ENDO-β-N-ACETYLGLUCOSAMINIDASE FROM FIG LATEX*

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SUMMARY: Commercially available fig latex contains several endo-β-N-acetylglucosaminidases which catalyze the reaction: $(Man)_nGlcNAc\beta 1\rightarrow 4GlcNAcAsn \longrightarrow (Man)_nGlcNAc + GlcNAcAsn$. Using $(NH_4)_2SO_4$ fractionation followed by chromatography on Sephadex G-100 and DEAE-Sephadex A-50, two distinct types of endo-β-N-acetylglucosaminidases have been partially purified and characterized. One, called F-I, hydrolyzes the di-N-acetylchitobiosyl linkage in the glycopeptide, $(Man)_3(GlcNAc)_2Asn$ prepared from human IgG, much faster than that linkage in the glycopeptides, $(Man)_5(GlcNAc)_2Asn$ and $(Man)_6(GlcNAc)_2Asn$ both from ovalbumin. The other, called F-II, hydrolyzes the same linkage in $(Man)_5(GlcNAc)_2$-Asn and $(Man)_6(GlcNAc)_2Asn$, but not that in $(Man)_3(GlcNAc)_2Asn$.

Proteases are widely used for preparing glycopeptides from glycoproteins. In order to obtain intact native saccharide chains, it is of the utmost importance that the protease employed be devoid of exo- and endoglycosidase activities.

In earlier work, Eylar (1), Arima et. al. (2) and Sharon et. al. (3) reported that during pronase digestion of glycoproteins, some oligosaccharides free of amino acids were released from glycoproteins by contaminating aspartyl-N-acetylglucosaminidase. While using ficin for proteolytic digestion of glycoproteins, we also observed contaminating endoglycosidase activity which released amino acid-free oligosaccharide. This communication reports the partial purification of two distinct endo-β-N-acetylglucosaminidases from crude fig latex. The first of these enzymes designated F-I, cleaves the di-N-acetylchitobiosyl linkage in the asparaginyl glycopeptide, $(Man)_3(GlcNAc)_2Asn$. This glycopeptide "trunk" is derived from sialic acid-containing glycopeptide prepared from IgG by exoglycosidases to remove sialic acid, galactose or N-acetylglucosamine at the "branches" or peripheral positions. The second type, F-II, ap-

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pears to cleave the same linkage in $(Man)_5(GlcNAc)_2Asn$ and $(Man)_6(GlcNAc)_2Asn$. These glycopeptides are derived from ovalbumin, and may contain the same "trunk" but different "branches" or peripheral structure(s); in the native state they contain neither sialic acid nor galactose. The general structure features of glycoproteins with these two types of saccharide chains have been recently reviewed by Montreuil (4).

EXPERIMENTAL

Materials--Crude fig latex, ficin, control no. 8284, and 2X crystalline ovalbumin were purchased from Nutritional Biochemicals. GlcNAcAsn was from Cyclo Chemical. [1-^{14}C] Acetic anhydride (1 mCi/mg) was from New England Nuclear. Asparaginyl glycopeptides from ovalbumin were prepared according to the procedure of Huang et. al. (5). [^{14}C]Acetyl-labeled glycopeptides were prepared by acetylation with [^{14}C] acetic anhydride (6). Radioactive glycopeptide was separated from the [^{14}C] acetate by Sephadex G-25 filtration using 0.1 M acetic acid as eluant. Dansyl derivatives of asparaginyl glycopeptides were prepared according to Gray (7), followed by Sephadex G-25 filtration to remove dansylsulfonic acid using 0.1 M acetic acid as eluant. The glycopeptide, $(Man)_3(GlcNAc)_2Asn$ from IgG was a gift of Dr. H. Schachter, University of Toronto, Canada. All other chemicals were obtained from commercial sources and were of the highest grade.

Methods--Endo-β-N-acetylglucosaminidase activity was assayed at pH 5.5 by using [^{14}C] acetyl-labeled $(Man)_3(GlcNAc)_2Asn$, $(Man)_5(GlcNAc)_2Asn$, and $(Man)_6(GlcNAc)_2Asn$ as substrates according to procedures previously described (8, 9). Exoglycosidases were assayed by using p-nitrophenyl glycosides as substrate (10, 11).

