Note

A Novel Endo- β -*N*-acetylglucosaminidase from Shoots of *Phyllostachys heterocycla* var. *pubescens*

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Together with sequential exoglycosidase hydrolysis, specific cleavage by endo- β -*N*-acetylglucosaminidase is useful in the characterization of asparagine-linked oligosaccharides of glycoproteins. The endo- β -*N*-acetylglucosaminidases from microorganisms have been extensively used for this purpose^{1,2)} and many microorganisms have been found to be sources of these enzymes.^{3–8)} Similar enzymes have also been purified and characterized from animal tissues^{9–12)} and fungi.^{13–15)} However, only a few enzymes from higher plants have been examined for their substrate specificities.^{16,17)}

When a crude extract of bamboo shoots was incubated with the dansyl derivative of ovalbumin glycopeptide GP-V Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-Ans,¹⁴⁾ a fluorescent product containing equimolecular amounts of *N*-acetylglucosamine and aspartic acid was found in the reaction mixture by paper chromatography.¹⁴⁾ Since a mannose-pentasaccharide having a *N*-acetylglucosamine residue at its reducing end was also found on the paper,¹⁴⁾ we concluded that the crude extract contains an endo- β -*N*-acetylglucosaminidase.

The endo- β -*N*-acetylglucosaminidase activity was assayed using the dansyl derivative of Taka-amylase A glycopeptide.¹⁴⁾ An enzyme solution (20 μ l) was mixed with a 0.02% substrate solution (10 μ l) in 0.1 M acetate buffer (pH 5.2). The reaction was allowed to proceed at 37°C, then stopped by heating at 100°C for 3 min. After being evaporated to dryness, the residue was dissolved in distilled water (10 μ l) and analyzed by HPLC. A Shimadzu model LC-6A chromatograph with a Rheodyne model 7125 injector and a Shimadzu model RF-530 fluorescence spectrometer were used. Chromatography was done on a reverse phase column (Cosmosil 5C18-P, 150 $\times$ 4.6 mm, Nacalai Tesque, Inc.) with 0.05 M ammonium acetate buffer (pH 4.0) containing 20% acetonitrile at a flow rate of 0.9 ml

per min. An excitation wavelength of 313 nm and an emission wavelength of 550 nm were used. One unit of the enzyme was defined as the amount which hydrolyzes 1 μ mol of the substrate per min. Other chemical and enzymatic analyses were done as described previously.¹⁸⁾

Throughout the purification of the enzyme, the temperature was maintained at 0–5°C. After being peeled, fresh bamboo shoots (*Phyllostachys heterocycla* var. *pubescens*, 3.8 kg) were homogenized in a blender with 0.15 M NaCl/0.05 M sodium phosphate buffer (pH 6.0, 3.8 l) and squeezed through two layers of cotton ward. The supernatant obtained by centrifugation of the filtrate was used as a crude enzyme. The protein precipitating between 30 and 75% saturation with ammonium sulfate was dissolved in 10 mM sodium phosphate buffer (pH 6.0, 120 ml) and dialyzed against the same buffer. After being concentrated to a small volume by ultrafiltration (Mini-module HC membrane, Asahi Kasei Industrial Co., Ltd.), the solution (20 ml, 0.40 g protein) was put on a DEAE-Sephacel column (1.4 $\times$ 92 cm) equilibrated with the same buffer. The column was washed with the same buffer (280 ml) and then eluted with a linear gradient of NaCl from 0 to 0.5 M in the same buffer (520 ml). Endo- β -*N*-acetylglucosaminidase was retained on the column and recovered in the fractions at 0.23–0.34 M NaCl. β -Mannosidase was also retained, but eluted at 0.08–0.20 M NaCl, much earlier than the major active fractions of endo- β -*N*-acetylglucosaminidase. β -Xylosidase and β -galactosidase passed through the column unbound. The pooled fractions of endo- β -*N*-acetylglucosaminidase were concentrated (8.5 ml, 95 mg protein), dialyzed against 0.2 M acetate buffer (pH 4.7) and put on a CM-Sephrose CL-6B column (1.6 $\times$ 36 cm) equilibrated with the same buffer. The column was washed with the buffer (150 ml) and eluted with a linear gradient of NaCl from 0 to 0.5 M in the same buffer (240 ml). The enzyme was recovered in fractions at 0.19–0.27 M NaCl, while the contaminating β -*N*-acetylhexosaminidase and α -mannosidase passed through the column unbound. However, when the endo- β -*N*-acetylglucosaminidase fractions were combined and concentrated to a small volume, a trace of β -*N*-acetylhexosaminidase activity was still detected in the concentrate. This solution (2.5 ml, 5 mg protein) was dialyzed against 0.05 M sodium phosphate buffer (pH 6.5) and then put on a hydroxylapatite¹⁹⁾ column (0.83 $\times$ 10 cm) equilibrated with the same buffer. The column was washed with the same buffer (10 ml) and then eluted with a linear gradient obtained with 35 ml of 0.05 M and 35 ml of 0.2 M sodium phosphate buffer (pH 6.5). The enzyme preparation, recovered in fractions at 0.07–0.12 M of the buffer, was free from other glycosidases, though it was still not electrophoretically homogeneous. The final recovery of the enzyme activity was 18% and the specific activity was increased 430-fold.

