The Role of Carbohydrate Moiety on Thermostability of Cellulases from *Humicola insolens* YH-8

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Received April 9, 1979

Two types of cellulases, Avicelase and CMCase, from a thermophilic fungus *Humicola insolens* YH-8 were glycoenzymes. Avicelase and CMCase contained 26.1% and 39.0%, respectively, of carbohydrate residues which consisted of mannose and *N*-acetylglucosamine. These enzymes, especially CMCase, showed remarkable thermal and pH-stability. The partial removal of the carbohydrate residues, 60% with mixed-glycosidase and 90% with periodate oxidation and Smith degradation, resulted in a significant decrease in thermal and pH-stability. The carbohydrate moiety was suggested to be a stabilizer of cellulases.

A thermophilic fungus, *Humicola insolens* YH-8 produced a remarkable amount of thermostable cellulases in culture in wheat bran medium at 50°C for 4 days. The culture extract hydrolyzed Avicel and newspaper without any physical or chemical pretreatment to the respective extents of 90% and 35% as glucose. Avicelase and CMCase* were purified from the culture extract and found to be homogeneous on polyacrylamide gel disc electrophoresis. Avicelase showed an optimum temperature at 50°C, and indicated 70 percent relative activity in incubation at 60°C for 24 hr. Avicelase was stable at pH 3.5–9.5 at 4°C for 24 hr. CMCase, which had the same optimum temperature as Avicelase, indicated 80 percent relative activity in incubation at 70°C for 24 hr and 45 percent original activity after heating at 95°C for 5 min. CMCase was stable at pH 3.0–11.0 at 4°C for 24 hr. It was suggested that the high stability of these cellulases contributed to the high saccharifying extents of cellulosic substrates at 50°C by use of culture extract.

In this communication, a proposal is advanced that carbohydrate residues covalently linked in the molecular structure of enzymes,

function as a protective factor against heat and acidic or alkaline pHs. This suggestion is made from the result that carbohydrate-split enzymes are remarkably less stable than native cellulases.

MATERIALS AND METHODS

Preparation of cellulases. *Humicola insolens* YH-8 was cultured in a 21-Erlenmeyer flask containing solid wheat bran medium to produce a remarkable amount of Avicelase and CMCase. Both enzyme preparations purified through consecutive column chromatography showed single protein bands on polyacrylamide gel disc electrophoresis.**

Assay of enzymes. (a) Avicelase activity: The reaction mixture containing 100 mg of Avicel (microcrystalline cellulose, Asahi Kasei Co., Tokyo). 5 ml of 0.1 M acetate buffer of pH 5.5 and 5 ml of enzyme solution, was shaken on a Monad-shaker at 90 strokes per minute at 50°C for appropriate periods. Reducing sugar formed was determined by the micro-Bertrand method¹⁾ and phenol-sulfuric acid method.²⁾ One unit of enzyme activity was defined as the amount of enzyme releasing 1 mg of reducing sugar from the substrate per hour. (b) CMCase activity: Incubation was made in the same manner as that described for the Avicelase assay. One unit of CMCase activity was defined as the amount of enzyme releasing 1 mg of reducing sugar from the substrate CMC (carboxymethylcellulose, Wako Junyaku Kogyo Co., Osaka) per minute.

* Avicelase hydrolyzes more strongly water-insoluble substrate (native cellulose and Avicel) than water-soluble substrate (CMC). CMCase hydrolyzes more strongly water-soluble substrate than water-insoluble substrate.

** A part of this work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan at Nagoya, Japan, on April 3, 1978.

Compositions of carbohydrate residues. The carbohydrate compositions were determined after hydrolyzing the purified enzyme preparations with 1N hydrochloric acid at 100°C for 1 hr. The constituent sugars of the carbohydrate residues were identified with paper chromatography, as described in the next paragraph. Mannose and *N*-acetylglucosamine contents were determined by the micro-Bertrand method¹⁾ and Morgan-Elson reaction method,²⁾ respectively.

