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Characterization of Two Forms of Glucoamylase from Traditional Korean *Nuruk* Fungi, *Aspergillus coreanus* NR 15-1

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Abstract Some characteristics of two forms of glucoamylase (glucan 1,4-α-glucosidase, EC 3. 2. 1. 3) purified from *Aspergillus coreanus* NR 15-1 were investigated. The enzymes were produced on a solid, uncooked wheat bran medium of *A. coreanus* NR 15-1 isolated from traditional Korean *Nuruk*. Two forms of glucoamylase, GA-I and GA-II, were purified to homogenity after 5.8-fold and 9.6-fold purification, respectively, judged by disc- and SDS-polyacrylamide gel electrophoresis. The molecular mass of GA-I and GA-II were estimated to be 62 kDa and 90 kDa by Sephadex G-100 gel filtration, and 64 kDa and 91 kDa by SDS-polyacrylamide gel electrophoresis, respectively. The optimum temperatures of GA-I and GA-II were 60°C and 65°C, respectively, and the optimum pH was 4.0. The activation energy (*Ea* value) of GA-I and GA-II was 11.66 kcal/mol and 12.09 kcal/mol, respectively, and the apparent Michaelis constants (K_m) of GA-I and GA-II for soluble starch were found to be 3.57 mg/ml and 6.25 mg/ml, respectively. Both enzymes were activated by 1 mM Mn^{2+} and Cu^{2+}, but were completely inhibited by 1 mM *N*-bromosuccinimide. The GA-II was weakly inhibited by 1 mM *p*-CMB, dithiothreitol, EDTA, and pyridoxal 5'-phosphate, but GA-I was not inhibited by those compounds. Both enzymes had significant ability to digest raw wheat starch and raw rice starch, and hydrolysis rates of raw wheat starch by GA-I and GA-II were 7.8- and 7.3-fold higher than with soluble starch, respectively.

Key words: *Aspergillus coreanus*, glucoamylase, *Nuruk* fungi

Nuruk has been used to brew a folk spirits in Korea. Traditional Korean *Nuruk* consists of uncooked raw barley and various grains. They are ground to paste and moistened, and then inoculated naturally by airborne microorganisms. Therefore, many kinds of microorganisms such as fungi, yeast, and some bacteria grow in *Nuruk*. In *Nuruk*, most fungi and raw grain produce amylase, which represents saccharification activity of *Nuruk*; alcohol fermentation ability is mainly due to yeast and some species of fungi. Dominant species of *Nuruk* fungi are *Aspergillus*, *Rhizopus*, and *Absidia*, and *Mucor* is less frequently found. *A.* sp. and *Rhizopus* sp. are known to play important roles in the saccharification of *Nuruk* [12, 29]. Most of the fungi isolated from traditional Korean *Nuruk* show better productivity of the saccharogenic and dextrinogenic enzymes in raw wheat bran medium than in cooked wheat bran medium. The majority of *A.* sp. have high cell growth and amylase activity in microflora of *Nuruk* [29]. Glucoamylase is a saccharogenic enzyme among various enzymes present in *Nuruk*, and is considered to be an important enzyme to form good taste and ethyl alcohol of Korean folk spirits. Glucoamylase (glucan 1,4-α-glucosidase, EC 3. 2. 1. 3) is an exo-acting carbohydrolase, which liberates glucose units from the nonreducing end of starch. Cooked starch is easily hydrolyzed by amylase, but uncooked starch is not. The enzymatic hydrolysis of raw starch has recently become the subject of much attention, because of energy and effective utilization of natural resources. In the course of conventional enzymatic hydrolysis, starch is first gelatinized by cooking. In order to reduce the cost of cooking, several studies have been undertaken on the enzymatic hydrolysis of raw starch. However, it was both technically and economically difficult. The production of an active enzyme depends on the selection of a suitable mould. Although the enzyme is well known to be produced by the genera of *Aspergillus* [4, 9, 14, 17–19, 30], *Penicillium* [25, 27], *Rhizopus* [11, 20], *Mucor* [25], and *Monascus* [28], it is also necessary to screen useful fungi for manufacturing *Nuruk*. This paper deals with the purification and some enzymatic characterization of glucoamylase from *A. coreanus* NR 15-1, which was isolated from traditional Korean *Nuruk*.

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MATERIALS AND METHODS

Materials

Molecular weight standard proteins for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were purchased from Pharmacia Co., Sweden. PGO enzymes (Sigma catalog No. 510-6), peroxidase (type 1, from horseradish), and glucose oxidase (from *Aspergillus niger*) were obtained from Sigma Co., U.S.A.

Organism and Growth Conditions

A. coreanus NR 15-1 [31] isolated from traditional Korean *Nuruk* was used. The medium composition and culture conditions were the same as those reported previously [30]. An uncooked medium was sterilized at room temperature by ethylene oxide gas (Ethylene oxide sterilizer, IKI-M, Japan). *A. coreanus* NR 15-1 was cultivated on an uncooked wheat bran with 35 w/w% of distilled water at 28°C for 4 days.

