl buffer (pH 7.0 to 8.0), and glycine-NaOH buffer (pH 9.0 to 12.0). (B) The enzyme activity was assayed under standard conditions using glycine-HCl buffer (pH 2.0 to 3.0), acetate buffer (pH 4.0 to 5.0), citrate phosphate buffer (pH 6.0), Tris-HCl buffer (pH 7.0 to 8.0), and glycine-NaOH buffer (pH 9.0 to 12.0).

### Effect of metal ions

In order to examine the effect of metal ions on enzyme activity, the enzyme were incubated in 0.1 M acetate buffer (pH 5.0) containing 1 mM concentration of various metal ions at 30°C for 30 min. Residual activity was then assayed under standard conditions.

As shown in Table 2, the enzyme was weakly inhibited by 1 mM  $\text{Hg}^{2+}$  and was not affected by



**Fig. 4.** Effect of temperature on stability (A) and activity (B) of the purified glucoamylase. (A) Enzyme solutions were kept in 0.05 M acetate buffers (pH 5.0), and incubated at the indicated temperature for 20 min. Residual activities were assayed under standard conditions. (B) The enzyme activity was assayed under standard conditions at indicated temperatures.

**Table 2.** Effect of metal ions on the activity of purified glucoamylase

Metal ions (1 mM)	Relative activity (%)
Control	100.0
MgCl <sub>2</sub>	101.0
CaCl <sub>2</sub>	100.5
FeCl <sub>2</sub>	99.0
MnCl <sub>2</sub>	96.0
ZnCl <sub>2</sub>	94.0
PbCl <sub>2</sub>	92.5
HgCl <sub>2</sub>	73.0

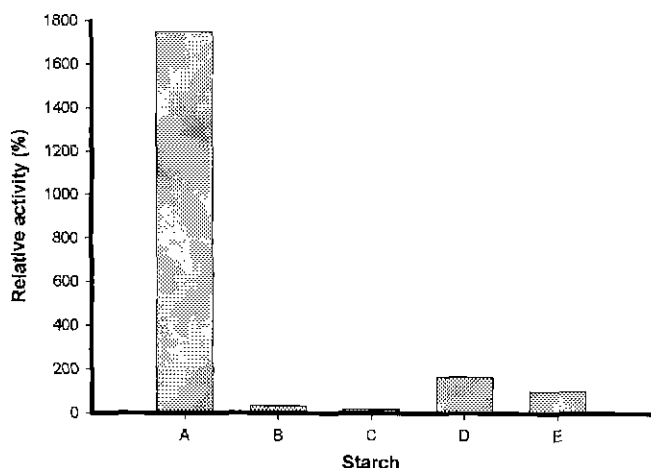
Glucoamylase activity was assayed under standard conditions in the presence of metal ions at the indicated concentration and expressed as relative activity to that of the control.

some metal ions such as Pb<sup>2+</sup>, Mg<sup>2+</sup>, Zn<sup>2+</sup>, Fe<sup>2+</sup>, Ca<sup>2+</sup> and Mn<sup>2+</sup> at a concentration of 1 mM. *A. usamii* glucoamylase (4) was not affected by 1 mM Hg<sup>2+</sup>, but enzymes from *R. oryzae* (10), and *A. oryzae* NR 3-6 used in this study were weakly inhibited by 1 mM Hg<sup>2+</sup>. Thermostability of the *A. usamii* enzyme (4) was greatly increased by the addition of Ca<sup>2+</sup>, however, the *R. oryzae* and *A. oryzae* enzyme were not greatly increased by Ca<sup>2+</sup> (data not shown).

### Raw starch digestion

Raw starch digestion by glucoamylase from *A. oryzae* NR 3-6 were carried out separately in 0.1 M acetate buffer (pH 5.0) at 40°C for 5 h.

As shown in Fig. 5, raw potato starch most difficult to hydrolyze and raw corn starch were digested at 10 and 25%, respectively, by the enzyme compared with 100% for soluble starch (control). Raw rice starch was digested 160% more than sol-

**Fig. 5.** Digestive activity of purified glucoamylase on raw starch. Various kinds of raw starch tested are as follows. A, raw wheat starch; B, raw corn starch; C, raw potato starch; D, raw rice starch; E, soluble starch.

uble starch. Furthermore, raw wheat starch was digested 1,750% more than the control. Based on these results, glucoamylase from *A. oryzae* NR 3-6 is believed to be unique because the enzyme digested raw wheat starch better than the starches used in this study.

### Product of soluble starch by the enzyme

In order to determine products of the substrate by the enzyme, soluble starch was digested by the enzyme at 40°C for 1 h, and then the products were assayed by TLC. As shown in Fig. 6, *R<sub>f</sub>* values of maltose and glucose were 0.32 and 0.47, respectively, and the *R<sub>f</sub>* value of the product of soluble starch was 0.47. From this result, purified enzyme from *A. oryzae* NR 3-6 was identified as glucoamylase because the product of soluble starch was mainly glucose by TLC.

Glucoamylase of *A. oryzae* showed negligible activities towards the digestion of raw starch (12). However, glucoamylase of *A. oryzae* NR 3-6 isolated from traditional Korean *Nuruk* had very strong digestive activities raw of wheat and rice starches.

In conclusion, these results suggest that glu-

**Fig. 6.** Thin layer chromatography of the reaction products of soluble starch by purified glucoamylase. S, soluble starch; M, maltose; G, glucose. SP, sample after 1 h of enzyme reaction.

coamylase come into use in ethanol fermentation of uncooked wheat starch.

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