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Specificity Towards Oligomannoside and Hybrid Type Glycans of the Endo-β-*N*-acetylglucosaminidase B from the Basidiomycete *Sporotrichum dimorphosporum*

OSSARATH KOL, COLETTE BRASSART, GENEVIÉVE SPIK, JEAN MONTREUIL and STÉPHANE BOUQUELET*

Laboratoire de Chimie Biologique, Unité Mixte de Recherche du CNRS no.111, Université des Sciences et Techniques de Lille Flandres-Artois, 59655 Villeneuve d'Ascq Cedex, France

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We have previously shown that an endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96) named "Endo B", isolated from culture filtrates of the basidiomycete *Sporotrichum dimorphosporum* cleaves asialo-, and to some extent, monosialylated bi-antennary glycans of the *N*-acetyllactosamine type linked to the asparagine residue of peptide or protein moieties [Bouquelet S, Strecker G, Montreuil J, Spik G (1980) Biochimie 62:43-49]. In the present paper, the substrate specificity of the enzyme towards oligomannoside and hybrid type glycans has been analyzed. The results obtained indicate that ovalbumin glycopeptides containing four to seven mannose residues and bovine lactotransferrin glycopeptides containing four to nine mannose residues were completely hydrolyzed by the enzyme. The degree of cleavage was variable among hybrid type structures, since glycopeptides containing the following glycans: $(Gal)_1(GlcNAc)_3(Man)_5(GlcNAc)_2$; $(GlcNAc)_3(Man)_5(GlcNAc)_2$; $(GlcNAc)_3(Man)_4(GlcNAc)_2$ were not hydrolyzed by the enzyme while the percentage of hydrolysis of a glycopeptide containing $(GlcNAc)_2(Man)_5(GlcNAc)_2$ glycan reached 90%. The bovine lactotransferrin was partially deglycosylated (40%) in the absence of non-ionic detergent while native ovalbumin glycoprotein was not hydrolyzed by the enzyme.

The oligomannoside- and the *N*-acetyllactosamine-type degrading activities present in the culture filtrates were not separated at any step of the purification procedure. Both activities were eluted as a single component with an apparent molecular mass of 89 kDa suggesting that they are located on the same enzyme molecule.

Abbreviations: Gal, D-galactose; Man, D-mannose; GlcNAc, *N*-acetyl-D-glucosamine; Con A, concanavalin A; Asn, asparagine; GLC, gas liquid chromatography; TLC, thin layer chromatography; Endo, endo-β-*N*-acetylglucosaminidase; Endo B, endo-β-*N*-acetylglucosaminidase isolated from *Sporotrichum dimorphosporum*; PBE, polybuffer exchanger; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

*Author for correspondence.

Endo B represents a powerful tool for removing oligomannoside- and *N*-acetyllactosamine-type glycans from *N*-glycopeptides and *N*-glycoproteins. Moreover, advantages in the use of Endo B in a soluble form as well as in an immobilized form result in its high activity and in its stability to heat denaturation and storage.

Endo-β-*N*-acetylglucosaminidases are powerful tools for removing the carbohydrate chains from glycoasparagines, glycopeptides and glycoproteins containing the *N*-acetylglucosaminyl-asparagine linkage [1-7]. In a previous paper we have described the properties of an endo-β-*N*-acetylglucosaminidase (Endo B) isolated from culture filtrates of the Basidiomycete *Sporotrichum dimorphosporum* slightly active on bi-antennary monosialoglycans and more active on asialo-glycans of the *N*-acetyllactosamine type [8]. In the present paper we describe the purification of the enzyme and, in order to complete the study on the substrate specificity, the enzyme activity towards oligomannoside and hybrid type substrates has been investigated.

Materials and Methods

Chemicals

p-Nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (*p*-NP-β-GlcNAc), *p*-nitrophenyl-α-D-mannopyranoside (*p*-NP-α-Man) and *p*-nitrophenyl-β-D-galactopyranoside (*p*-NP-β-Gal) were purchased from Koch Light Ltd (Haverhill, Suffolk, England). ^{13}C-Acetic anhydride (6 mCi/mmol) and potassium borotritide (20 Ci/mmol) were from CEA (Saclay, France) and 2-acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine from Sigma Chemical Co. (St Louis, MO, USA). All other reagents were of analytical grade.