Isolation of Endo-β-N-Acetylglucosaminidases--All operations were carried out at 0 to 5°.

Step 1: Extraction and $(NH_4)_2SO_4$ fractionation - One hundred grams of crude fig latex was stirred for 1 hour in 500 ml of 0.05 M sodium phosphate buffer, pH 7.0 and then centrifuged at 8,000 x g for 30 min. The clear brown extract was subsequently dialyzed against the same buffer overnight. The protein which precipitated during dialysis was removed by centrifugation and discarded. The supernatant was adjusted to 30% saturation with solid $(NH_4)_2SO_4$ and let stand overnight. After centrifugation the supernatant was adjusted to 60% saturation with $(NH_4)_2SO_4$ and let stand overnight. The precipitated protein was collected by centrifugation and dissolved in 240 ml of 0.05 M sodium phosphate buffer, pH 7.0.

Step 2: Sephadex G-100 chromatography - A 30 ml-portion of the enzyme solution (containing 6 g of protein) obtained in Step 1 was applied to a Sephadex G-100 column (5 x 90 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.0 and was eluted with the same buffer at 30 ml per hr. As shown in Fig. 1, three peaks, A, B, and C, of endo-β-N-acetylglucosaminidase activities were detected. All three peaks contained activities toward both $(Man)_3(GlcNAc)_2Asn$ and $(Man)_5(GlcNAc)_2Asn$. Protein in peak A was recovered by reverse dialysis against 100% $(NH_4)_2SO_4$ and dissolved in 6 ml of 0.1 M sodium phosphate buffer, pH 7.0. The endoglycosidase activities in peaks B and C were found to be rather unstable. Because of this we were not successful in

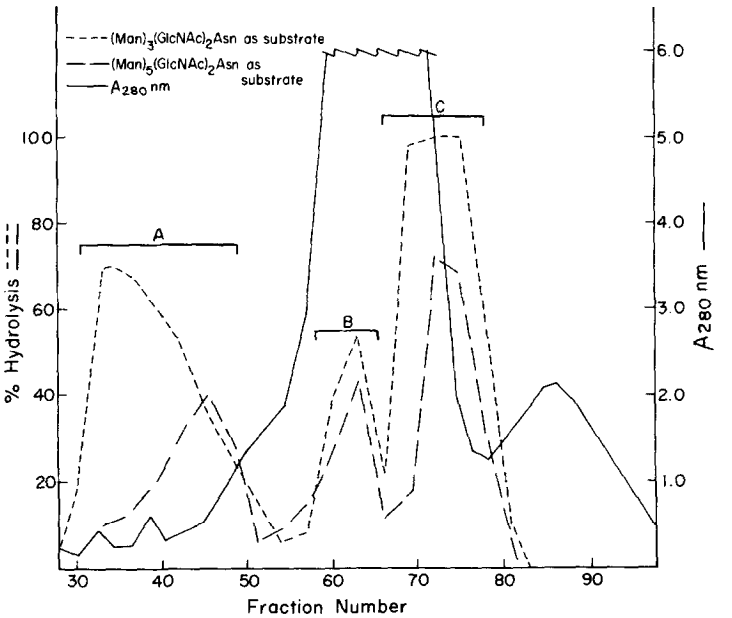

Fig. 1. Sephadex G-100 filtration of enzyme preparation obtained at Step 1. Detailed conditions are described under "Isolation of Endo-β-N-acetylglucosaminidases"; 19 ml per fraction were collected; for assaying the endoglycosidase activity, 0.1 ml each of the fraction were incubated with $(Man)_5(GlcNAc)_2$-Asn-N-Ac- [^{14}C] or $(Man)_3(GlcNAc)_2$Asn-N-Ac [^{14}C]for 16 hours. The assays were carried out as previously described (8, 9).

carrying out further studies on these two activities. Upon observing the partial separation of the two activities evident in peak A of Fig. 1, we rechromatographed peak A on Sephadex G-100 using a narrower column (1.6 x 100 cm). The results of this procedure are shown in Fig. 2. The endo-β-N-acetylglucosaminidase activity which preferentially cleaves $(Man)_3(GlcNAc)_2$Asn was designated F-I and that which cleaves $(Man)_5(GlcNAc)_2$Asn was called F-II. The proteins in pooled fractions of F-I and F-II were separately precipitated by reverse dialysis against 100% $(NH_4)_2SO_4$, and then dissolved in 0.8 ml of 0.05M sodium phosphate buffer, pH 7.0, and dialyzed against the same buffer.

