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Note

A sensitive assay for endo-β-*N*-acetylglucosaminidase H using a native glycoenzyme as substrate*

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Endoglycosidases of defined substrate specificities are valuable tools for the study of glycoprotein structure and function[1,2]. Endo-β-*N*-acetylglucosaminidase H (Endo-H), which hydrolyzes the chitibiosyl linkage of many asparagine-linked, high-mannose glycoproteins[3,4], is commonly used and is available from several suppliers. Because many investigators obtain the enzyme in lyophilized form from commercial sources, it is desirable that they be able to verify and quantitate its activity independently, thus avoiding possible misinterpretation of data resulting from the lack of a positive experimental control.

Previous assay-methods for Endo-H[4–6] have disadvantages for those who do not conduct the assays routinely and wish to use the enzyme analytically. Some of these procedures require preparation of radioactive or fluorescent substrates that are not available commercially, and involve analysis of product by rather complex chromatographic or electrophoretic means. In addition, standard methods of analysis for Endo-H have not used intact glycoproteins as substrates. The use of oligosaccharide or small glycopeptide substrates may not allow an accurate quantitative assessment of the enzyme's ability to deglycosylate native glycoproteins[7].

We have devised a simple, rapid, and sensitive method for the detection and comparison of endoglycosidase activities in various samples of Endo-H using RNase B, a commercially available glycoprotein, as substrate. A quantitative conversion of the native glycoenzyme into its partially deglycosylated counterpart, RNase B′, occurs readily when it is incubated with Endo-H, and the enzymic activity of the RNase is not affected by this deglycosylation. RNase B binds to immobilized concanavalin A (Con A-Sepharose)[8], whereas RNase B′ does not. Thus, any RNase activity converted into a form that does not bind to Con A-Sepharose represents the product of the reaction catalyzed by Endo-H. The assay is quite sensitive because the product is measured on the basis of its enzymic activity. Under

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the conditions described here, less than 0.2 pmol of product (RNase B′) can be accurately measured.

The validity of the assay is demonstrated in Fig. 1. The amount of product formed is directly proportional to time of incubation and enzyme concentration under the conditions tested. Linearity was observed up to ~10% conversion of RNase B into B′. The specific activity of highly purified Endo-H measured by this method, 0.012 μmol product/min/mg protein, is 2500-fold less than that obtained with an assay utilizing [^{14}C]acetyl-Asn(GlcNAc)$_2$(Man)$_5$ as substrate[5]. Although the specific activity measured with RNase B by the method decribed here is much lower than that obtained with glycopeptide substrates, the overall sensitivities of the assays for Endo-H are comparable because of the highly sensitive method for detecting the catalytic activity of RNase B′. The present method is capable of detecting 0.2 ng of highly purified Endo-H. This sensitivity is approximately 10-fold lower than the assay of Tarentino *et al.*[4], which employs a radioactive substrate, and 5- to 10-fold greater than that of Iwase *et al.*[6], who used a dansylated glycopeptide as substrate and measured the reaction product with a high-pressure liquid chromatograph equipped with a fluorescence detector. Most of the difference in specific activity may be attributed to the nonsaturating substrate-concentration used in the present assay. The concentration of RNase B in the mixture is 0.43μM, compared to a K_m of 0.3mM reported by Tarentino *et al.*[4] using dansyl-Asn(GlcNAc)$_4$(Man)$_6$ as substrate. It should be noted that none of the standard assay methods previously described for Endo-H[4–6] employ a saturating amount of substrate. The use of saturating levels of RNase B in our assay system is precluded by the excessive consumption of the glycoprotein substrate and by the elevated blank readings that result from leakage of some of the RNase B from the Con A-Sepharose columns when high concentrations of the glycoenzyme are used as substrate. Under the present conditions, the Endo-H product in the Con A-Sepharose

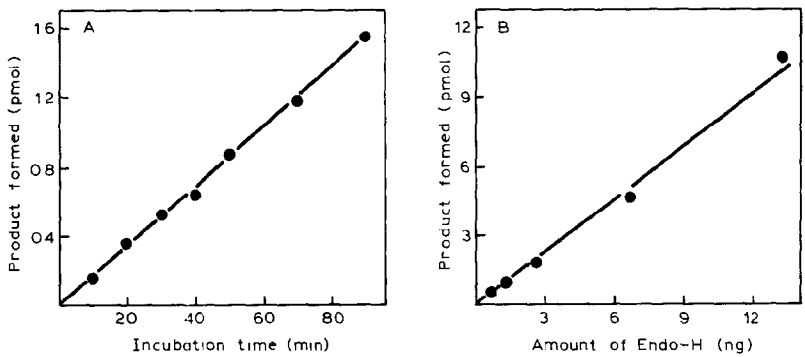

Fig. 1. Effect of time of incubation and enzyme concentration on the determination of Endo-H activity. RNase was deglycosylated and analyzed as described in the Experimental section. Panel A shows linearity with respect to time of incubation: 1.3 ng of Endo-H was incubated with RNase B for various times. Panel B shows linearity with respect to Endo-H concentration: various amounts of Endo-H were incubated with RNase B for 60 min.