Materials

Con A-Sepharose and supplies for chromatofocusing Polybuffer exchanger 94 (PBE 94) were from Pharmacia Fine Chemicals (Uppsala, Sweden); Dowex 50-X8 (50-100 mesh, H^+ form), Dowex 1-X8 (50-100 mesh, formate form) and Dowex 50-X2 (200-400 mesh, acetate form) were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Hydroxyapatite-Ultrogel and DEAE-Trisacryl M were from IBF (Villeneuva-La-Garenne, France) and Eupergit C from Rohm Pharma Gmbh (Darmstadt, West Germany). Authentic standard oligosaccharides from urine of patients with G_{M1} gangliosidosis, Mannosidosis and Sandhoff's disease were gifts from Dr. Gérard Strecker. Immobilized neuraminidase (EC 3.2.1.18) from *Clostridium perfringens* and galactose oxidase (EC 1.1.3.9) from *Dactylium dendroides* were obtained from Sigma.

Substrates

Azocoll was purchased from Calbiochem (San Diego, CA, USA). Hen ovalbumin (Grade III) obtained from Sigma was separated into three fractions (F-1 to F-3) by Con A-Sepharose chromatography according to the method of Shepherd *et al.* [9]. Ovalbumin glycoasparagines obtained by exhaustive pronase digestion of ovalbumin were fractionated by ion-exchange chromatography on Dowex 50-X2 [10]. Ovalbumin glycopeptides GP-1, GP-IIA,

GP-IIB, GP-IIIA, GP-IIIB and GP-IIIC were gifts from Dr. A. Kobata. Glycopeptides containing bi-, tri- and tri'-antennary glycans in the relative proportions of 85:6.8:8.2 [11, 12] were isolated from pronase digest of human serotransferrin (Behringwerke AG, Marburg, W. Germany). Bovine lactotransferrin prepared according to the method of Chéron *et al.* [13] was submitted to pronase digestion and the mixture of glycopeptides was fractionated on Con A-Sepharose column into glycopeptides containing *N*-acetyllactosamine type glycans and glycopeptides containing a mixture of oligomannoside type glycans [14, 15].

Labeling of Glycopeptides

N-Acetylation of glycopeptides with ^{14}C-acetic anhydride was carried out as described by Koide and Muramatsu [16]. Desialylation of glycopeptides was performed by using immobilized neuraminidase from *Clostridium perfringens* [17] and the galactose residues of the desialylated glycopeptides were labeled by reduction with potassium borotritide after oxidation by galactose oxidase [18].

Enzyme Assays

Protease activities were determined by using Azocoll [19] or ^{125}I-ovalbumin, bovine serum albumin or human serotransferrin as substrates [20].

Exoglycosidase activities were assayed with appropriate *p*-NP-glycosides. β-D-Galactosidase, α-D-mannosidase and β-*N*-acetylglucosaminidase activities were also tested by using as substrates oligosaccharides isolated from urine of patients with G_{M1}-gangliosidosis, Mannosidosis, and Sandhoff's disease, respectively. The procedure was as follows: 60 μg of oligosaccharides were mixed with 10 μl of 0.2 M sodium phosphate-0.1 M sodium citrate buffer pH 5.5 (Buffer 1) and 10 μl of the enzyme preparation. The reaction mixture was incubated at 37°C for 24 h. The hydrolysis products were analyzed by TLC using the solvent *n*-butanol/acetic acid/water, 2/1/1 by vol (Solvent 1). Neutral sugars were stained as previously described [8].

The activity of endo-β-*N*-acetylglucosaminidase towards glycoproteins was measured as follows: 3 mg of glycoprotein were dissolved in 300 μl of buffer 1 and 100 μl of the enzyme preparation were added. The reaction mixture was incubated at 40°C overnight with a few drops of toluene. Proteins were precipitated by adding 650 μl of absolute ethanol. The sugar composition was determined by GLC [21]. The supernatant was freeze-dried and analyzed by GLC and by TLC using Solvent 1 with three consecutive runs.

The endo-β-*N*-acetylglucosaminidase activity towards glycopeptides was determined as follows: 3 nmol (20,000 cpm) of *N*-^{14}C-acetylated glycopeptides in 20 μl of buffer 1 were incubated with 10μl of the enzyme preparation from 30 min to 6 h at 60°C. The hydrolysates were analyzed by descending paper chromatography on Whatman no. 3 paper. The chromatography was carried out with the solvent: pyridine/ethyl acetate/acetic acid/water, 5/5/1/3 by vol, (Solvent 2) for 16 h. The released GlcNAcβ(1-*N*)[^{14}C-acetyl]Asn was quantified by liquid scintillation. The enzyme activity was also determined by using 40-60 μg of unlabeled glycopeptides. The assay conditions were the same as described above, except that the hydrolysis products were analyzed by TLC using solvent 1.