Step 3: DEAE-Sephadex A-50 chromatography - The enzyme F-I was strongly adsorbed on DEAE Sephadex A-50 column which had been equilibrated with 0.05 M sodium phosphate buffer, pH 7.0 and was eluted in high purity by 0.05 M sodium citrate buffer, pH 6.0 containing 0.05 M NaCl (see Figure 3A). As shown in Fig. 3B, F-II was only slightly retarded by the same column under the same conditions. Both F-I and F-II were precipitated from the eluate by reverse dialysis with saturated $(NH_4)_2SO_4$, and dissolved in the minimal volumes of phosphate buffer, 0.05 M, pH 7.0. The enzymes were stable under this condition in the refrigerator for several months. The two enzymes isolated by this procedure were free from α-mannosidase, β-N-acetylhexosaminidase, and α-galactosidase. These two preparations were used in the following studies of their specificities.

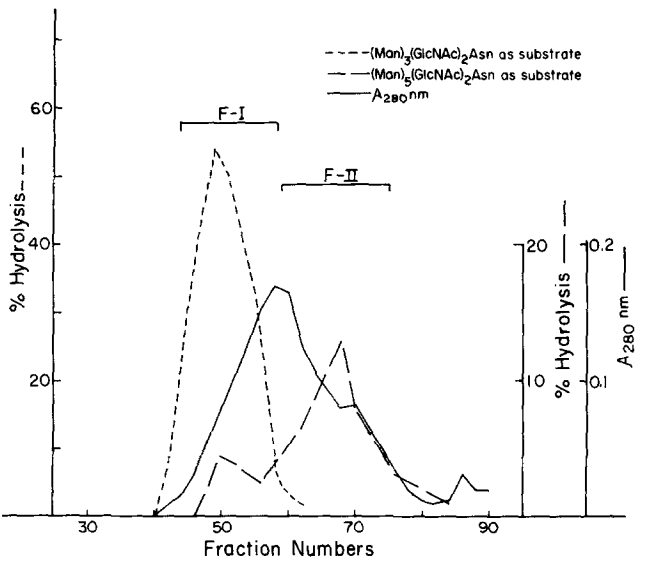

Fig. 2. Rechromatography of peak A as shown in Fig. 1 on Sephadex G-100. The enzyme solution, 2 ml containing 74 mg of protein, was applied to a Sephadex G-100 column (1.6 x 80 cm). The column was eluted at 5 ml per hour; 2 ml per fraction were collected; for assaying the endoglycosidase activity, 20 µl each of the fraction were incubated with $(Man)_5(GlcNAc)_2Asn$-N-Ac[^{14}C]or $(Man)_3$-$(GlcNAc)_2Asn$-N-Ac[^{14}C]at 37° for 4 hours. The proteins in pooled fractions of F-I and F-II were separately precipitated by reverse dialysis against 100% $(NH_4)_2SO_4$ and then dissolved in 0.8 ml of 0.05 M sodium phosphate buffer, pH 7.0 and dialyzed against the same buffer.