Preparation of Crude Extract

The enzyme was extracted from cultivated materials with 0.5% NaCl solution. After maceration of the cultivated materials overnight at 4°C, the suspension was filtered with a steel net. The materials were removed by centrifugation at 13,000 ×*g* for 10 min, and the resulting supernatant was used as the crude enzyme preparation.

Assay of Glucoamylase Activity

Glucoamylase activities were assayed by the release of glucose from boiled soluble starch by the method reported previously [22]. 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 hour at 40°C.

Protein Determination

Protein concentration was determined by the method of Bradford [3] using a protein assay kit (Bio-Rad Co., U.S.A.) with bovine serum albumin as a standard protein or by measuring absorbance at 280 nm.

Purification of Glucoamylase

The glucoamylase from *A. coreanus* NR 15-1 was thermostable, but, all operations were done at temperature below 4°C.

Step 1: Ammonium Sulfate Fractionation. Solid ammonium sulfate was added to crude enzyme suspension (500 ml) to give 0.4 saturation, and pH was adjusted to 5.0. After standing for 12 h, the precipitate at 15,000 ×*g* centrifugation for 30 min was discarded. The ammonium sulfate concentration in the supernatant was then increased to 0.8 saturation by the addition of solid ammonium sulfate. After standing overnight, the resulting precipitate was collected by centrifugation at the same above conditions and dissolved in 0.02 M Na-acetate buffer (pH 5.0). The solution (30 ml) was dialyzed overnight at 4°C against 50 volumes of 0.02 M acetate buffer (pH 5.0), and the precipitate formed was removed by centrifugation at 15,000 ×*g* for 15 min.

Step 2: CM-Cellulose Column Chromatography. The dialyzed enzyme solution was applied to a CM-cellulose column (3.5×15 cm) equilibrated with 0.02 M acetate buffer (pH 5.0). The column was thoroughly washed with the same buffer. After removing much of the inactive protein, elution was carried out with the same buffer containing a linear gradient of 0.2 to 0.4 M NaCl at a flow rate of 15 ml per hour. Active fractions were pooled and concentrated by an Amicon ultrafiltration kit (Model 8050, Grace company, U.S.A.). The concentrated active fractions were dialyzed overnight at 4°C against 4 changes of 10 volumes of the same buffer.

Step 3: DEAE-Cellulose Column Chromatography. The dialyzed enzyme solution was applied to a DEAE-cellulose column (3.5×15 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 8.0). The column was thoroughly washed with the same buffer. An elution was carried out with the same buffer containing a linear gradient of 0.0 to 0.6 M NaCl at flow rate of 15 ml per·h, and active fractions were pooled and concentrated by an Amicon ultrafiltration kit.

Step 4: Sephadex G-100 Column Chromatography. The dialyzed enzyme solution was applied to a Sephadex G-100 column (1.5×70 cm) equilibrated with 0.02 M acetate buffer (pH 5.0). The active fractions were combined and concentrated by an Amicon ultrafiltration kit.

Step 5: Hydroxyapatite Column Chromatography. Glucoamylase from the concentrated active fractions of step 4 was finally purified by hydroxyapatite column (2.0× 10 cm) chromatography. The concentrated enzyme solution was introduced into the column equilibrated with 1 mM phosphate buffer (pH 6.8), the column was washed thoroughly with the same buffer, and elution was done by a linear gradient, formed with 0.01 M to 0.04 M phosphate buffer, at a flow rate of 15 ml per h.

Electrophoresis

Polyacrylamide gel electrophoresis was performed by a modified version of Davis' method [6]. Stacking and running gels were polymerized in a test tube (0.5×10 cm). After running with a constant current of 8 mA per gel, 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 [23] on a 10% gel with the normal amount of cross-linker at 5 mA per gel. After running, the gel was

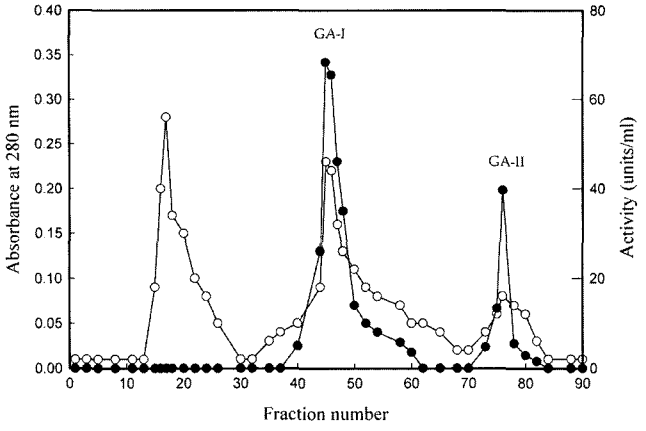

Fig. 1. Elution profile of the enzyme on hydroxyapatite column chromatography.
The column was equilibrated with 0.001 M phosphate buffer (pH 6.8). The absorbed enzyme was eluted by stepwise addition of 0.01, 0.02, and 0.04 M phosphate buffer (pH 6.8) at a flow rate of 15 ml/h, and each fraction volume was 3.5 ml. Symbols: ○, Absorbance at 280 nm; ●, Glucoamylase activity.

stained with Coomassie brilliant blue R-250 (Sigma Co., U.S.A.), electrophoretically destained, and stored in 7% acetic acid.

