Effects of Size of Carbohydrate Chain on Protease Digestion of *Aspergillus niger* Endo-β-1,4-glucanase

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Three different carbohydrate-depleted enzymes were prepared from an endo-β-1,4-glucanase of *Aspergillus niger*IFO31125 by treatment with endo-β-N-acetylglucosaminidase or α-mannosidase. They were purified by Concanavalin A-Sepharose affinity and DEAE ion-exchange column chromatographies. The molecular sizes of these enzymes had been decreased from 40 kDa containing 9.0% carbohydrate to 39, 38, and 37 kDa with carbohydrate at 4.5, 1.3, and 0.8% (wt/wt), respectively. The native and these carbohydrate-depleted enzymes were compared in their enzymatic properties, and it was found that they were identical in their catalytic activities and both thermal and pH stabilities. However, the 37-kDa enzyme was more susceptible to proteolysis by Savinase, proteinase K, and Pronase E. On the other hand, the specific protease trypsin showed no such effect on activity of all enzymes. These results suggested that the core structure of the asparagine-linked sugar chain, which consisted of three monosaccharide residues, contributed to the high stability of the endo-1,4-glucanase against protease digestion.

Although many glycoproteins are widely distributed in nature, the biological significance of their constituent oligosaccharides is not clear. The discovery of the role of the oligosaccharides in the structure and function of enzymes of glycoproteins is most intriguing. The term glycoenzymes has been suggested by Pazur et al. to be appropriately descriptive of enzymes containing covalently-linked carbohydrates in their molecular structure,\textsuperscript{11} i.e., enzymes that are glycoproteins. Many glycoenzymes have been identified from various animals, plants, and microorganisms. However, there is little information concerning the role(s) of the carbohydrate moieties in such glycoenzymes. Protection and stabilization against proteolysis and heat inactivation by the carbohydrate moieties have been reported in some glycoenzymes such as porcine ribonuclease, yeast invertase, and mold glucamylase.\textsuperscript{1-8} Bernard et al. described the effects of size and location of the carbohydrate chain in glycoenzymes on protease degradation using bovine pancreatic ribonuclease, and suggested that the protection conferred by the carbohydrate chain might be mediated by steric hindrance of the protease-substrate interaction.\textsuperscript{9}

Recently, we found an endo-1,4-glucanase from a culture filtrate of *Aspergillus niger*IFO31125 with very high thermal stability and high resistance to surfactants and proteases.\textsuperscript{10} Although some workers have isolated several endo-1,4-glucanases from *A. niger* to homogeneity, their enzymes did not show high thermal stability, which is very different from our enzyme. Moreover, no endo-1,4-glucanase showed the high degree of protease resistance observed in our enzyme. The amino acid composition of this endo-1,4-glucanase was similar to that of other enzymes. However, it contained 8.9% carbohydrate, which was the highest content reported among these enzymes from *A. niger*.\textsuperscript{11-15} Thus, we supposed that the high thermal stability and high degree of resistance against protease digestion were due to the carbohydrate moiety of this glycoenzyme.

In this study, we tried to discover the effects of the size of the carbohydrate chain on resistance to proteolysis of the enzyme.

**Materials and Methods**

**Materials.** Endo-β-N-acetylglucosaminidase from *Flavobacterium* sp. was purchased from Seikagaku Corp. α-Mannosidase from jack bean and proteinase K were from Wako Pure Chemical Industries, Ltd. Pronase E was obtained from Kakenkagaku Corp., and Savinase, which is a commercial laundry alkaline protease, was from Novo Nordisk. Trypsin was obtained from Sigma Chemical Co. Concanavalin A-Sepharose was obtained from Pharmacia Fine Chemicals. Carboxymethylcellulose (CM-cellulose) (A01MC, degree of substitution 0.68) was used as substrate for the cellulase activity assay was obtained from Sanyo Kokusaku Pulp. All other chemicals were of the highest grade commercially available.

**Microorganism and cultivation.** *Aspergillus niger* IFO31125 was obtained from the Institute of Fermentation, Osaka. The fungus was cultivated in a liquid culture medium containing 3% rice bran, 0.05% yeast extract, 0.5% NaNO\textsubscript{3}, 0.1% KH\textsubscript{2}PO\textsubscript{4}, and 0.02% MgSO\textsubscript{4}·7H\textsubscript{2}O at 30°C with a 30-liter jar fermentor.