With the dansyl derivative of Taka-amylase A glycopeptide¹⁴⁾ as the substrate, the maximal enzyme ac-

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Table 1. ACTION OF ENDO- β -N-ACETYLGUCOSAMINIDASES ON VARIOUS OLIGOSACCHARIDES

Oligosaccharide		Hydrolysis ^c				Oligosaccharide		Hydrolysis ^c			
Name ^a	Structure ^b	H	D	C _{II}	P	Name ^a	Structure ^b	H	D	C _{II}	P
GP-I	$ \begin{array}{c} \text{M}\alpha_6\text{M}\alpha_3\text{GN}\beta_1 \\ \text{M}\alpha_3 \\ \text{G}\beta_4\text{GN}\beta_2\text{M}\alpha_4 \\ \text{GN}\beta_2\text{M}\alpha_3 \\ \text{M}\beta_3\text{GN}\beta_4\text{R}_1 \end{array} $	+	- ^e	-	-	GP-V	$ \begin{array}{c} \text{M}\alpha_6\text{M}\alpha_3\text{GN}\beta_1 \\ \text{M}\alpha_3 \\ \text{M}\beta_3\text{GN}\beta_4\text{R}_1 \end{array} $	+	+	+	+
GP-IIA	$ \begin{array}{c} \text{M}\alpha_3\text{M}\alpha_3\text{GN}\beta_1 \\ \text{G}\beta_4\text{GN}\beta_2\text{M}\alpha_4 \\ \text{GN}\beta_2\text{M}\alpha_3 \\ \text{M}\beta_3\text{GN}\beta_4\text{R}_1 \end{array} $	+	-	-	-	GP-VI	$ \begin{array}{c} \text{M}\alpha_3\text{M}\alpha_3\text{GN}\beta_1 \\ \text{M}\alpha_3 \\ \text{M}\beta_3\text{GN}\beta_4\text{R}_1 \end{array} $	+	+	+	+
GP-IIB	$ \begin{array}{c} \text{M}\alpha_6\text{M}\alpha_3\text{GN}\beta_1 \\ \text{M}\alpha_3 \\ \text{GN}\beta_4\text{GN}\beta_2\text{M}\alpha_4 \\ \text{GN}\beta_2\text{M}\alpha_3 \\ \text{M}\beta_3\text{GN}\beta_4\text{R}_1 \end{array} $	+	-	-	-	GP-GG	$ \begin{array}{c} \text{G}\beta_4\text{GN}\beta_2\text{M}\alpha_4 \\ \text{GN}\beta_2\text{M}\alpha_3 \\ \text{M}\beta_3\text{GN}\beta_4\text{R}_2 \end{array} $	-	-	-	-
GP-IIIA	$ \begin{array}{c} \text{M}\alpha_6\text{M}\alpha_3\text{GN}\beta_1 \\ \text{M}\alpha_3 \\ \text{GN}\beta_4\text{GN}\beta_2\text{M}\alpha_4 \\ \text{M}\beta_3\text{GN}\beta_4\text{R}_1 \end{array} $	+	-	+	+	GP-GM	$ \begin{array}{c} \text{G}\beta_4\text{GN}\beta_2\text{M}\alpha_4 \\ \text{M}\alpha_3 \\ \text{M}\beta_3\text{GN}\beta_4\text{R}_2 \end{array} $	-	+	-	-

