Relationship between Carbohydrate Moiety and Thermostability of β -Glucosidase from *Mucor miehei* YH-10

Hajime YOSHIOKA and Shinsaku HAYASHIDA

Department of Agricultural Chemistry, Kyushu University,
Fukuoka 812, Japan

Received July 8, 1980

Two forms of thermostable β -glucosidase (a more thermostable G-a and less thermostable G-b) were purified to homogeneity from the culture filtrate of the thermophilic fungus *Mucor miehei* YH-10. G-a and the G-b were glycoenzymes, and they contained 23.4% and 13.0% carbohydrate residues, respectively. The carbohydrate residues were mannose and *N*-acetylglucosamine. The carbohydrate residues in G-a had a high content of *N*-acetylglucosamine. In the culture filtrate, a high activity of β -*N*-acetylglucosaminidase was observed. The carbohydrate content of native G-a decreased to 13.0% when sufficiently digested with a commercial preparation of β -*N*-acetylglucosaminidase instead of β -*N*-acetylglucosaminidase produced by this fungus. The digested enzyme was identical with native G-b in carbohydrate content, molecular weight, amino acid composition, isoelectric point, hydrolysis curve for cellobiose, *K_m* value for β -glucosides, and in both thermo- and pH-dependency and stability. Therefore, the liberation of carbohydrates from more thermostable G-a by β -*N*-acetylglucosaminidase in culture filtrate led to the formation of less thermostable G-b.

As previously reported,¹⁾ a thermophilic fungus identified as *Mucor miehei* YH-10 produced a remarkable amount of thermostable β -glucosidase when cultured in wheat bran medium (50°C, 4 days). During the purification of β -glucosidase produced by *Mucor miehei* YH-10, two forms of β -glucosidase (G-a and G-b) were observed in the wheat bran culture filtrate. G-a and the G-b were further purified from the mold bran filtrate by consecutive column chromatography to a homogeneous state on polyacrylamide gel disc electrophoresis. G-a retained 56% of original activity after heating at 95°C for 5 min, whereas G-b was completely inactivated after heating at 80°C for 5 min.

In the study here, α -mannosidase, β -*N*-acetylglucosaminidase and protease activities were measured in the culture filtrate and were found to be high in β -*N*-acetylglucosaminidase activity. To elucidate the formation mechanism of the two forms of β -glucosidase, native G-a was first digested with a commercial preparation of *Turbo*- β -*N*-acetylglucosaminidase instead of the β -*N*-acetylglucosaminidase produced by this fungus. The digested G-a

showed similar properties of native G-b. This paper describes the formation mechanism of the two forms of β -glucosidase and the role of the carbohydrate moiety on thermostability of β -glucosidase from *Mucor miehei* YH-10.

MATERIALS AND METHODS

Preparation of β -glucosidase. *Mucor miehei* YH-10 was cultured in a 21-Erlenmeyer flask containing solid wheat bran medium to produce a remarkable amount of thermostable β -glucosidase. In the process of purification, two forms of β -glucosidase (G-a and G-b) were fractionated by Sephadex G-100 gel filtration. Both enzyme preparations were further purified by DEAE-Sephadex A-50 column chromatography and Sephadex G-200 gel filtration until a homogeneous state was attained on polyacrylamide gel disc electrophoresis, as previously reported.¹⁾

Chemicals. Cellobiose and salicin were obtained from Nakarai Chemical Co., Kyoto. *p*-Nitrophenyl- β -D-glucopyranoside, *p*-nitrophenyl- α -D-mannoside, *p*-nitrophenyl- β -D-*N*-acetylglucosaminide and gentiobiose were from Sigma Chemical Co., St. Louis.