**Enzyme purification.** The endo-1,4-glucanase was purified from the culture fluid of *A. niger* IFO31125 by the previously described method.\textsuperscript{10} The purified enzyme, which was shown to be homogeneous on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), was used for preparation of various carbohydrate-depleted enzymes.

**Enzyme assay.** The cellulolytic activity of the endo-1,4-glucanase toward CM-cellulose was measured by the previously described method.\textsuperscript{10} 0.1 ml of enzyme solution and 0.9 ml of 1.1% (wt/vol) CM-cellulose in 110 mM potassium phosphate buffer (pH 6.0) were mixed and incubated for 20 min at 40°C, and then the reducing sugar formed was measured as glucose by the method using dinitrosalicylic acid.\textsuperscript{16}

One unit of enzymatic activity was defined as the amount of protein that produced 1.0 μmol of product per min under the standard assay.

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**Abbreviations:** Man, Mannose; GlcNAc, N-Acetylglucosamine.
Role of Carbohydrate Chain in Protease-Resistant Cellulase

Analytical methods. The carbohydrate contents of enzyme preparations were measured by the method of Dubois et al. using the phenol-sulfuric acid[18] with mannose as the standard. Protein content was measured by the method of Lowry et al. [19] with bovine serum albumin as the standard.

Results

Preparation of various carbohydrate-depleted enzymes

From the purified endo-β-1,4-glucanase, various forms of carbohydrate-depleted enzymes were prepared by treatment with endo-β-N-acetylglucosaminidase or z-mannosidase and subsequent purification by Concanavalin A-Sepharose affinity and DEAE ion-exchange column chromatographies.

The CD-1 preparation, which was treated with endo-β-N-acetylglucosaminidase and then passed through the Concanavalin A-Sepharose column, and the CD-2 preparation, which was treated with z-mannosidase and then passed through the Concanavalin A-Sepharose column, were obtained with yields of 8 and 41%, respectively. These enzyme preparations each showed a single band of protein on SDS–PAGE (Fig. 2). On the other hand, the PD preparation, which was treated with z-mannosidase and adsorbed on a Concanavalin A-Sepharose column, was obtained with a yield of 59%.

Molecular sizes, carbohydrate contents, and specific activities of various enzyme preparations

Molecular sizes of native enzyme, PD, CD-2, and CD-1, were measured by SDS–PAGE, and were about 40, 39, 38, and 37 kDa, respectively (Fig. 2). These enzymes contained 9.0, 4.5, 1.3, and 0.8% carbohydrate, respectively, measured by the phenol-sulfuric acid method (Table I).

Specific activities of these enzymes toward CM-cellulose as the substrate are shown in Table I. All enzymes had almost the same specific activity, ranging from 190 to 210 units/mg of protein.

Effects of pH on the enzymatic activities and stabilities of various enzyme preparations

The activities of various enzyme preparations toward CM-cellulose were examined at various pHs between 3.0 to

![Fig. 1](image)

**Fig. 1.** The Estimated Form of the Carbohydrate Chain on the Various Enzyme Preparations.

![Fig. 2](image)

**Fig. 2.** Silver-stained SDS–Polyacrylamide Gel Electrophoretogram of Various Enzyme Preparations.

The proteins were electrophoresed on a 12.5% polyacrylamide gel. Lanes 1 and 6, molecular mass markers; lane 2, 96 ng of native cellulase; lane 3, 104 ng of PD; lane 4, 340 ng of CD-2; lane 5, 112 ng of CD-1.
Table  Comparison of Carbohydrate Content and Specific Activity of Various Enzyme Preparations

<table>
<thead>
<tr>
<th>Enzyme Preparation</th>
<th>Specific act. (U/mg)</th>
<th>Carbohydrate cont. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>194</td>
<td>8.95</td>
</tr>
<tr>
<td>Partially carbohydrate-depleted enzyme (PD)</td>
<td>199</td>
<td>4.47</td>
</tr>
<tr>
<td>Carbohydrate-depleted enzyme 2 (CD-2)</td>
<td>212</td>
<td>1.32</td>
</tr>
<tr>
<td>Carbohydrate-depleted enzyme 1 (CD-1)</td>
<td>191</td>
<td>0.76</td>
</tr>
</tbody>
</table>

The methods of production of each enzyme preparation are described in the text.