RESULTS AND DISCUSSION

For both F-I and F-II, the optimum pH was found to be between pH 4.0 and 6.0. As shown in Fig. 4A, F-I hydrolyzes the di-N-acetylchitobiosyl linkage in $(Man)_3(GlcNAc)_2Asn$ isolated from human IgG much faster than that linkage in $(Man)_5(GlcNAc)_2Asn$ and $(Man)_6(GlcNAc)_2Asn$, both from ovalbumin. F-II, on the other hand, hydrolyzes the di-N-acetylchitobiosyl linkage in $(Man)_5(GlcNAc)_2Asn$ and $(Man)_6(GlcNAc)_2Asn$, but not that in $(Man)_3(GlcNAc)_2Asn$ (Fig. 4B). Thus, the specificity of F-I appears to be similar to that of the endo-β-acetylglucosaminidase D from Diplococcus pneumoniae (8) and F-II resembles the endo-β-N-acetylglucosaminidase H from Streptomyces plicatus (12). However, ovalbumin glycopeptides larger than $(Man)_6(GlcNAc)_2Asn$, such as $(Man)_6(GlcNAc)_4Asn$, $(Man)_5(GlcNAc)_5Asn$ and $(Man)_6(GlcNAc)_5Asn$, were resistant to both F-I and F-II.

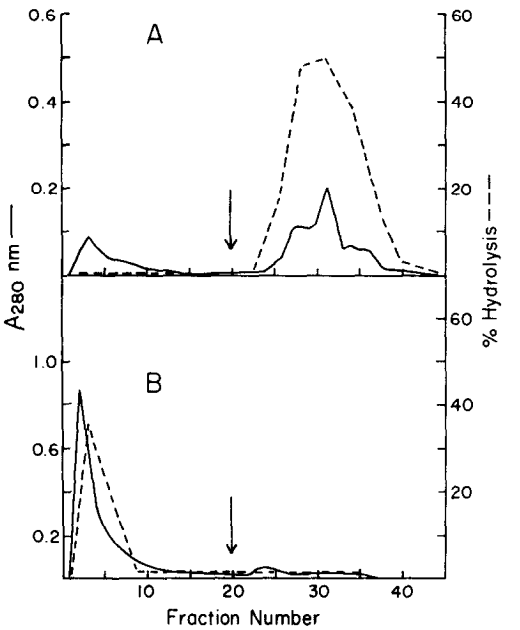

Fig. 3. DEAE-Sephadex A-50 column chromatography of endo-β-N-acetylglucosaminidase F-I and F-II as shown in Fig. 2. A. Enzyme F-I, 1.4 ml containing 5 mg of protein was applied to a DEAE-Sephadex A-50 column (1.2 x 6 cm) which had been previously equilibrated with 0.05 M sodium phosphate buffer, pH 7.0; 1.5 ml per fraction were collected; for assaying the endoglycosidase activity, 0.1 ml each of the fraction were incubated with $(Man)_5(GlcNAc)_2$-Asn-N-Ac[^{14}C]or $(Man)_3(GlcNAc)_2$Asn-N-Ac[^{14}C]at 37° for 16 hours. B. Enzyme F-II, 1.5 ml containing 10 mg of protein was applied to a DEAE-Sephadex A-50 column (1.2 x 6 cm). All other conditions were identical to that described for A.

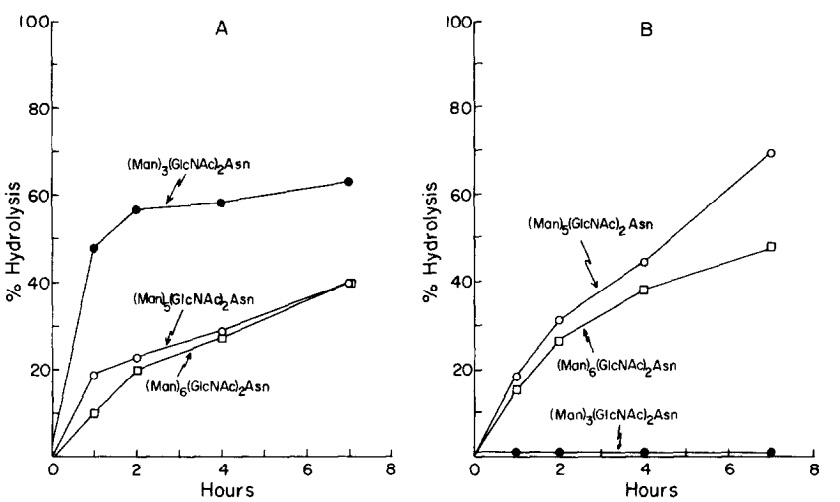

Fig. 4. Hydrolysis of various [^{14}C]-acetyl labeled asparaginyl oligosaccharide by endo-β-N-acetylglucosaminidase F-I (A) and F-II (B). The designated substrate (50 n moles) were incubated for the indicated time with 100 μg of either enzyme F-I or F-II. The assays were carried out as previously described (8, 9).