Preparations of cellulase- and protease-free glycosidase. Trace activities of cellulase and protease in mixed-glycosidase preparation (*Turbo*, Seikagaku Kogyo Co., Tokyo; 74 U/mg of α -mannosidase, 133 U/mg of *N*-acetyl- β -glucosaminidase) were removed by DEAE-Sephadex A-50 column chromatography. The mixed-glycosidase preparation (400 mg) was dissolved in 10 ml of 0.05 M phosphate buffer (pH 5.5), applied on a DEAE-Sephadex A-50 column (1.5 $\times$ 4.5 cm) previously buffered with 0.05 M phosphate buffer of pH 5.5, and was eluted with a linear gradient from 0.05 M to 1 M phosphate buffer of pH 5.5 at 4°C. The flow rate was 10 ml per hr, and the eluate collected in 5 ml fractions. Glycosidase activity appeared in fraction No. 10–15. These fractions were combined, concentrated, completely desalted by filtration through a Sephadex G-50 column and lyophilized. The lyophilized samples had no cellulase or protease activity and was designated as *Turbo*-glycosidase.

Digestions of native cellulases with *Turbo*-glycosidase. Each purified Avicelase and CMCase sample (500 mg) was dissolved in 10 ml of 0.2 M citrate-phosphate buffer of pH 4.0, and incubated at 30°C for 2 days with 3 mg of *Turbo*-glycosidase.

Mild Smith degradation. Each purified Avicelase and CMCase sample (500 mg) was dissolved in 100 ml of 0.02 M sodium metaperiodate in 0.05 M sodium acetate buffer (pH 5.0) by standing for 24 hr at 4°C in the dark.⁴⁾ After desalting by filtration through Sephadex G-15, the periodate-oxidized enzymes were adjusted to pH 9.5 with sodium borohydride by standing for 16 hr at 15°C. The reaction mixture was desalted with filtration through Sephadex G-15, and adjusted to pH 1.0 with hydrochloric acid by standing for 8 hr at 15°C.

Determination of molecular weights. Molecular weights were determined with SDS-polyacrylamide gel electrophoresis. About 1 mg of each cellulase preparation was incubated at 4°C for 10 hr in 0.01 M phosphate buffer (pH 7.2), containing 1% sodium dodecyl sulfate and 5% 2-mercaptoethanol in a final volume of 1 ml. After addition of BPB, solution containing 10 μ g of enzyme was layered on the top of the gel. Electrophoresis was carried out at 8 mA per tube (0.5 $\times$ 10 cm) for 5 hr with 0.1 M phosphate buffer (pH 7.2), containing 0.1% SDS. The protein band was

detected by staining with 0.5% amido black solution.

Paper chromatography. Paper chromatography was carried out at room temperature on Whatman No. 1 filter paper by ascending technique in a solvent of *n*-butanol-pyridine-water (6:4:3). Sugars were detected by the dipping method with silver nitrate-sodium hydroxide reagent.⁵⁾

RESULTS

Composition of carbohydrate residues in Avicelase and CMCase

As shown in Fig. 1, carbohydrate residues in Avicelase and CMCase were composed of mannose and *N*-acetylglucosamine. Avicelase and CMCase contained 14.0 and 20.0 percent, respectively, of mannose, and 12.1 and 19.0 percent, respectively, of *N*-acetylglucosamine. The total content of carbohydrates in Avicelase and CMCase was 26.1 and 39.0 percent, respectively. The carbohydrate residues of both enzymes had abundant *N*-acetylglucosamine.

FIG. 1. Paper Chromatogram of Constituent Sugars of Avicelase and CMCase.

1, glucose marker; 2, galactose marker; 3, mannose marker; 4, *N*-acetylglucosamine-HCl marker; 5, constituent sugars of Avicelase; 6, constituent sugars of CMCase.

Partial removals of carbohydrate moieties from native cellulases

Digested samples of both cellulases with *Turbo*-glycosidase were adjusted to pH 3.0 with 1 N hydrochloric acid, followed by standing at 4°C overnight. Then centrifugation was performed to remove the precipitates formed. The supernatants were adjusted to pH 5.5 with ammonia water, and applied to DEAE-

FIG. 2. DEAE-Sephadex A-50 Column Chromatographies of Digests of Avicelase and CMCase with *Turbo*-glycosidase.