Assay of enzymes. (a) β -Glucosidase: β -Glucosidase was assayed in the reaction mixture containing 0.5% cellobiose in 5 ml 0.1 M phosphate buffer (pH 8.0) and 5 ml enzyme solution. The mixture was incubated

at 60°C for 1 hr. The glucose formed was estimated with glucose-oxidase-colorimetric reagents (glucose-K, Katayama Chemical Industry Co., Osaka). The enzyme unit was defined by the amount of enzyme which forms 1 mg of glucose from the substrate per min under the assay conditions. (b) α -Mannosidase and β -*N*-acetylglucosaminidase: *p*-Nitrophenyl- α -D-mannoside and *p*-nitrophenyl- β -D-*N*-acetylglucosaminide were used as substrates for the assay of α -mannosidase and β -*N*-acetylglucosaminidase, respectively. The reaction mixture contained 0.1 ml enzyme solution and 0.9 ml of 0.01% substrate suspended in 0.2 M citrate-phosphate buffer, pH 4.2. The mixtures were incubated at 50°C for 30 min before the addition of 2 ml 0.25 M sodium carbonate. The liberated *p*-nitrophenol was determined spectrophotometrically at 400 nm by the method of Norkans.²⁾ The enzyme unit was defined as the amount of enzyme liberating 1 μ mol *p*-nitrophenol from the substrate per min at 50°C. (c) Protease: Protease activity was determined by incubating a 1 ml enzyme solution with 5 ml of 0.6% Hammersten's casein (buffered at pH 3, 7 and 9) for 10 min. One unit of protease activity was defined as the amount of enzyme which liberated 1 μ g tyrosine equivalent from the substrate for 1 min at 50°C, measured by Folin's procedure.³⁾

Composition of carbohydrate residues. The carbohydrate composition was determined after hydrolyzing the purified enzyme preparation with 1 N hydrochloric acid at 100°C for 1 hr. The constituent sugar of the carbohydrate residues was identified with paper chromatography. Mannose and *N*-acetylglucosamine contents were determined by the phenol-sulfuric acid method⁴⁾ and Morgan-Elson reaction method,⁵⁾ respectively.

Preparation of β -glucosidase- and protease-free β -*N*-acetylglucosaminidase. Trace activities of β -glucosidase and protease in β -*N*-acetylhexosaminidase (*Turbo*, Seikagaku Kogyo Co., Tokyo; 50.1 Umg of β -*N*-acetylglucosaminidase) were removed by DEAE-Sephadex A-50 column chromatography. The β -*N*-acetylhexosaminidase preparation (40 mg) was dissolved in 10 ml 0.05 M phosphate buffer, pH 5.5, applied on a DEAE-Sephadex A-50 column (15 $\times$ 4.5 cm) previously buffered with 0.05 M phosphate buffer pH 5.5, and was eluted with a linear gradient from 0.05 M to 1 M phosphate buffer, pH 5.5 at 4°C. The flow rate was 10 ml per hr, and the eluate was collected in 5 ml fractions. β -*N*-acetylglucosaminidase activity appeared in fraction No. 12~16. These fractions were combined, concentrated, completely desalted by filtration through a Sephadex G-50 column and lyophilized. The lyophilized sample showed no β -glucosidase or protease activity and was designated as *Turbo*- β -*N*-acetylglucosaminidase.

Digestion of native G-a with *Turbo*- β -*N*-acetylglucosaminidase. Purified G-a (500 mg) was dissolved in 10 ml 0.2 M citrate-phosphate buffer, pH 4.2, and incubated at 30°C for 2 days with 3 mg of β -*N*-acetylglucosaminidase.

Hydrolysis of cellobiose. The hydrolysis rate (%) was calculated from the amount of glucose formed with enzyme divided by the amount of glucose after acid hydrolysis with 2.5% sulfuric acid at 100°C for 5 hr by the method of Seaman *et al.*⁶⁾

Other analytical techniques. Determination of protein and molecular weight, paper chromatography, isoelectric focusing and amino acid analysis were carried out according to methods described previously.⁷⁾

Determination of Michaels constants for substrate. *K_m* value was calculated from Lineweaver-Burk plots. The velocity of the enzyme reaction was measured under standard assay conditions for β -glucosidase (2.00 $\times$ 10⁻² units). The substrate concentration was varied in the range from 0.15 mM to 3.50 mM. When *p*-nitrophenyl- β -D-glucopyranoside was used as substrate, the liberated *p*-nitrophenol was determined spectrophotometrically at 400 nm. When salicin was used as substrate, the liberated glucose was determined by the glucose-oxidase-colorimetric reagent.

