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ISOLATION OF TWO ENDO-β-*N*-ACETYLGLUCOSAMINIDASES FROM FIG LATEX

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Two endo-β-*N*-acetylglucosaminidases (mannosyl-glycoprotein 1,4-*N*-acetamidodeoxy-β-D-glycohydrolase, EC 3 2 1 96) (type F-I and type F-II) have been isolated from fig latex At pH 7 0, type F-1 was retained by the DEAE-Sephadex A-50 column, whereas type F-II was not adsorbed by the column The optimum pH of type F-I was found to be pH 5 9 and type F-II, pH 5 4 Type F-I enzyme hydrolyzes the tri-mannosyl derivatives di-*N*-acetylglucosaminylasparagine faster than the penta- or hexa-mannosyl compounds Type F-II hydrolyzes the penta- and hexa-mannosyl derivatives, but not the tri-mannosyl compound

Introduction

Endo-β-*N*-acetylglucosaminidase (mannosyl-glycoprotein 1,4-*N*-acetamidodeoxy-β-D-glycohydrolase, EC 3 2 1 96) is one of the most studied endo-glycosidases useful for the structural analysis of glycoconjugates This enzyme has been isolated from microorganisms [1–5] and animal tissues [6–9] Although there have been reports of the activities resembling endo-β-*N*-acetylglucosaminidase in plants [10], so far, fig latex is the only plant source which has been found to contain this enzyme using ovalbumin glucopeptides as substrates [11,12] In this report, we describe the isolation and characterization of two types of endo-β-*N*-acetylglucosaminidases from this source

Materials and Methods

Materials

The following compounds and materials were obtained from commercial sources crude fig latex (Ficin, control No 8284) and twice-crystallized ovalbumin, Nutritional Biochemicals, Cleveland, OH, *N*-acetylglucosaminylasparagine, Vega-Fox Biochemicals, Tucson, AZ, [^{14}C]acetic anhydride (1 mCi/mg), New England Nuclear, Boston, MA, Con A-Sepharose (10 mg concanavalin A/ml packed gel), Pharmacia Fine Chemicals, Piscataway, NJ Asparaginyl oligosaccharides, derived from ovalbumin, were prepared according to the procedure of Huang et al [13] [^{14}C]Acetyl glycopeptides were prepared by acetylation with [^{14}C]acetic anhydride [14], and were separated from [^{14}C]acetate by using Sephadex G-200 column filtration eluted with 0 1 M acetic acid [11] Dansyl derivatives of asparaginyl glycopeptides were prepared according to the method of Gray [15], followed by Sephadex G-25 filtration, to remove dansylsulfonic acid, using 0 1 M acetic acid [11] The glycopeptide, $(Man)_3(GlcNAc)_2Asn$, derived from IgG was a gift of Dr H Schachter, University of Toronto, Toronto, Canada

Methods

The reaction mixture for assaying endo-β-*N*-acetylglucosaminidase contained the following components in 100 μl 20 nmol $(Man)_5(GlcNAc)_2$-Asn-[^{14}C]Ac (8 000–10 000 cpm), sodium acetate buffer (pH 5 0, 5 μmol acetate) and an appropriate amount of enzyme After incubation at 37°C for various times, the reaction mixture was heated in a boiling water-bath for 3 min to stop the reaction To this mixture, 0 5 ml Con A-Sepharose gel (containing 5 mg bound concanavalin A) suspended in an equal volume of

0 05 M sodium phosphate buffer (pH 7 0) was added The mixture was mixed for 30 s, and allowed to stand for 1 h at room temperature After centrifugation, the amount of radioactivity in a 0 2 ml aliquot of the supernatant that contained GlcNAc-Asn-[^{14}C]Ac was measured by a Beckman Model LS-150 scintillation counter The mixture containing the substrate without the enzyme was used as a control The principle of this method is to remove the mannose-containing glycopeptide and oligosaccharide by the immobilized concanavalin A, and to leave the liberated GlcNAc-Asn-[^{14}C]Ac in the solution [16] Under these conditions the radioactivity in a 0 2-ml aliquot of the control supernatant was about 200 cpm Fig latex was found to be free from α-mannosidase activity However, in order to ensure that GlcNAc-Asn-[^{14}C]Ac was not formed by the combined action of α-mannosidase, β-mannosidase and β-*N*-acetylhexosaminidase, the liberation of $(Man)_5GlcNAc$ was confirmed using TLC [17]

1 unit of enzyme was defined as the amount of enzyme required to release 1 nmol substrate/h The specific activity of the enzyme was expressed as units/mg protein Protein was determined by the method of Lowry et al [18], using bovine serum albumin as standard