(a) Avicelase digested with *Turbo*-glycosidase. (b) CMCase digested with *Turbo*-glycosidase. The experimental details are described in the text. $\blacktriangle$ — $\blacktriangle$, Enzyme activity; $\circ$ — $\circ$, absorbance at 280 nm; $\bullet$ — $\bullet$, absorbance at 490 nm with phenol-sulfuric acid reagent.

Sephadex A-50 columns (2.3×57 cm) at 4°C by procedures described in MATERIALS AND METHODS. Activities of *Turbo*-glycosidase-digested Avicelase and CMCase were found in fraction No. 42–49 and fraction No. 44–57, respectively (Fig. 2). The eluted positions were almost the same as those of native cellulases. The total content of carbohydrates in digested preparations of Avicelase and CMCase decreased to 9.3 percent and 13.6

percent, respectively, when sufficiently digested. These digested enzymes were designated as 65%-carbohydrate-split enzymes.

The periodate-oxidized- and Smith-degraded-samples of both cellulases were adjusted to pH 5.5 with hydrochloric acid, concentrated and then entirely desalted by filtration through a Sephadex G-15 column. The Smith-degraded-samples were further purified with DEAE-Sephadex A-50 column and then desalted by filtration through Sephadex G-50. The total content of carbohydrates in degraded preparations of Avicelase and CMCase decreased to 2.5 and 4.0 percent, respectively. These digested enzymes were designated as 90%-carbohydrate-split enzymes.

Molecular weight of native and carbohydrate-split cellulases

Though the molecular weights of native Avicelase and CMCase were 72,000 and 57,000, respectively, those of 65%-carbohydrate-split cellulases became 60,000 and 43,000, respectively. On the other hand, those of 90%-carbohydrate-split cellulases became 55,000 and 37,000, respectively (Fig. 3).

FIG. 3. Determination of Molecular Weights of Native and Carbohydrate-split Cellulases.

SDS-polyacrylamide gel electrophoresis was performed as described in the text. 1, cytochrome C (MW 12,400); 2, chymotrypsinogen A (MW 25,000); 3, 90%-carbohydrate-split CMCase [MW 37,000]; 4, 65%-carbohydrate-split CMCase [MW 43,000]; 5, 90%-carbohydrate-split Avicelase [MW 55,000]; 6, native CMCase [MW 57,000]; 7, 65%-carbohydrate-split Avicelase [MW 60,000]; 8, bovine serum albumin (MW 67,000); 8, native Avicelase [MW 72,000].

FIG. 4. Hydrolysis Curves of 65%-Carbohydrate-split Cellulases on Avicel.

The reaction mixture consisting of 100 mg of Avicel, 5 ml of 0.1 M acetate buffer (pH 5.5) and 5 ml of enzyme solution (100 μ g/ml) was incubated at 50°C. The amount of reducing sugar formed was converted into the amount of cellobiose, and then the hydrolysis rate was calculated as percentage of the amount of cellobiose formed with enzyme per the amount of glucose formed with acid hydrolysis. ○—○, Native Avicelase; △—△, native CMCase; □—□, native Avicelase plus native CMCase; ●—●, 65%-carbohydrate-split Avicelase; ▲—▲, 65%-carbohydrate-split CMCase; ■—■, 65%-carbohydrate-split Avicelase plus 65%-carbohydrate-split CMCase.

Hydrolysis on Avicel with native and carbohydrate-split cellulases

As shown in Fig. 4, hydrolysis curves on Avicel with 65%-carbohydrate-split cellulases were the same of those of native cellulases. The 65%-carbohydrate-split Avicelase and CMCase hydrolyzed Avicel to 40 percent and 15 percent, respectively. Synergistic incubation of 65%-carbohydrate-split Avicelase and CMCase hydrolyzed Avicel to 90 percent, the same as native enzymes. On the other hand, by successive chemical treatment by mild periodate oxidation and mild Smith degradation, Avicelase and CMCase showed somewhat weak activities, as seen in Fig. 5.

Hydrolysis products of Avicel with native and carbohydrate-split cellulases

Hydrolysis products of Avicel with carbohydrate-split cellulases incubated at 50°C for 24 hr were the same of those of native cel-

FIG. 5. Hydrolysis curves of 90%-Carbohydrate-split Cellulases on Avicel.