RESULTS

Enzyme productivity in wheat bran medium

During cultivation of *Mucor miehei* YH-10 for 5 days at 50°C in wheat bran medium, the culture filtrate was examined for enzyme activity and changes in pH. The peak of β -glucosidase activity was at about 4 days, as previously reported.¹⁾ As shown in Fig. 1, high β -*N*-acetylglucosaminidase and low protease activities were exhibited by the culture filtrate, whereas no α -mannosidase activity was observed throughout the culture period.

Composition of carbohydrate residues in native β -glucosidase

As shown in Fig. 2, carbohydrate residues in native β -glucosidase (G-a and G-b) were composed of mannose and *N*-acetylglucosamine. Native G-a and G-b contained 10.3 and 8.0 percent, respectively, of mannose, and 13.1 and 5.0 percent, respectively, of *N*-acetylglucosamine. The total carbohydrate content of native G-a and G-b was 23.4 and 13.0 percent,

FIG. 1. Time Courses of Enzyme Productions in Wheat Bran Medium.

Enzyme activities were determined as described in MATERIALS AND METHODS. $\circ$ — $\circ$, β -glucosidase activity; $\bullet$ — $\bullet$, β -N-acetylglucosaminidase activity; $\triangle$ — $\triangle$, α -mannosidase activity; $\blacktriangle$ — $\blacktriangle$, protease activity; $\square$ — $\square$, pH of culture filtrate.

FIG. 2. Paper Chromatogram of Constituent Sugars of β -Glucosidase G-a and G-b.

Developed by ascending technique on Whatman No. 1 paper, using *n*-butanol-pyridine-water (6:4:3, v/v) as solvent. Sugars were detected by the dipping method with silver nitrate-sodium hydroxide reagent. 1, glucose marker; 2, galactose marker; 3, mannose marker; 4, *N*-acetylglucosamine-HCl marker; 5, constituent sugars of G-a; 6, constituent sugars of G-b.

respectively. Carbohydrate residues in native G-a had more abundant *N*-acetylglucosamine than those in native G-b.

FIG. 3. DEAE-Sephadex A-50 Column Chromatography of the Digest of β -Glucosidase G-a with Turbo- β -N-acetylglucosaminidase.

The experimental details are described in the text. $\circ$ — $\circ$, absorbance at 280 nm; $\bullet$ — $\bullet$, β -glucosidase activity; $\triangle$ — $\triangle$, absorbance at 490 nm with phenol-sulfuric acid reagent.

Partial removal of carbohydrate moieties from native β -glucosidase

The digested sample of native G-a with Turbo- β -N-acetylglucosaminidase was adjusted to pH 3.0 with 1 N hydrochloric acid and left to stand overnight at 4°C. The precipitate formed was removed by centrifugation. The pH of the supernatant was adjusted to 5.5 with ammonia water, and applied to DEAE-Sephadex A-50 column (2.3 $\times$ 57 cm) at 4°C, as described previously in the text. The activity of the digested G-a was found in fraction No. 67~76, as shown in Fig. 3. The eluted position was almost the same as that of native β -glucosidase. The total carbohydrate content of the digested G-a decreased to 13.0 percent when sufficiently digested.

Composition of carbohydrate residues in digested β -glucosidase

Carbohydrate residues in the digested G-a were composed of mannose and *N*-acetylglucosamine. The digested enzyme contained 8.0 percent of mannose and 5.0 percent of *N*-acetylglucosamine, which was the same as the carbohydrate content of native G-b. The liberated carbohydrate moiety after digestion of native G-a with Turbo- β -N-acetylglucosaminidase was also composed of mannose and *N*-

FIG. 4. Determination of Molecular Weights of Native and Digested β -Glucosidase.