Molecular Weight

The molecular mass of the enzyme was estimated by 7.5% polyacrylamide gel electrophoresis in the presence of 0.1% SDS according to Weber and Osborn [23]. The standard proteins used were myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamate dehydrogenase (53 kDa). The molecular mass of the enzyme was also estimated by gel filtration, according to Andrews [2]. Gel filtration was performed in a Sephadex G-100 column (1.8×81 cm) previously equilibrated with 0.02 M Na-acetate buffer (pH 5.0), and was eluted at a flow rate of 10 ml per h. Each standard protein was applied as a solution of 1 mg in 1 ml of the buffer. V_e was the elution volume, and the void volume (V_0) was determined by elution of blue dextran. The standard proteins used were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa),

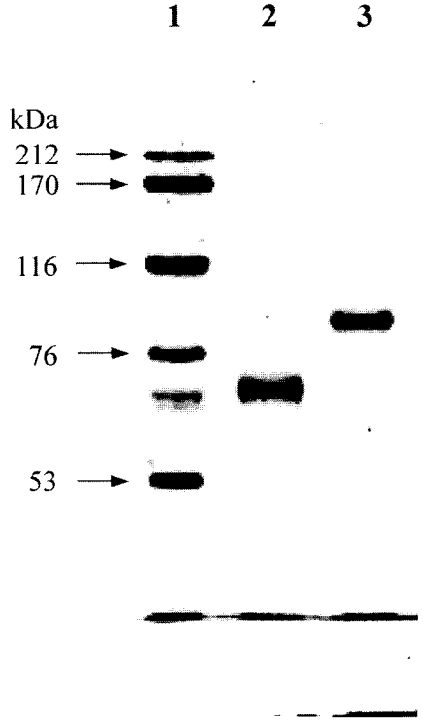

Fig. 2. SDS-polyacrylamide gel electrophoresis of the purified enzyme.
Lane 1, standard protein size markers: myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), glutamic dehydrogenase (53 kDa); lane 2, purified glucoamylase GA-I; lane 3, purified glucoamylase GA-II.

bovin serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

Analysis of Hydrolysis Products

The reaction mixture was centrifuged, and the amount of reducing sugar in the supernatant was determined as glucose by thin layer chromatography (TLC). One μl of the supernatant was developed on TLC plate (Silica gel 60 aluminium plate, E. Merck, Darmstadt, Germany) with a solvent (acetone:H_2O=9:1) at room temperature for 30 min. The TLC plate was dried, and

Table 1. Summary of purification of glucoamylase from *Aspergillus coreanus* NR 15-1.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	225	19,826	88	100.0	1.0
$(NH_4)_2SO_4$ (40-80%)	64.9	10,868	167	54.8	1.9
CM-cellulose chromatography	45.5	8,869	194	44.7	2.2
DEAE-cellulose chromatography	15.2	4,069	267	20.5	3.0
Sephadex G-100 gel filtration	8.3	3,637	436	18.3	4.9
Hydroxyapatite chromatography					
GA-I	2.1	1,073	511	5.41	5.8
GA-II	0.24	204	851	1.03	9.6

spots were detected by spraying aniline-diphenylamine [7].

Raw Starch Digestion

The reaction mixture contained 0.2 g each of various raw starch, such as wheat starch, corn starch, potato starch, rice starch, and soluble starch, 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 while stirring occasionally [21], and 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.