Purification of endo-β-N-acetylglucosaminidase

Extraction and $(NH_4)_2SO_4$ fractionation Unless otherwise indicated, all operations were carried out at 0–5°C A 50 g portion of Ficin was stirred gently with 250 ml buffer I (0 05 M sodium phosphate buffer, pH 7 0) for 1 h at 25°C The mixture was centrifuged at 10000 rev /min for 20 min, and the supernatant was dialyzed against 3 l of the same buffer overnight The precipitate forming at 30–60% satn $(NH_4)_2SO_4$ was prepared and dissolved in 120 ml buffer I

Sephadex G-100 chromatography A 30 ml portion of the above solution was applied to a Sephadex G-100 column (5 X 75 cm) equilibrated and eluted with buffer I (Fig 1A) The active fractions were pooled and precipitated by dialyzing against saturated $(NH_4)_2SO_4$ The precipitate was collected by centrifugation, dissolved and dialyzed in buffer I

DEAE-Sephadex A-50 chromatography The fraction was applied to a DEAE-Sephadex A-50 column (2 6 X 30 cm) equilibrated with buffer I and was first eluted with this buffer, followed by 0 05 M sodium citrate buffer (pH 6 0)/0 05 M NaCl As shown in Fig 1B, the endo-β-*N*-acetylglucosaminidase activity was resolved into two peaks The peak not retained by the column was called type F-II, and the one

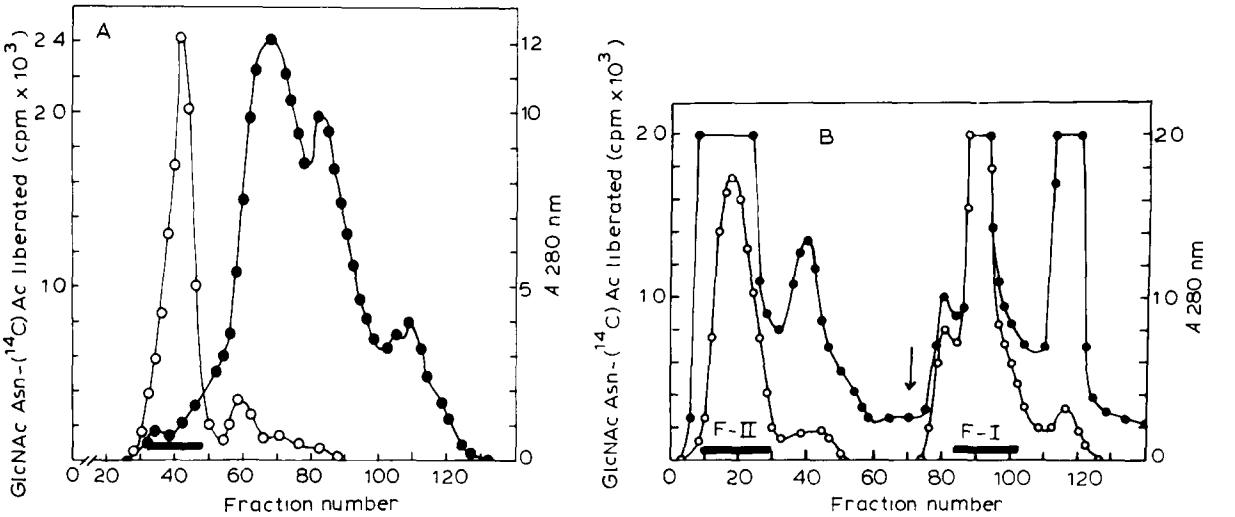

Fig 1 A Sephadex G-100 chromatography of crude Ficin extract The size of the column was 5 X 75 cm and the buffer was 0 05 M sodium phosphate buffer, pH 7 0 B DEAE-Sephadex A-50 chromatography of the product from 1A The size of the column was 2 6 X 30 cm The column was first eluted with 0 05 M sodium phosphate buffer, pH 7 0, then with 0 05 M sodium citrate buffer, pH 6 0/0 05 M NaCl The horizontal bars indicate the fractions which were pooled and the vertical arrow indicates the place where the buffer was changed ○———○, GlcNAc Asn-[^{14}C], ●———●, absorbance

retained by the column and subsequently eluted by 0 05 M sodium citrate buffer (pH 6 0)/0 05 M NaCl was called type F-I The types F-I and F-II were pooled separately, and precipitated by dialyzing against saturated $(NH_4)_2SO_4$ The precipitate of type F-I was dissolved in a minimum volume of buffer I and applied again to Sephadex G-100 column (2 6 X 87 cm) equilibrated and eluted with the same buffer Type F-I enzyme, which appeared in the first protein peak, was pooled and precipitated by dialysis as above The precipitate was dissolved in, and dialyzed against buffer II (0 05 M sodium acetate buffer, pH 4 5) for further purification