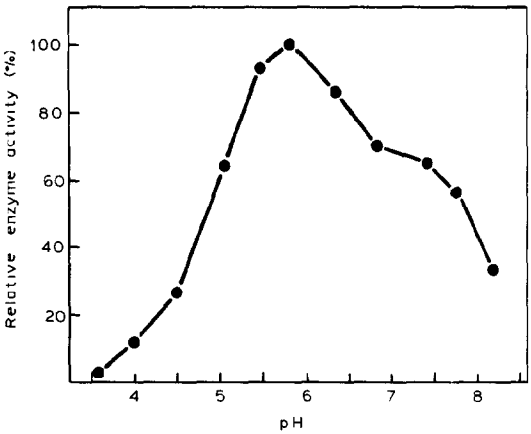

Fig. 2. Effect of pH on the deglycosylation of RNase B by Endo-H. Samples containing 2.6 ng of Endo-H were incubated with RNase B for 30 min, and then chromatographed and analyzed as described in the Experimental section.

pass-through fraction is sufficiently dilute that 10–100 μL volumes may be used directly for RNase assay. The activities measured with this assay may better reflect the quantity of Endo-H needed for deglycosylation of a small amount of a high-molecular-weight glycoprotein than assays employing larger concentrations of glycopeptide substrates.

The effect of pH on the deglycosylation of RNase B by Endo-H has also been examined. A pH optimum of 5.8 was observed (Fig. 2). This is in agreement with the pH optimum determined previously using dansyl-Asn-$(GlcNAc)_4(Man)_6$ as substrate[4], suggesting that charged groups or other structural features associated with the glycoprotein substrate do not significantly alter this catalytic property of Endo-H.

In summary, the assay method for Endo-H described in this communication employs a commercially available, inexpensive glycoprotein substrate, and it utilizes equipment available in most laboratories. This procedure may have greatest utility as a rapid and facile means of determining the activity of endoglycosidase samples intended for use in the analysis of glycoprotein structure. In view of the structure[9] of the carbohydrate moiety of native ribonuclease B and its accessibility to the substrate-binding region of Endo-H, it is likely that this method can also be employed for the assay of several other enzymes acting on glycoproteins and glycopeptides, including[1,10] endo-β-*N*-acetylglucosaminidases D, C_I, C_{II}, and F.

EXPERIMENTAL

Materials. — Endo-H [specific activity of 30 μmol/min/mg protein with [^{14}C]acetyl-Asn$(GlcNAc)_2(Man)_5$] was obtained from Miles Laboratories. Con A-Sepharose was supplied by Pharmacia. Bovine pancreatic RNase B (Type XII-B)

and yeast RNA (Type II-S) were purchased from Sigma. The RNA was purified by phenol extraction and gel filtration[11]. Commercial RNase B preparations are contaminated with the nonglycosylated isozyme[8], RNase A. The glycoenzyme was separated by Con A-Sepharose chromatography[11]; methyl α-D-mannoside in the eluate was removed by gel filtration with Sephadex G-25 equilibrated with 0.1M NaCl.

Ribonuclease assay. — The incubation mixtures (1.0 mL) contained 50mM Tris-HCl buffer (pH 7.5), 0.35 mg of RNA, and enzyme. After incubation for 30 min at 37°, the reactions were terminated by chilling to 0°, followed by addition of 1.0 mL of ice-cold 0.1% bovine serum albumin (BSA) in 10mM Na_3EDTA and 1.0 mL of cold M $HClO_4$. The samples were kept for 10 min at 0° prior to removal of precipitated RNA by centrifugation. The absorbances of the supernatant solutions at 260 nm were then determined. The RNase assay was linear to an A_{260} value of 1.5, and product formation was directly proportional to time of incubation for at least 60 min at 37° A value of 10.0 was used as the millimolar extinction coefficient of the acid-soluble oligonucleotides[12]. One unit of RNase was defined as the amount needed to produce 1.0 μmol of acid-soluble nucleotides per min under the conditions already described. The specific activity of purified RNase B was 5330 units/mg protein (0.081 unit/pmol of protein).

Endoglycosidase assay. — The standard mixture (0.20 mL) contained 1.3 μg (85.5 pmol) of RNase B, 20mM Bis-Tris-HCl buffer (pH 5.8), 0.04 mg of BSA, and enzyme (equivalent of 0.2–10 ng of highly purified Endo-H). The samples were incubated for 60 min at 37°, and the reactions were terminated by chilling on ice and adding 0.05 mL of a cold solution containing 0.8M NaCl in 0.8M Tris-HCl buffer (pH 8.0). The mixtures were immediately applied to small columns (Pasteur pipets) containing 1-mL bed-volumes of Con A-Sepharose. Each column was washed with a total of 2.0 mL of solution containing 0.2M NaCl in 0.02M Tris-HCl buffer (pH 7.5) to elute unadsorbed RNase B′, and a portion of the eluate was assayed for RNase activity as already described. Appropriate blanks were included. Endo-H samples assayed in duplicate agreed within 4%.

For study of the effect of pH on Endo-H activity, 20mM Bis-Tris-HCl buffer (pH 5.8) in the standard mixture was replaced with the same concentration of each of the following buffers: sodium formate (pH 3.5), sodium acetate (pH 4.0–5.5), Bis-Tris-HCl (pH 5.8-6.8), and Tris-HCl (pH 7.5–8.2).

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