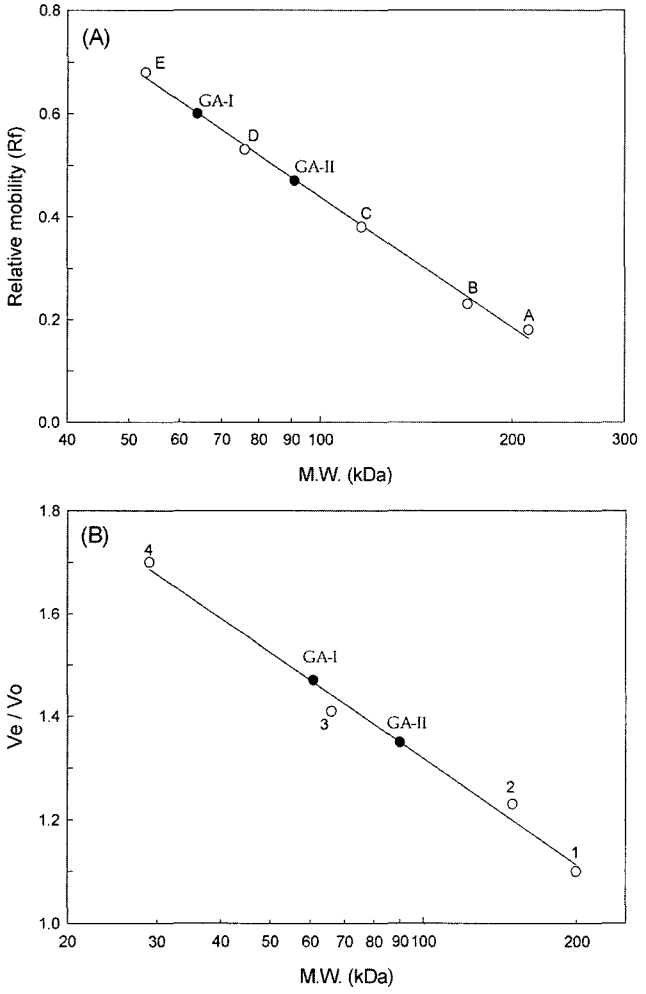

Fig. 3. Determination of molecular mass of the enzymes by SDS-PAGE (A) and by gel filtration (B).
The electrophoresis was performed in 7.5% polyacrylamide gel containing 0.1% SDS. Molecular masses of standard proteins were as follows: A, myosin (212 kDa); B, α_2-macroglobulin (170 kDa); C, β-galactosidase (116 kDa); D, transferrin (76 kDa); E, glutamate dehydrogenase (53 kDa). Symbols: ○, standard proteins; ●, Glucoamylase. Sephadex G-100 column equilibrated with 0.02 M Na-acetate buffer (pH 5.0) was eluted at a flow rate of 10 ml per h. Each standard protein was applied as a solution of 1 mg in 1 ml of the buffer. Ve is the elution volume. The void volume (V_0) was determined by elution of blue dextran. Molecular sizes of standard proteins were as folows: 1, β-amylase (200 kDa); 2, alcohol dehydrogenase (150 kDa); 3, bovine serum albumin (66 kDa); 4, carbonic anhydrase (29 kDa).

Results and Discussion

Purification of Glucoamylase

Elution profile of the enzyme on hydroxyapatite column chromatography, the final preparation step, showed a single and symmetrical protein peak, and the enzyme activity was entirely associated with these two protein peaks (Fig. 1). The purification procedure is summarized in Table 1. By these procedures, glucoamylase GA-I and GA-II from *A. coreanus* NR 15-1 were apparently homogeneously purified about 5.8- and 9.6-fold with 5.41% and 1.03% yields, respectively.

Homogeneity and Molecular Size

The homogeneity of the purified glucoamylase was investigated by disc- and SDS-polyacrylamide gel electrophoresis. The final preparation (GA-I and GA-II) showed a single band on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 2). As shown in Fig. 3(A), the molecular sizes of the GA-I and GA-II were estimated to be 64 kDa and 91 kDa by polyacrylamide gel electrophoresis in the presence of SDS, and 62 kDa and 90 kDa by Sephadex G-100 gel filtration, respectively [Fig. 3(B)]. The molecular mass of the GA-I is similar to those of *A. niger* (56 kDa) [4], *A. usami* AM 2185 (67 kDa) [5], and

Table 2. Amino acid composition of glucoamylase GA-I and GA-II from *Aspergillus coreanus* NR 15-1.

Amount (%)	GA-I	GA-II	GA-I/GA-II
Cys	0.19	1.22	0.16
Asp	10.14	8.39	1.21
Glu	10.55	11.30	0.93
Ser	13.26	13.25	1.00
Gly	11.88	13.05	0.91
His	0.79	1.03	0.77
Arg	3.19	2.72	1.17
Thr	10.05	11.07	0.90
Ala	9.41	9.07	1.04
Pro	5.41	5.56	0.97
Tyr	0.14	0.24	0.58
Val	5.55	5.69	0.98
Met	0.17	0.20	0.85
Cys2	0.76	0.39	1.95
Ile	4.24	3.71	1.14
Leu	7.41	6.51	1.14
Phe	3.70	3.16	1.17
Trp	2.01	2.21	0.91
Lys	1.13	1.22	0.93

A. oryzae (68.4 kDa) [14], and that of the GA-II is similar to those of *A. saitoi* (90 kDa) [19] and *A. awamori* (90 kDa) [8]. Based on the above results, the glucoamylases (GA-I and GA-II) from *Aspergillus coreanus* NR 15-1 appeared to be a monomer.

Amino acid composition analysis of the GA-I and GA-II showed that they contained a large proportion of serine and glycine, and a small fraction of basic amino acids, but high acidic amino acids content. Therefore, the GA-I and GA-II enzyme proteins were found to be acidic.

Furthermore, the GA-II contained a larger proportion of cysteine (GA-I/GA-II contents=0.16%) than that of the GA-I (Table 2).