CM-Sephadex C-50 chromatography The type F-I was applied to a CM-Sephadex C-50 column (2 6 X 19 cm) equilibrated with and eluted with buffer II The column was further eluted with buffer II at pH 5 and buffer I As shown in Fig 2A, type F-I was eluted at pH 5 The active fractions were precipitated by dialysis as above The precipitate was recovered by centrifugation, and dissolved in a minimum volume of buffer II at pH 5

Type F-II was applied to CM-Sephadex C-50 column (2 6 X 22 cm) equilibrated and eluted with buffer I Type F-II enzyme was not retained by the column under these conditions, however, most of the contaminating proteins were retained on the column (Fig 2B) The active fractions were pooled, and dialyzed as above The precipitate was dissolved in buffer I

Con A-Sepharose chromatography Type F-II obtained from CM-Sephadex column was dialyzed against buffer I and applied to a Con A-Sepharose column (1 2 X 25 cm), equilibrated and eluted with the same buffer The enzyme was not retained by the column, while the proteins having exoglycosidase activities were adsorbed The active fractions were pooled and dialyzed as above The resultant precipitate was dissolved in buffer II at pH 5, and kept at −20°C

Results and Discussion

A summary of a typical purification from 50 g fig latex is presented in Table I

Purity The final preparations of types F-I and F-II were free from exoglycosidases such as α-mannosidase, α- and β-galactosidases However, both enzymes still contained some exo-β-*N*-acetylhexosaminidase and protease activities Attempts to remove these two activities were unsuccessful These two activities would not affect the use of types F-I and F-II in the hydrolysis of glycopeptides, but, due to the presence of protease activity, they may not be

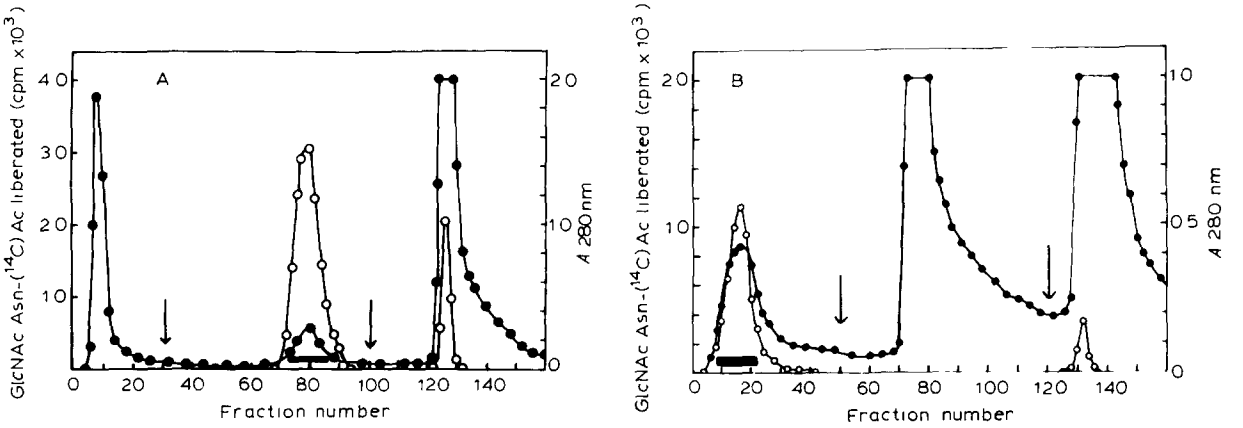

Fig 2 CM-Sephadex C-50 chromatography of type F-I (2A) and type F-II (2B) obtained from DEAE-Sephadex A-50 chromatography In 2A, the size of the column was 2 6 X 19 cm The column was eluted first with 0 05 M sodium acetate, pH 4 5, second with 0 05 M sodium acetate, pH 5 0 and third with 0 05 M sodium phosphate, pH 7 0 In 2B, the size of the column was 2 6 X 22 cm The column was first eluted with 0 01 M sodium phosphate, pH 7 0, second with 0 1 M sodium phosphate, pH 7 0, and third with 0 1 M sodium phosphate, pH 8 0/0 1 M NaCl The vertical arrows indicate where the buffers were changed ○———○, GlcNAc Asn-[^{14}C], ●———●, absorbance