Electrophoresis was performed in 5% polyacrylamide gel in the presence of 0.1 M phosphate buffer, pH 7.2 containing 0.1% SDS. Samples containing 0.01 M phosphate buffer (pH 7.2), 1% SDS, 5% 2-mercapto-ethanol, 50% glycerol and 1 mg of protein were left standing overnight at room temperature. Symbols and molecular weights of standard proteins: 1, cytochrome *c* (12,400); 2, chymotrypsinogen (25,000); 3, ovalbumin (45,000); 4, γ -globulin (160,000); 5, native G-a [250,000]; 6, native G-b [220,000]; 7, digested G-a [220,000]; 8, apo-ferritin (480,000).

acetylglucosamine.

Molecular weight of native and digested β -glucosidase

The molecular weight of native G-a and G-b was 250,000 and 220,000, respectively. On the other hand, the molecular weight of the digested G-a was 22,000, the same as that of native G-b (Fig. 4).

Amino acid composition of native and digested β -glucosidase

The amino acid composition of digested G-a was the same as that of native G-a and G-b, as shown in Table I. Aspartic acid, glycine and glutamic acid were characteristically abundant.

Isoelectric point of native and digested β -glucosidase

As shown in Fig. 5, the isoelectric point of native G-a was pH 4.30. The isoelectric point

TABLE I. AMINO ACID COMPOSITION OF NATIVE AND DIGESTED β -GLUCOSIDASE

Amino Acid	Native G-a	Digested G-a	Native G-b
	Molar Ratio (%)		
Lysine	3.62	3.63	3.62
Histidine	2.10	2.11	2.11
Arginine	4.45	4.45	4.44
Cysteine	1.13	1.13	1.13
Aspartic acid	12.52	12.50	12.51
Threonine	5.73	5.73	5.72
Serine	5.41	5.41	5.41
Glutamic acid	8.59	8.60	8.60
Proline	6.42	6.41	6.42
Glycine	11.93	11.91	11.93
Alanine	7.98	7.97	7.98
Valine	7.62	7.63	7.62
Methionine	1.55	1.55	1.55
Isoleucine	3.69	3.70	3.69
Leucine	7.27	7.28	7.28
Tyrosine	3.41	3.41	3.41
Phenylalanine	3.81	3.81	3.81
Tryptophan	2.77	2.77	2.77

FIG. 5. Isoelectrofocusing of β -Glucosidase G-a.

Electrofocusing electrophoresis was performed for 48 hr on Carrier ampholite (pH ranges 2~8) in a column made by LKB-Produkter AB. $\circ$ — $\circ$, absorbance at 280 nm; $\bullet$ — $\bullet$, β -glucosidase activity; —, pH of eluted fractions.

of native G-b and digested G-a was also pH 4.30.

Hydrolysis action of native and digested β -glucosidase on cellobiose

Cellobiose hydrolysis and its by-products are shown in Figs. 6 and 7, respectively.

Native G-a and G-b almost completely hydrolyzed cellobiose into glucose in 9 hr in 1%

FIG. 6. Hydrolysis Curves of Cellobiose by Native and Digested β -Glucosidase.

The reaction mixture (10 ml) containing cellobiose in 0.1 M phosphate buffer, pH 8.0 and 5 ml of enzyme solution (6.50 units) was incubated at 60°C. The hydrolysis rate (%) is calculated from the amount of glucose formed with enzyme divided by the amount of glucose after acid hydrolysis. (a) Hydrolysis of 1% cellobiose. (b) Hydrolysis of 5% cellobiose. $\circ$ — $\circ$, native G-a; $\bullet$ — $\bullet$, native G-b; $\triangle$ — $\triangle$, digested G-a.

FIG. 7. Paper Chromatograms of the Hydrolysis Products Obtained from Cellobiose.