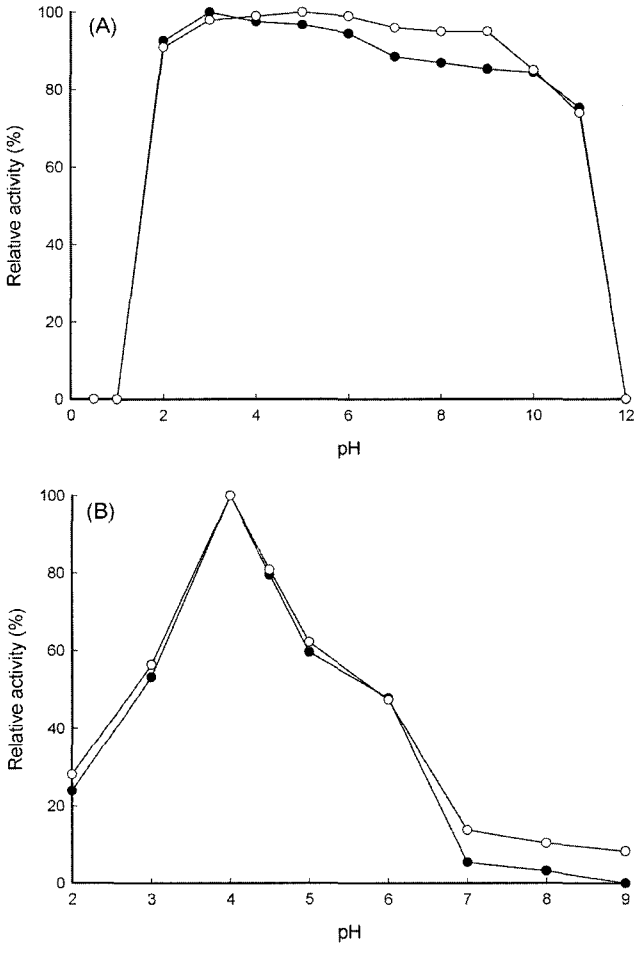

Fig. 4. pH stability (A) and optimum pH (B) of the purified enzyme.

Enzyme suspensions were kept in 0.2 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). Symbols: ●, GA-I; ○, GA-II. The enzyme activity was assayed under standard reaction conditions using glycine-HCl buffer (pH 2.0 to 3.0), Na-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). Symbols: ●, GA-I; ○, GA-II.

Effect of pH and Temperature on the Enzyme Stability and Activity

In order to examine the effect of pH on the enzyme stability, the enzymes were dialyzed for 24 h at 4°C in various 0.2 M buffers of pH 0.5 to 12.0, and the residual activity was assayed under the standard conditions. As shown in Fig. 4(A), about 55% and 40% of both enzyme activities remained in the pH 2.0 and 11.0 at 4°C for 24 h, respectively, and were stable in the pH range of 3.0 to 10.0. The above results suggest that glucoamylase of *A. coreanus* NR 15-1 is very stable in acidic and alkaline pH, compared with those of *A. niger* [4], *A. oryzae* [14], and *R. oryzae* [10]. The optimum pH of both enzyme activities were found to be 4.0, as shown in Fig. 4(B).

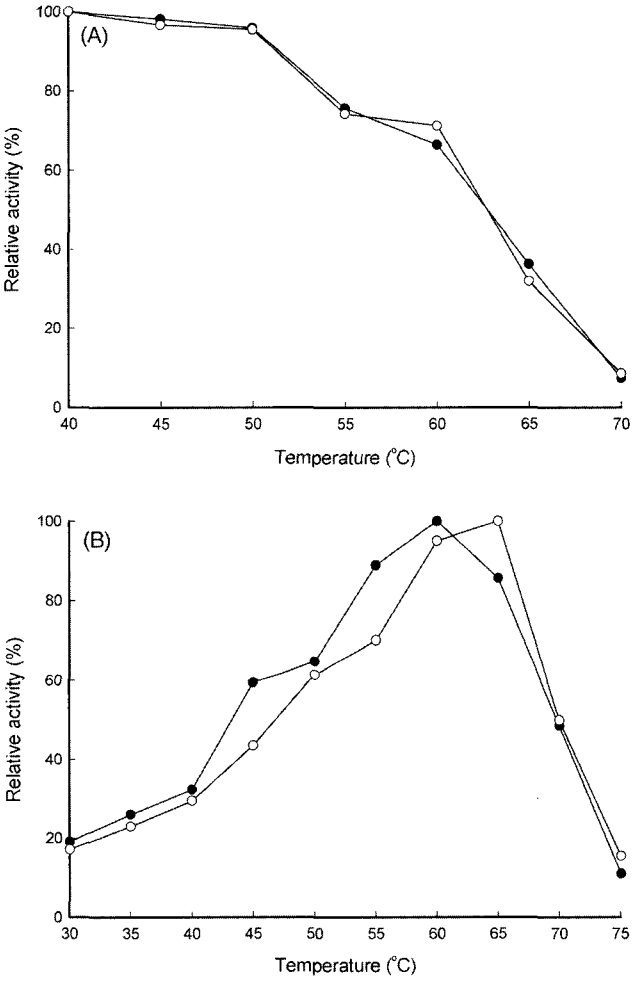

Fig. 5. Thermal stability (A) and optimum temperature (B) of the purified enzyme activity.

Enzyme suspensions were kept in 0.2 M Na-acetate buffers (pH 5.0) and incubated at the indicated temperatures for 20 min. The residual activity was assayed under standard reaction conditions. Symbols: ●, GA-I; ○, GA-II. The enzyme activity was assayed under standard reaction conditions at various temperatures. Symbols: ●, GA-I; ○, GA-II.