The reaction mixture consisting of 100 mg of Avicel, 5 ml of 0.1 M acetate buffer (pH 5.5) and 5 ml of enzyme solution (100 μ g/ml) was incubated at 50°C. The amount of reducing sugar formed was converted into the amount of cellobiose, and then the hydrolysis rate was calculated as percentage of the amount of cellobiose formed with enzyme per the amount of glucose formed with acid hydrolysis. ○—○, Native Avicelase; △—△, native CMCase; □—□, native Avicelase plus native CMCase; ●—●, 90%-carbohydrate-split Avicelase; ▲—▲, 90%-carbohydrate-split CMCase; ■—■, 90%-carbohydrate-split Avicelase plus 90%-carbohydrate-split CMCase.

FIG. 6. Paper Chromatogram of Avicel Hydrolysates with Native and Carbohydrate-split Cellulases Incubated at 50°C for 24 hr.

1, glucose marker; 2, cellobiose marker; 3, cellotriose marker; 4, hydrolysates with native Avicelase; 5, hydrolysates with native CMCase; 6, hydrolysates with 65%-carbohydrate-split Avicelase; 7, hydrolysates with 65%-carbohydrate-split CMCase; 8, hydrolysates with 90%-carbohydrate-split Avicelase; 9, hydrolysates with 90%-carbohydrate-split CMCase.

lulases, as shown in Fig. 6. The products of Avicel with carbohydrate-split Avicelases were cellobiose and glucose. The products of Avicel with carbohydrate-split CMCase were cellobiose, cellotriose and glucose. With the synergistic action of carbohydrate-split preparations of Avicelase and CMCase, Avicel was hydrolyzed as cellobiose and glucose.

Activities of native and carbohydrate-split cellulases

The specific activities of 65%-carbohydrate-split cellulases were almost the same of those of native cellulases, whereas 90%-carbohydrate-split Avicelase and CMCase were found to retain 70 percent and 90 percent, respectively, of original activity, as shown in Table I.

TABLE I. SPECIFIC ACTIVITY OF NATIVE AND CARBOHYDRATE-SPLIT CELLULASES

	Native	65% Carbo- hydrate-split	90% Carbo- hydrate-split
Avicelase	17.3	17.4	11.9
CMCase	39.2	39.4	34.5

Specific activity was expressed as units per mg of protein.

Adsorbabilities of native and carbohydrate-split Avicelases onto Avicel

As shown in Fig. 7, 65%-carbohydrate-split Avicelase was completely adsorbed onto Avicel as native enzyme, whereas 90%-carbohydrate-split Avicelase also retained 73 percent of

FIG. 7. Adsorption of Native and Carbohydrate-split Cellulases onto Avicel.

Each enzyme (10 μ g/ml) in 5 ml of 0.1 M acetate buffer, pH 5.5, was applied to 1 g of Avicel, followed by standing at 4°C for 10 min. After centrifugation, Avicelase activity in the supernatant fluid was assayed, and the adsorption rate was calculated.

adsorbability onto Avicel.

Thermal stability of native and carbohydrate-split cellulases

The thermal stabilities of native cellulases, 65%-carbohydrate-split cellulases and 90%-carbohydrate-split cellulases were compared in

FIG. 8. Thermal Stability of Native and Carbohydrate-split Avicelases.

Avicelase activity was measured after allowing the reaction mixture to stand at pH 5.5 for 5 min at various temperatures. The residual activity was expressed as percent of untreated control. ○—○, Native Avicelase; ●—●, 65%-carbohydrate-split Avicelase; ▲—▲, 90%-carbohydrate-split Avicelase.

FIG. 9. Thermal Stability of Native and Carbohydrate-split CMCases.

CMCase activity was measured after allowing the reaction mixture to stand at pH 5.5 for 5 min at various temperatures. The residual activity was expressed as percent of untreated control. ○—○, Native CMCase; ●—●, 65%-carbohydrate-split CMCase; ▲—▲, 90%-carbohydrate-split CMCase.