The specificity of F-I for $(Man)_3(GlcNAc)_2Asn$ and F-II for both $(Man)_5(GlcNAc)_2$-Asn and $(Man)_6(GlcNAc)_2Asn$ may be due to a preference for size of oligomannosyl side chain as in the cases of endoglycosidases from D. pneumoniae (8) and S. plicatus (12). The actual reason for the different specificities of these two enzymes is not known at this moment.

The production of GlcNAc-N-dansyl-Asn from two types of dansylglycopeptides was examined by incubating 30 n moles each of $(Man)_3(GlcNAc)_2$-N-dansyl-Asn and $(Man)_5(GlcNAc)_2$-N-dansyl-Asn separately with 50 μg each of F-I and F-II respectively in 0.1 ml of 0.05 M sodium acetate buffer, pH 5.5 at 37° for 1 hour. The incubation mixtures were then analyzed by paper chromatography (9). In both cases, a fluorescent spot with identical Rf value as that of the authentic GlcNAc-N-dansyl-Asn was detected. In order to isolate the oligosaccharide from the enzymic digest in a quantity sufficient for the subsequent elucidation of its structure, 5 mg of $(Man)_3(GlcNAc)_2Asn$ and $(Man)_6(GlcNAc)_2Asn$ were each incubated with 2 mg each of F-I and F-II respectively in 1.5 ml of 0.05 M sodium acetate buffer, pH 5.5 at 37° for 16 hours. The reaction mixtures were then heated at 100° for 5 minutes. The precipitated protein removed by centrifugation, and the clear supernatant were separately applied to Bio-Rad AG50W X2 (H^+ form, 200-400 mesh, 1.6 x 15 cm) columns which had previously been equilibrated with H_2O. The GlcNAcAsn and the undigested substrates were firmly adsorbed by the resin while the amino acid-free oligosaccharides were eluted from the columns with H_2O. The oligosaccharide released from $(Man)_3(GlcNAc)_2Asn$ by F-I was established to contain GlcNAc and Man in the ratio of 1:3 while that produced from $(Man)_6(GlcNAc)_2Asn$ by F-II had the ratio of 1:6. Reduction of both oligosaccharides with $NaBH_4$ and subsequent analysis of their acid hydrolyzates by the amino acid analyzer (13) resulted in the detection of glucosaminitol as the sole amino sugar. α-Mannosidase liberated two residues of mannose from $(Man)_3GlcNAc$ and five residues from $(Man)_6GlcNAc$. These results indicated that the oligosaccharides contain N-acetylglucosamine at the reducing end. Therefore, it can be concluded that endo-β-N-acetylglucosaminidase F-I and F-II

catalyze the hydrolysis of di-N-acetylchitobiosyl linkage in $(Man)_3(GlcNAc)_2Asn$ and $(Man)_6(GlcNAc)_2Asn$ respectively.

The data presented above indicate that fig latex contains two types of endo-β-N-acetylglucosaminidases useful for the structural analysis of complex carbohydrates. The most serious hazards in using exo- and endoglycosidases for the structural analysis of complex carbohydrates is the possibility of unrecognized contamination by other glycosidases in the enzyme preparation being used. We have found that α-galactosidase purified from fig latex (14) is contaminated with endo-β-N-acetylglucosaminidase activity. Recently this endo-glycosidase activity was also found in the commercial neuraminidase type V isolated from Clostridium perfringens (9) and exo-β-N-acetylglucosaminidase prepared from Diplococcus pneumoniae (15). When using glycosidases for the structural analysis of complex carbohydrates, it is important to be alerted against the possible presence of unanticipated enzyme contamination.

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