Moreover, as shown in Fig. 5(B), the optimum temperatures for the GA-I and GA-II were found to be 60°C and 65°C, respectively, and both enzyme activities were decreased to half after 20 min at 63°C [Fig. 5(A)]. Activation energies (*Ea*) of the GA-I and GA-II were 11.66 kcal/mol and 12.09 kcal/mol, respectively, determinated by Arrhenius plot (data not shown) [16]. Glucoamylase from *A. coreanus* NR 15-1 was very thermo-unstable at higher than 60°C, unlike that from *A. niger* [4] and *A. oryzae* [14]; however, it was relatively stable at room temperature. Optimum temperatures of glucoamylase from *A. oryzae* [14], *A. usamii* [5], and *A. niger* [4] were found to be 56, 60, and 65°C, respectively. Optimum temperature of *A. coreanus* NR 15-1 enzymes was similar to other *A.* spp. enzymes [4, 5].

Michaelis Constants

The effect of concentration of the substrate on the enzyme activities was examined with soluble starch as substrate. Lineweaver-Burk double reciprocal plots gave 3.57 mg/ml and 6.25 mg/ml of the apparent Michaelis constant K_m of the GA-I and GA-II, respectively (Fig. 6).

Effect of Metal Ions and Inhibitors

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

As shown in Table 3, the enzymes were weakly inhibited by 1 mM Hg^{2+}, but not affected by some metal ions, such as Pb^{2+}, Zn^{2+}, Fe^{2+}, Ca^{2+}, and Cd^{2+}, at 1 mM concentration. They were activated by Mn^{2+} and Cu^{2+}. *A. usamii* glucoamylase [5] was not affected by 1 mM Hg^{2+}, but enzymes from *R. oryzae* [11] and *A. coreanus* NR 15-1 were weakly inhibited by 1 mM Hg^{2+}. Thermostability of *A. usamii* enzyme [5] has been shown to be greatly increased by the addition of Ca^{2+}, however, thermostability of the present two enzymes were not increased by Ca^{2+}.

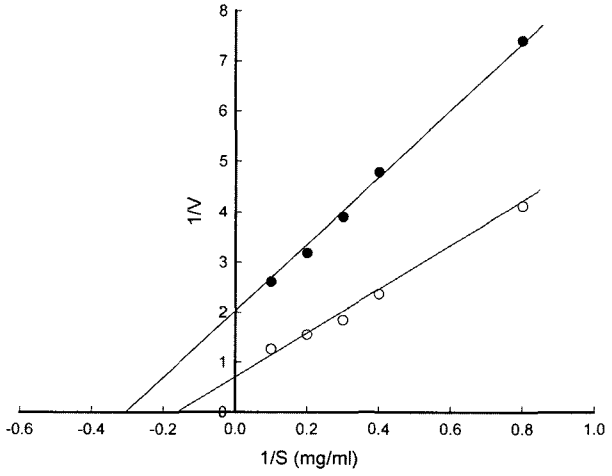

Fig. 6. Determination of K_m value for the purified enzyme by Lineweaver-Burk plot.

The plot is based on the rearrangement of the Michaelis-Menten equation into a linear form. Reaction mixtures of purified glucoamylase and various concentrations of substrate were incubated at pH 5.0 and 40°C to determine the K_m values. Symbols: ●, GA-I; ○, GA-II.

Table 3. Effect of metal ions and inhibitors on the purified glucoamylase activity.

Metal ion and inhibitor (1 mM)	Relative activity* (%)	
	GA-I	GA-II
$PbCl_2$	104.0	95.7
$MnCl_2$	137.5	152.5
$HgCl_2$	96.1	94.1
$ZnCl_2$	104.6	99.1
$FeCl_2$	105.2	103.3
$CaCl_2$	101.7	100.0
$CuCl_2$	115.6	128.5
$CdCl_2$	100.0	100.0
p-CMB	97.8	58.7
N-Bromosuccinimide	0	0
DTT	98.5	67.1
EDTA	100.5	69.5
2-Mercatoethanol	98.1	93.9
o-Phenanthroine	101.1	88.3
PMSF	101.1	82.6
Pyridoxal 5'-phosphate	102.1	66.5
None	100	100

*The enzyme activity was assayed under the standard condition.
p-CMB, *p*-chloromercuribenzoate; DTT, dithiothreitol; EDTA, ethylene diaminetetraacetic acid; PMSF, phenylmethyl sulfonyl fluoride.

The GA-II was inhibited by *p*-chloromercuribenzoate, dithiothreitol, ethylene diaminetetraacetic acid, and pyridoxal 5'-phosphate, and weakly inhibited by *o*-phenanthroline and phenylmethyl sulfonyl fluoride; however, the GA-I was not inhibited by them. *N*-Bromosuccinimide has been used to oxidize tryptophan residues of enzyme protein, thereby decreasing the enzyme activity [13]. The GA-I and GA-II were completely inhibited by 1 mM *N*-bromosuccinimide, suggesting that the tryptophan residue might be located in or near the active site of the GA-I and GA-II enzyme proteins.