TABLE I

PURIFICATION OF ENDO-β-*N*-ACETYL-D-GLUCOSAMINIDASE I ROM 50 g FIG POWDER

Procedure	Protein (mg)	Total unit	Specific activity	Recovery (%)	Purification factor
Extraction	42 639	2 086 0	0 049	100	1
Dialysis	32 192	1 750 2	0 054	84	1 1
$(NH_4)_2SO_4$ fractionation	10 843	1 619 9	0 149	78	3 0
Sephadex G-100	1 527 5	1 531 4	1 00	73	20 4
DEAE-Sephadex A-50					
F-I	138 2	467 3	3 38	22	69 0
F-II	835 0	433 3	0 52	21	10 6
Sephadex G-100					
F-I	53 6	377 4	7 04	18	143 7
CM-Sephadex C-50					
F-I	1 5	263 8	175 9	14	3 589 8
F-II	33 1	264 7	8 0	13	163 3
Con-A-Sepharose					
F-II	6 7	253 9	37 9	12	773 5

suitable for the structural analysis of sugar chains in intact glycoproteins

Molecular weight, pH optimum and stability The molecular weights of types F-I and F-II were determined to be 52 000 and 17 500, respectively, by gel filtration using a calibrated Sephadex G-100 column according to the method of Andrews [19] The effects of pH on the activities of types F-I and F-II

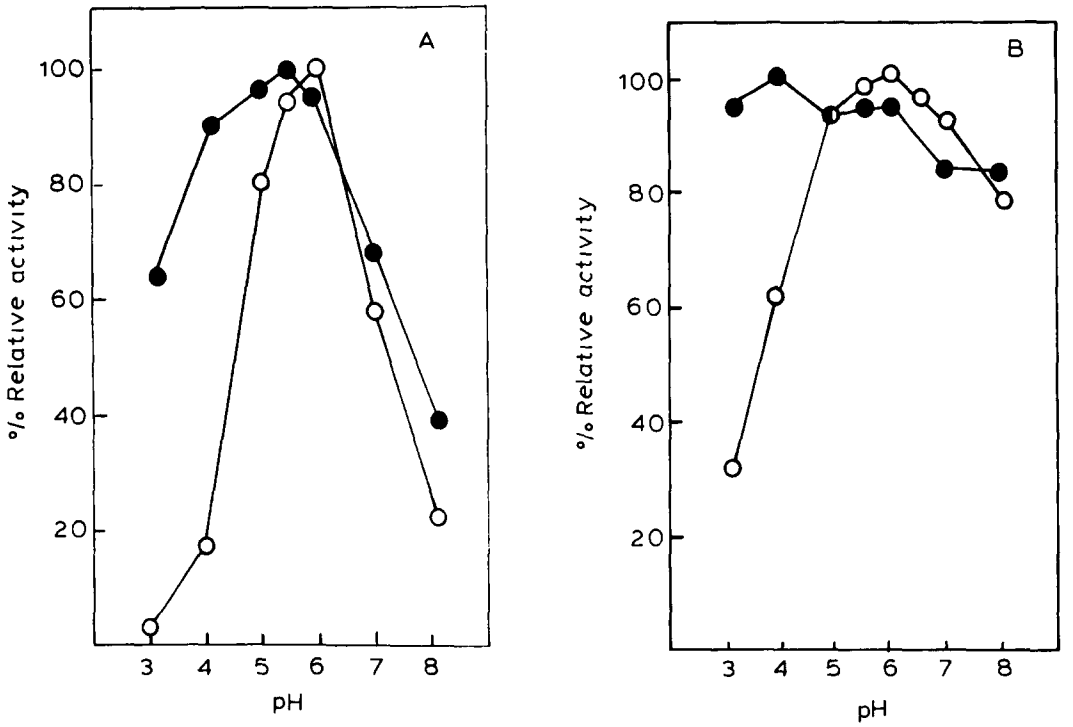

Fig 3 The effects of pH on the activities (3A) and on the stabilities (3B) of types F-I (○———○) and F-II (●———●) enzymes

are shown in Fig 3A Type F-II was found to have a broader pH optimum than type F-I Using $(Man)_5(GlcNAc)_2$-Asn-[^{14}C]Ac as substrate, the highest activity for type F-I occurred at pH 5 9, whereas for type F-II, it was at pH 5 4 The pH stabilities of types F-I and F-II were studied by placing the enzymes in 0 05 M citrate phosphate buffers (pH 2 8–8 0) at room temperature for 24 h, and the activities assayed at pH 5 5 using $(Man)_5(GlcNAc)_2$-Asn as substrate As shown in Fig 3B, type F-II enzyme retained 80–100% of the activity at pH 3 2–8, whereas type F-1 enzyme was only stable at pH 5–8 and unstable at pH values below 5