11.0 under the standard assay conditions. All enzyme preparations showed a similar profile of activity over a pH range of 3.0 to 10.0 (Fig. 3), although the optimum pH for activity seemed to be slightly shifted to the acidic side according to the decrease in carbohydrate contents in enzyme preparations.

Next, these enzyme preparations were incubated at various pHs from 3 to 11 in various 50 mm buffers at 40°C, and then the residual activities were assayed at 40°C for 20 min under the standard assay conditions. These enzymes were stable at pH 4.0 to 11.0 on incubation for at least 2 h. However, after incubation for 24 h, these enzymes tended to decrease their activities at acidic and alkaline pHs. CD-1 was slightly less stable than other enzymes below pH 5 and above pH 10.

Effects of temperature on enzymic activities and stabilities of various enzyme preparations

The optimum temperature for the activity of the various enzyme preparations was measured by incubating for 20 min at various temperatures at pH 6.0. All enzymes were most active at 70°C, showing approximately 7 times higher activity than at 40°C.

The various enzyme preparations in 50 mm potassium phosphate buffer (pH 7.0) were incubated at various temperatures ranging from 30°C to 80°C for 60 min, and then the residual activities were assayed at 40°C for 20 min under the standard assay conditions. The activities of all enzymes were not lost even after heating at 60°C (Fig. 4), and they showed similar profiles of thermal stability.

Effects of protease on enzymic stabilities of the various enzyme preparations

To examine the protease resistance of the various enzyme preparations, they were incubated at 40°C with a protease such as 0.25% (vol/vol) Savinase, 0.025% (wt/vol) pro-
teinase K, 0.025% (wt/vol) Pronase E, and 0.25% (wt/vol) trypsin in 50 mM Tris buffer (pH 7.5). As shown in Fig. 5, only CD-1 was inactivated with Savinase, proteinase K, and Pronase E for 3 d, while the activities of native cellulase, PD, and CD-2 were not abolished on incubation with each protease even for 3 d. The specific protease trypsin had no such effect on activities of any of these enzyme preparations.

Discussion

To elucidate the role of the carbohydrate chains of glycoenzymes, we prepared various forms of endo-\(\beta\)-1,4-glucanase containing different-sized carbohydrate chains by enzymatic cleavage. From the results of SDS-PAGE and carbohydrate content, and also considering the specificity of each enzyme used, their forms were estimated as follows: the CD-1 preparation was taken to be the enzyme form containing only GlcNAc, and the CD-2 preparation was that containing Man (GlcNAc), carbohydrate chain. The PD preparation was partially depleted of carbohydrate. These enzymes showed similar specific activities, indicating that the carbohydrate moiety of the endo-\(\beta\)-1,4-glucanase did not affect enzyme activity.

Some reports have suggested that the carbohydrate moieties in glycoenzymes are not essential participants in catalysis.\(^{6,7,20,21}\) Our results concerning endo-\(\beta\)-1,4-glucanase agreed with these previous reports. In this study, however, it was suggested that the optimum pH of these enzymes shifted slightly to the acidic side with decreases in carbohydrate content (Fig. 3). Therefore, the carbohydrate chains of the enzyme seem to affect some interaction(s) between the enzyme and the CM-cellulose substrate.

Hayashida et al. reported that the partially deglycosylated CM-cellulases of \textit{Humicola insolens} YH-8 had significantly decreased thermal and pH stabilities,\(^{22}\) Takegawa et al.,\(^{7}\) and Hayashida et al.,\(^{23}\) also found the same phenomena in fungal glucoamylases. However, no form of our enzyme showed greatly altered thermal stability (Fig. 4). Only CD-1, which was the most deglycosylated form, was found to have a slightly decreased pH stability. These results suggested that the sugar chain of the enzyme had no effect on the pH or thermal stabilities of the enzyme.

On the other hand, this study also showed the effect of carbohydrate chain on protection against proteolysis by nonspecific proteases; only CD-1 was inactivated by incubation with proteases (Fig. 5), while the activities of the other enzymes were not abolished under our conditions. These results suggest that core-oligosaccharides consisting of only three monosaccharide residues in the asparagine-linked sugar chain on the enzyme contributed to protection against proteolysis of the endo-\(\beta\)-1,4-glucanase. This finding seems to suggest a mechanism of protease resistance, and further investigations are in progress in our laboratory.

References