Raw Starch Digestion

Raw starch digestion by *A. coreanus* NR 15-1 glucoamylase was carried out in 0.1 M acetate buffer (pH 5.0) at 30°C for 5 h. As shown in Fig. 7, about 10% of raw potato starch and 25% of raw corn starch were digested by the enzyme, compared with 100% for soluble starch (control). Raw rice starch was digested 205% and 370% better than soluble starch by the GA-I and GA-II, respectively. Furthermore, raw wheat starch was digested 780% and 730% better than soluble starch by the GA-I and GA-II, respectively. Based on these results, the glucoamylase from *A. coreanus* NR

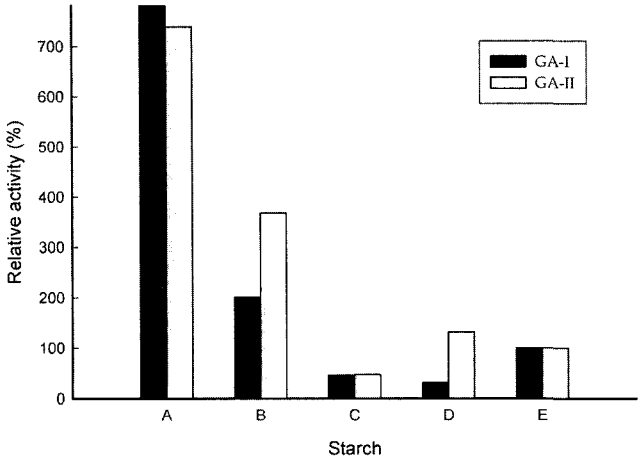

Fig. 7. Digestion of raw starches by the purified enzyme.
The various kinds of raw starch were as follows: A, Raw wheat starch; B, Raw rice starch; C, Raw potato starch; D, Raw corn starch; E, Soluble starch.

15-1 appears 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 substrate by the enzyme, 2% soluble starch was digested by both enzymes at 40°C for 1 h, and the products were then assayed by TLC. As shown in Fig. 8, R_f values of maltose and glucose were 0.32 and 0.47, respectively, and the R_f value of the product of soluble starch was 0.47. From this result, purified GA-I and GA-II enzymes from *A. coreanus* NR 15-1 were identified as glucoamylase, because the reaction product of soluble starch was mainly glucose, as determined by TLC.

Glucoamylase of *A. coreanus* showed negligible activity towards raw starch [15]. Furthermore, raw potato starch and raw wheat starch have been shown to be most difficult to be hydrolyzed by glucoamylase from *A. niger* [4], *R. oryzae* [11], and *Corticium rolfsii* [24]. In the present study, however, glucoamylase of *A. coreanus* NR 15-1 isolated from traditional Korean *Nuruk* showed very strong digesting activities towards raw wheat and rice starches. These results suggest that the glucoamylase is extremely useful in ethanol fermentation of uncooked wheat starch.

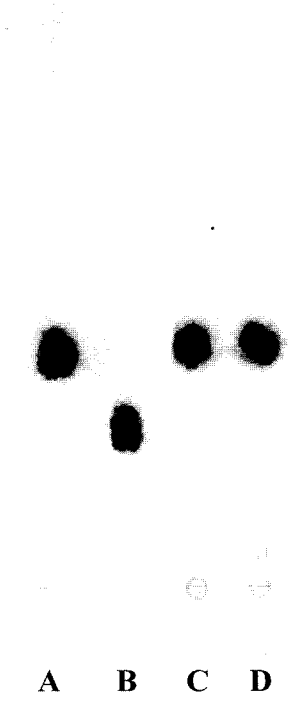

Fig. 8. Thin-layer chromatography of the reaction product of soluble starch hydrolyzed by the purified enzyme.
Standard sugars, glucose (A), maltose (B), sugar produced by hydrolysis by purified glucoamylase GA-I (C), and sugar produced by hydrolysis by purified glucoamylase GA-II (D).

Acknowledgments

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References

1. Allain, C. C., L. S. Poon, C. S. G. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20:** 470-475.
2. Andrews, P. 1964. Estimation of the molecular weights of proteins by Sephadex gel filtration. *J. Biochem.* **91:** 222-233.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
4. Chung, M. J. 1997. Purification and characteristics of raw starch hydrolyzing enzyme from *Aspergillus niger*. *Kor. J. Appl. Microbiol. Biotechnol.* **25:** 166-172.
5. Chung, M. J., W. N. Hou, J. H. Jeong, and H. Taniguchi. 1990. Studies on the development and the characteristics of the powerful raw starch digesting enzyme. *Kor. J. Appl. Microbiol. Biotechnol.* **18:** 251-259.
6. Davis, B. J. 1964. Disc electrophoresis II. Method and application to human serum protein. *Ann. New York Acad. Sci.* **121:** 404-427.
7. Hansen, S. A. 1975. Thin-layer chromatographic method for identification of oligosaccharides in starch hydrolysates. *J. Chromatogr.* **105:** 388-390.
8. Hayashida, S., S. Kunisaki, M. Nakao, and P. Q. Flor. 1982. Evidence for raw starch-affinity site on *Aspergillus awamori* glucoamylase I. *Agric. Biol. Chem.* **46:** 83-89.
9. Ji, J.-H, J.-S. Yang, and J.-W. Hur. 2003. Purification and characterization of the exo-B-D-glucosaminase from *Aspergillus flavus* IAM2044. *J. Microbiol. Biotechnol.* **13:** 269-275.