GP-III B	$\begin{array}{c} \text{M}\alpha\text{-}_2\text{M}\alpha\text{-}_6\text{M}\alpha \\ \text{M}\alpha\text{-}_3\text{M}\alpha \\ \text{M}\alpha\text{-}_2\text{M}\alpha \end{array} \begin{array}{c} \text{M}\beta\text{-}_4\text{GN}\beta\text{-}_4\text{R}_1 \\ \text{M}\beta\text{-}_4\text{GN}\beta\text{-}_4\text{R}_1 \end{array}$	+	-	+	+	GP-MM	$\begin{array}{c} \text{M}\alpha \\ \text{M}\alpha \end{array} \begin{array}{c} \text{M}\beta\text{-}_4\text{GN}\beta\text{-}_4\text{R}_2 \\ \text{M}\beta\text{-}_4\text{GN}\beta\text{-}_4\text{R}_2 \end{array}$	-	+	-	-
GP-III C	$\begin{array}{c} \text{M}\alpha\text{-}_3\text{M}\alpha \\ \text{GN}\beta\text{-}_4\text{M}\alpha \\ \text{GN}\beta\text{-}_2\text{M}\alpha \end{array} \begin{array}{c} \text{GN}\beta \\ \text{M}\beta\text{-}_4\text{GN}\beta\text{-}_4\text{R}_1 \end{array}$	+	-	-	-	M3X	$\begin{array}{c} \text{M}\alpha \\ \text{M}\alpha \end{array} \begin{array}{c} \text{M}\beta\text{-}_4\text{GN}\beta\text{-}_4\text{R}_3 \\ \text{X}\beta \end{array}$	-	-	nd ^f	+
GP-IV	$\begin{array}{c} \text{M}\alpha\text{-}_6\text{M}\alpha \\ \text{M}\alpha\text{-}_3\text{M}\alpha \\ \text{M}\alpha\text{-}_2\text{M}\alpha \end{array} \begin{array}{c} \text{M}\beta\text{-}_4\text{GN}\beta\text{-}_4\text{R}_1 \\ \text{M}\beta\text{-}_4\text{GN}\beta\text{-}_4\text{R}_1 \end{array}$	+	-	+	+	M4X	$\begin{array}{c} \text{M}\alpha\text{-}_3\text{M}\alpha \\ \text{M}\alpha \end{array} \begin{array}{c} \text{M}\beta\text{-}_4\text{GN}\beta\text{-}_4\text{R}_3 \\ \text{X}\beta \end{array}$	-	-	nd	+

Abbreviations: H, *Streptomyces griseus* endo-β-N-acetylglucosaminidase; D, *Streptococcus pneumoniae* endo-β-N-acetylglucosaminidase; C_{II}, *Clostridium perfringens* endo-β-N-acetylglucosaminidase; P, *Phyllostachys heterocycla* endo-β-N-acetylglucosaminidase.

^a See ref. 14.

^b M, Man; G, Gal; X, Xyl; GN, GlcNAc; R₁, GlcNAc-Asn; R₂, (Fucα1→6)GlcNAc-Asn; R₃, GlcNAc.

^c Dansylated glycopeptides (ca. 1 μmol) or pyridilaminated oligosaccharides (M3X and M4X, ca. 0.1 nmol) were incubated for 2 hr with the enzyme (2 munit) in 10 μl of 0.1 M acetate buffer (pH 5.5).

^d +, Hydrolyzed.

^e -, Not hydrolyzed.

^f nd, Not determined.

tivity was found at around pH 5.5, using citrate buffer or acetate buffer. The enzyme was stable for at least 6 months in the pH range of 4.0 to 5.8 at 2–4°C.

The substrate specificity of this enzyme was examined by using various dansylated glycopeptides and pyridilaminated oligosaccharides. Dansylated glycopeptides of ovalbumin and bovine IgG were prepared as described previously,¹⁴⁾ and pyridilamino derivatives of Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)(Xyl β 1 $\rightarrow$ 2)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc and Man α 1 $\rightarrow$ 3Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)(Xyl β 1 $\rightarrow$ 2)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc (M3X and M4X, respectively) were supplied by Dr. S. Hase of Osaka University.^{20,21)} Enzymatic hydrolysis of the dansyl derivatives was analyzed by HPLC as described above. Enzymatic action on the pyridilamino derivatives was also followed by HPLC as described previously.¹⁸⁾ The substrate specificity of this endo- β -N-acetylglucosaminidase was found to be similar to that of *Clostridium perfringens* endo- β -N-acetylglucosaminidase C_{II}²⁾ with respect to the relative activity toward the substrates prepared from ovalbumin and bovine IgG (Table I). It is noteworthy, however, that this enzyme, contrary to endo- β -N-acetylglucosaminidase H and D (Seikagaku Kogyo Co., Ltd.), hydrolyzed M3X and M4X, but whether or not C_{II} enzyme acts on M3X and M4X is unknown because this enzyme was not available to us. In any event, this report describes, for the first time, an endo- β -N-acetylglucosaminidase acting on the xylose-containing type of oligosaccharides. The precise specificity of this enzyme can be identified only after further examination of its activity on different glycopeptides. This type of endo- β -N-acetylglucosaminidase may be common among plants in which these xylose-containing sugar chains are widely distributed.

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