Fig. 8 and Fig. 9, respectively. Native Avicelase was stable after heating at 65°C for 5 min, and native CMCase retained 45 percent of original activity after heating at 95°C for 5 min. When 90 percent of the carbohydrate residues of each enzyme was liberated, Avicelase was

inactivated after heating at 65°C for 5 min, and CMCase was inactivated after heating at 75°C for 5 min.

pH-Stability of native and carbohydrate-split cellulases

Native Avicelase and CMCase were stable at pH 3.5–9.5 and pH 3.0–11.0, respectively, at 4°C for 24 hr. As shown in Fig. 10 and Fig. 11, however, the stability of 65%-carbohydrate-split Avicelase and CMCase was lowered to pH 4.0–8.5 and pH 3.5–9.5, respectively, and the stability of 90%-carbohydrate-split Avicelase and CMCase was lowered to pH 4.5–8.0 and pH 4.0–9.0, respectively.

DISCUSSION

This investigation found that the thermal and pH-stabilities of cellulases from *Humicola insolens* YH-8 had a correlation with their carbohydrate moieties.

In order to check the possible influence of carbohydrate moieties on thermal and pH-stabilities, the carbohydrate residues of purified enzymes were partially removed with mixed-glycosidase or mild Smith degradation. By mixed-glycosidase digestion and Smith degradation, 65 percent and 90 percent, respectively, of carbohydrate moieties were liberated from cellulase. The comparison of the properties of native cellulases with the properties of carbohydrate-split cellulases have shown that the removal of carbohydrates from cellulase does not exert a gross influence on specific activity, hydrolysis curves on Avicel, hydrolysis products of Avicel and adsorbability onto Avicel. Quite clearly, however, partial removal of carbohydrate moieties from both cellulases resulted in a significant decrease in thermal and pH-stability.

Some reports^{6,7)} have indicated that removal of carbohydrate residues from enzyme with periodate oxidation and Smith degradation has exerted a gross influence not only on the stability of the enzyme but also on enzyme activity. For example, shown in yeast invertase, invertase activity towards sucrose and

FIG. 10. pH-Stability of Native and Carbohydrate-split Avicelases.

Avicelase activity was measured after allowing the reaction mixture to stand at 4°C for 24 hr at various pHs. The residual activity at optimal condition was expressed as percent of untreated control. ○—○, Native Avicelase; ●—●, 65%-carbohydrate-split Avicelase; ▲—▲, 90%-carbohydrate-split Avicelase.

FIG. 11. pH-Stability of Native and Carbohydrate-split CMCases.

CMCase activity was measured after allowing the reaction mixture to stand at 4°C for 24 hr at various pHs. The residual activity at optimal condition was expressed as percent of untreated control. ○—○, Native CMCase; ●—●, 65%-carbohydrate-split CMCase; ▲—▲, 90%-carbohydrate-split CMCase.

raffinose dropped to about 38% and 35%, respectively, of original activity, during periodate oxidation. The thermostable cellulases from *Humicola insolens* YH-8, however, have been found to retain considerable catalytic activity even when 90 percent of carbohydrates was removed from protein with our technique of mild Smith degradation.

It is suggested that the characteristically high stability of native Avicelase and CMCase is attributed to an abundance of carbohydrate moieties which characteristically have high content of *N*-acetylglucosamine. The relationship between high stability and the chemical structure of the carbohydrate moieties of these cellulases will be reported in next papers.

REFERENCES

- 1) G. Bertrand, *Bull. Soc. Chim. Paris*, **35**, 1285 (1906).
 - 2) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
 - 3) W. T. J. Morgan and L. A. Elson, *Biochem. J.*, **28**, 988 (1934).
 - 4) H. Yamaguchi, T. Ikenaka and Y. Matsushima, *J. Biochem.*, **65**, 793 (1969).
 - 5) W. E. Trevelyan, D. P. Procter and J. S. Harrison, *Nature*, **166**, 444 (1950).
 - 6) J. H. Pazur, H. R. Knull and D. L. Simpson, *Biochem. Biophys. Res. Commun.*, **40**, 110 (1970).
 - 7) Y. Tashiro and J. R. Trevithick, *Can. J. Biochem.*, **55**, 249 (1977).
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