The experimental details are described in the legend to Fig. 2. (a) Hydrolyzates from 1% cellobiose. (b) Hydrolyzates from 5% cellobiose. M, designated markers; 1, hydrolyzates with native G-a; 2, hydrolyzates with native G-b; 3, hydrolyzates with digested G-a.

solution, but a trace of gentiobiose was detected during the reaction. Moreover, when the concentration of cellobiose increased up to 5%, native G-a and G-b hydrolyzed it into glucose in 24 hr to the extent of 60%. Gentiobiose and cellobiose were also detected during the reaction. The hydrolysis curves and its by-products of digested G-a were the same as those of native G-b.

Hydrolysis action of native and digested β -glucosidase on other substrates

Native G-a and G-b had no action on celulosic substrates, such as Avicel and CMC, but the enzymes attacked not only cellobiose but also some β -glucosides, such as *p*-nitrophenyl- β -D-glucopyranoside and salicin. From Lineweaver-Burk plots, the K_m values of native β -glucosidase (G-a and G-b) were: cellobiose, 1.04 mM; *p*-nitrophenyl- β -D-glucopyranoside, 0.58 mM; and salicin, 0.72 mM. Digested G-a also hydrolyzed other β -glucosides. The K_m value of digested G-a for β -glucosides was the same as that of native G-b.

Activities of native and digested β -glucosidase

Table II shows the specific activities of native and digested β -glucosidase. The specific activity of digested G-a was the same as that of native G-b.

TABLE II. SPECIFIC ACTIVITY OF NATIVE AND DIGESTED β -GLUCOSIDASE

β -Glucosidase	Specific activity
Native G-a	93.1
Native G-b	82.3
Digested G-a	82.3

Specific activity was expressed as units per mg of protein.

Thermo- and pH-dependencies of native and digested β -glucosidase

The effect of temperature on the activity of native and digested β -glucosidase is shown in Fig. 8. The optimal temperature of native and digested β -glucosidase was 60°C. The effect of pH on the activity of native and

digested β -glucosidase is shown in Fig. 9. The optimal pH of native and digested β -glucosidase was 8.0.

FIG. 8. Thermo-dependency of Native and Digested β -Glucosidase.

The reaction mixture (10 ml), containing 0.5% cellobiose in 0.1 M phosphate buffer, pH 8.0 and 5 ml of enzyme solution (4.50×10^{-3} units) was incubated at each temperature for 1 hr. $\circ$ — $\circ$, native G-a; $\bullet$ — $\bullet$, native G-b; $\triangle$ — $\triangle$, digested G-a.

FIG. 9. pH-Dependency of Native and Digested β -Glucosidase.

The reaction mixture (10 ml) containing 0.5% cellobiose in 5 ml of each buffer solution and 5 ml of enzyme solution (4.50×10^{-3} units) was incubated at 60°C for 1 hr. 0.1 M Hydrochloric acid-sodium acetate buffer, 0.1 M acetate buffer, 0.1 M phosphate buffer and 0.1 M ammonia water-ammonium chloride buffer were used at pH 2.0~3.0, pH 3.0~5.0, pH 5.0~8.0 and pH 8.0~11.0, respectively. $\circ$ — $\circ$, native G-a; $\bullet$ — $\bullet$, native G-b; $\triangle$ — $\triangle$, digested G-a.

Thermo- and pH-stabilities of native and digested β -glucosidase

The thermal stability of native and digested β -glucosidase is compared in Fig. 10. Native G-a was stable after heating at 70°C for 5 min, and 56% of original activity remained after

FIG. 10. Thermo-stability of Native and Digested β -Glucosidase.

β -Glucosidase activity was measured after allowing the reaction mixture to stand at pH 8.0 for 5 min at various temperatures. The residual activity was expressed as a percentage of the untreated control. $\circ$ — $\circ$, native G-a; $\bullet$ — $\bullet$, native G-b; $\triangle$ — $\triangle$, digested G-a.

FIG. 11. pH-Stability of Native and Digested β -Glucosidase.