Substrate specificities The specificities of types F-I and F-II enzymes towards different glycopeptides were very different from each other Type F-I hydrolyzed $(Man)_3(GlcNAc)_2$-Asn-[^{14}C]Ac much faster than $(Man)_5(GlcNAc)_2$-Asn-[^{14}C]Ac and $(Man)_6(GlcNAc)_2$-Asn-[^{14}C]Ac (Fig 4A) On the other hand, type F-II hydrolyzed $(Man)_5(GlcNac)_2$-Asn-[^{14}C]Ac and $(Man)_6(GlcNAc)_2$-Asn-[^{14}C]Ac, but not $(Man)_3(GlcNAc)_2$-Asn-[^{14}C]Ac (Fig 4B) Other glycopeptides isolated from ovalbumin were not good substrates for these two endoglycosidases The substrate specificity of type F-I seems to be similar to that of the endo-β-*N*-acetylglucosaminidase-D from *Diplococcus pneumoniae* [2], and of endo-β-*N*-acetylglucosaminidase C-I from *Clostridium perfringens* [3] The specificity of type F-II is similar to that of endo-β-*N*-acetylglucosaminidase-H from *Streptomyces plicatus* [1], and of endo-β-*N*-acetylglucosaminidase C-II from *Cl perfringens* [3]

The conventional method for assaying endo-β-*N*-acetylglucosaminidase involved the use of ^{14}C-labeled or dansylated asparaginyl oligosaccharides, such as $(Man)_5(GlcNAc)_2$-Asn-[^{14}C]Ac or $(Man)_5(GlcNAc)_2$-dansyl-Asn, as the substrate The reaction was measured by the production of GlcNAc-Asn-[^{14}C]Ac or GlcNAc-dansyl-Asn using high voltage paper electrophoresis or paper chromatography [1–3] These methods are laborious and time consuming We have used Con A-Sepharose to selectively adsorb the unreacted substrate and the oligosaccharide containing mannosyl group The product, GlcNAc-Asn-[^{14}C]Ac, which was not adsorbed by Con A-Sepharose was measured by a scintillation counter This method is simpler and more convenient than the conventional methods In order to ensure that the GlcNAc-Asn-[^{14}C]Ac was not produced by the concerted action of exo-α- and β-mannosidase and exo-β-*N*-acetylglucosaminidase, the liberation of a hexasaccharide, $(Man)_5GlcNAc$, not the free monosaccharide, was demonstrated by the TLC [17]

Since fig latex is very rich in proteolytic activity, the possibility that type F-I enzyme might be a degradation product of type F-I cannot be excluded

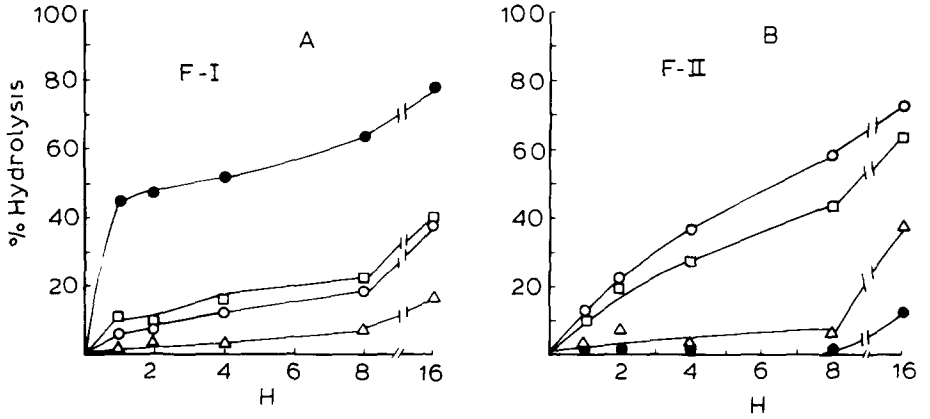

Fig 4 The specificity of types F-I (4A) and F-II (4B) towards different glycopeptides •——•, IgG the glycopeptide $(Man)_3(GlcNAc)_2$Asn prepared from IgG, △——△, C □——□, D and ○——○, E, the glycopeptide prepared from ovalbumin Their structures are C, the mixture of $(Man)_5(GlcNAc)_4$Asn, $(Man)_4(GlcNAc)_5$Asn and $(Man)_7(GlcNAc)_2$Asn D, $(Man)_6(GlcNAc)_2$Asn, E, $(Man)_5(GlcNAc)_2$Asn

In view of the wide occurrence of endo-β-*N*-acetylglucosaminidases, they may be important in the catabolism of sugar chains of glycoproteins

Acknowledgments

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