10. Kim, C. J., M. J. Oh, and J. S. Lee. 1985. Studies on digestion of raw starch by *Rhizopus oryzae*. *Kor. J. Appl. Microbiol. Bioeng.* **13:** 329-337.
11. Kim, C. J., M. J. Oh, and J. S. Lee. 1986. Purification characterization of raw starch digesting enzyme from *Rhizopus oryzae*. *Korean J. Food Sci. Technol.* **18:** 288-293.
12. Kim, H. S., J. S. Hyun, J. Kim, H. P. Ha, and T. S. Yu. 1998. Enzymological characteristics and identification of useful fungi isolated from traditional Korean *Nuruk*. *Kor. J. Appl. Microbiol. Biotechnol.* **26:** 456-464.
13. Nakasaka, Y., K. Kurosawa, A. Yokota, and F. Tomita. 1998. Purification and properties of the raw-starch-digesting glucoamylases from *Corticium rolfsii*. *Appl. Microbiol. Biotechnol.* **50:** 323-330.
14. Ono, K., S. Shigeta, and S. Oka. 1988. Effective purification of glucoamylase in koji, a solid culture of *Aspergillus oryzae* on steamed rice, by affinity chromatography using an immobilized acabose (Bay g-5421). *Agric. Biol. Chem.* **52:** 1707-1714.
15. Razzaque, A. and S. Ueda. 1978. Glucoamylase of *Aspergillus oryzae*. *J. Ferment. Technol.* **56:** 296-302.
16. Segel, I. H. 1975. *Biochemical Calculations* (2nd Ed.), New York, John Wiley and Sons, p. 203.
17. Sohn, C. B. 1983. Studies on the raw starch saccharifying enzyme from *Aspergillus niger* and its mutant. The graduate school of Chungnam National University. Thesis for the degree of Doctor.
18. Song, M. H., K. Selvam, H.-Y. Jeong, and K.-S. Chae. 2003. Inhibition of asexual sporulation and growth of *Aspergillus niger* and *Aspergillus oryzae* by propylamine. *J. Microbiol. Biotechnol.* **13:** 146-148.
19. Takahashi, T., N. Inokuchi, and M. Irie. 1981. Purification and characterization of a glucoamylase from *Aspergillus saitoi*. *J. Biochem.* **89:** 125-134.
20. Takahashi, T., Y. Tsuchida, and M. Irie. 1978. Purification and some properties of three forms of glucoamylase from a *Rhizopus* species. *J. Biochem.* **84:** 1183-1194.
21. Taniguch, H., F. Odashima, M. Igarashi, Y. Maruyama, and M. Nakamura. 1982. Characterization of a potato starch-digesting bacterium and its production of amylase. *Agric. Biol. Chem.* **46:** 2107-2115.
22. The Brewing Society of Japan. 1993. *The Annotation of the Official Method of Analysis of the National Tax Administration Agency.* 4th Ed., Tokyo: 218-226.
23. Weber, K. and M. Osborn. 1969. The reliability of molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406-4412.
24. Witkop, B. 1961. Nonenzymatic methods for the preferential and selective cleavage and modification of protein, pp. 221-321. *In* C. B. Anfinsen, Jr., M. L. Anson, K. Bailey, and J. T. Edsall (eds.), *Advances in Protein Chemistry*, Vol. **16**. Academic Press Inc., New York, U.S.A.
25. Yamasaki, Y., Y. Suzuki, and J. Ozawa. 1977. Purification and properties of two forms of glucoamylase from *Penicillium oxalicum*. *Agric. Biol. Chem.* **41:** 755-762.
26. Yamasaki, Y., A. Tsuboi, and Y. Suzuki. 1977. Two forms of glucoamylase from *Mucor rouxianus*. *Agric. Biol. Chem.* **41:** 2139-2148.
27. Yamasaki, Y., Y. Suzuki, and J. Ozawa. 1977. Properties of two forms of glucoamylase from *Penicillium oxalicum*. *Agric. Biol. Chem.* **41:** 1443-1449.
28. Yasuda, M., M. Kuwae, and H. Matsushita. 1989. Purification and properties of two forms of glucoamylase from *Monasocus* sp. No. 3403. *Agric. Biol. Chem.* **53:** 247-249.
29. Yu, T. S., H. S. Kim, J. J. Hong, J. P. Ha, T. Y. Kim, and I. W. Yoon. 1996. Bibliographical study on microorganisms of *Nuruk* (until 1945). *J. Korean Soc. Food. Nutri.* **25:** 170-179.
30. Yu, T. S., T. H. Kim, and C. Y. Joo. 1999. Purification and characteristics of glucoamylase in *Aspergillus oryzae* NR 3-6 isolated traditional Korean *Nuruk*. *J. Microbiol.* **37:** 80-85.
31. Yu, T. S., S. H. Yeo, and H. S. Kim. 2004. A new species of Hyphomycetes, *Aspergillus coreanus* sp. nov., isolated from traditional Korean *Nuruk*. *J. Microbiol. Biotechnol.* **14:** 182-187.