β -Glucosidase activity was measured after allowing the reaction mixture to stand at 4°C for 24 hr at various pH. The residual activity at optimal condition (pH 8.0 and 60°C) was expressed as a percentage of the untreated control. $\circ$ — $\circ$, native G-a; $\bullet$ — $\bullet$, native G-b; $\triangle$ — $\triangle$, digested G-a.

heating at 95°C for 5 min. In contrast to G-a, native G-b was completely inactivated after heating at 80°C for 5 min, as previously reported.¹⁾ When the carbohydrate residues of native G-a were partially removed, G-a was inactivated after heating at 80°C for 5 min as native G-b. The thermal stability of digested G-a was the same as that of native G-b. The pH-stability of native and digested β -glucosidase is compared in Fig. 11. Native G-a and G-b were stable at pH 2.5~10.0 and pH 3.0~9.5, respectively, at 4°C for 24 hr. The stability of digested G-a was lowered to pH 3.0~9.5, the same as the activity of native G-b.

DISCUSSION

During the purification procedure of thermostable β -glucosidase from the thermophilic fungus *Mucor miehei* YH-10, two protein peaks with β -glucosidase activity were observed. The highly purified preparation of the two forms of β -glucosidase, G-a and G-b, had the same optimum temperature and pH, amino acid composition, isoelectric point, hydrolysis curves for cellobiose and K_m values for β -glucosides, but differed in molecular weight, carbohydrate content and thermo- and pH-stabilities. On the other hand, *Mucor miehei* YH-10 also exhibited high β -*N*-acetylglucosaminidase and low protease activities but no α -mannosidase activity throughout the cultivation period.

To elucidate the formation mechanism of the two forms of β -glucosidase (G-a and G-b), native G-a was sufficiently digested by *Turbo*- β -*N*-acetylglucosaminidase instead of the β -*N*-acetylglucosaminidase produced by this fungus. During digestion of native G-a, the carbohydrate residues in the enzyme were partially removed. The liberated carbohydrate moiety was also composed of mannose and *N*-acetylglucosamine. Properties of digested G-a were compared with native β -glucosidase. These results showed that digested G-a was identical with native G-b in elution

pattern of DEAE-Sephadex A-50 column, hydrolysis curves for cellobiose, K_m values for β -glucosides, molecular weight, isoelectric point, amino acid composition, carbohydrate content and in thermo- and pH-dependencies and stabilities. Digested G-a was composed of 8.0 percent mannose and 5.0 percent *N*-acetylglucosamine. The fact that digested G-a and native G-b had the same carbohydrate composition and content indicates that specific glycoside bonds located apart from the active site on the molecule of native G-a are susceptible to β -*N*-acetylglucosaminidase. Therefore, the multiple formation of β -glucosidase (G-a and G-b) might be due to liberation of the carbohydrate moiety from the prototype of β -glucosidase (G-a) with β -*N*-acetylglucosaminidase in the culture filtrate during the extraction or purification of β -glucosidase. In addition, the fact that highly stable native G-a became less stable after liberation of the carbohydrate moiety suggested that the high stability of β -glucosidase could be attributed to an abundance of carbohydrate moieties with a high content of *N*-acetylglucosamine. This suggestion was previously reported⁸⁾ in the case of thermostable cellulases produced by *Humicola insolens* YH-8. The relationship between high stability and the chemical structure of the carbohydrate moieties of β -glucosidase will be reported in subsequent papers.

REFERENCES

- 1) H. Yoshioka and S. Hayashida, *Agric. Biol. Chem.*, **44**, 2817 (1980).
- 2) B. Norkans, *Physiol. Plant*, **10**, 198 (1957).
- 3) O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 627 (1927).
- 4) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
- 5) W. T. J. Morgan and L. A. Elson, *Biochem. J.*, **28**, 350 (1934).
- 6) J. F. Seaman, J. L. Buhl and E. E. Harris, *Ind. Eng. Chem. (Anal. Edition)*, **17**, 35 (1945).
- 7) H. Yoshioka and S. Hayashida, *Agric. Biol. Chem.*, **44**, 1729 (1980).
- 8) S. Hayashida and H. Yoshioka, *Agric. Biol. Chem.*, **44**, 481 (1980).