Table 1. Purification of endo-β-*N*-acetylglucosaminidase B from *Sporotrichum dimorphosporum.*

	Volume (ml)	Protein (mg)	Activity (mU)		Specific activity (mU/mg)			Recovery (%)		Purification factor	
			(a)	(b)	(a)	(b)	(b)/(a)	(a)	(b)	(a)	(b)
Culture filtrate	5,000	6,750	140	324	0.02	0.05	2.3	100	100	1	1
Step 1: Ultrafiltration	200	1,160	116	265	0.10	0.23	2.3	81	82	5	4.7
Step 2: Hydroxylapatite-Ultrogel	125	471	68	185	0.14	0.39	2.8	47	57	6.8	8.2
Step 3: DEAE-Trisacryl M	57	13.5	34.5	94	2.56	6.99	2.7	24	29	121	145
Step 4: Chromatofocusing	15	3.2	23.2	58	7.25	18.2	2.5	16	18	345	364
Step 5: Affinity chromatography	5	2.3	22	55	9.56	23.9	2.5	15	17	450	480

(a) With $(Gal)_2(GlcNAc)_2(Man)_3(GlcNAc)_2Asn$ as substrate.
(b) With $(Man)_6(GlcNAc)_2Asn$ as substrate.

One milliunit (mU) of Endo B was the amount of enzyme able to split one nanomole of substrate per minute under standard conditions of pH and temperature.

Purification of Endo-β-N-acetylglucosaminidase B

The culture filtrate of *Sporotrichum dimorphosporum* was a gift from Gist Brocades (Seclin, France).

Step 1: 5 l of the culture filtrate were concentrated to 0.2 l by using an Amicon ultrafiltration apparatus equipped with a hollow fiber cartridge H1DP10 and then dialysed exhaustively against 6 l of 10 mM sodium phosphate buffer pH 6.8.

Step 2: The dialysed enzyme fraction from step 1 was applied to a hydroxyapatite-Ultrogel column (5.4 x 22.5 cm) previously equilibrated with 10 mM sodium phosphate buffer, pH 6.8. After washing the column with the same buffer, the adsorbed products were eluted with 0.2 M sodium phosphate buffer pH 6.8. The flow rate was 25 ml/h and 15 ml fractions were collected. The active fractions were pooled and concentrated by ultrafiltration.

Step 3: The enzyme fraction from step 2 was exhaustively dialysed against 10 mM sodium phosphate buffer pH 6.8 and then concentrated by ultrafiltration. The obtained fraction was applied to a column of DEAE-Trisacryl M (3.2 x 42 cm) previously equilibrated with 10 mM sodium phosphate buffer pH 6.8. The column was washed with the same buffer. The enzyme was eluted with a linear gradient from 0 to 1 M NaCl. The flow rate was 25 ml/h and 15 ml fractions were collected. The fractions containing endoglycosidase activities were pooled and concentrated by ultrafiltration.

Step 4: The enzyme fraction from step 3 was dialysed against water and equilibrated in 25 mM imidazole/HCl buffer pH 4.0. After concentration by ultrafiltration, the solution was subjected to chromatofocusing on a PBE 94 column (1.2 x 25 cm) previously equilibrated with 25 mM imidazole/HCl pH 4.0 buffer. The column was washed with the same buffer. Contaminating proteins were eluted with buffer containing 0.3 M NaCl and the Endo B was eluted with a linear gradient of NaCl from 0.3 M to 0.7 M.

Step 5: The enzyme fraction from step 4 was dialysed against water and concentrated by ultrafiltration. The solution was equilibrated in 0.02 M sodium phosphate 0.01 M sodium citrate pH 5.0. The equilibrated enzyme solution was applied to an ε-aminocaproyl-*N*-acetylglucosaminylamine-Sepharose 6 B column (1.5 x 35 cm). Endo B was eluted with a 0.1 M sodium phosphate-0.05 M sodium citrate pH 5.0 buffer.

Immobilization of Endo B on Eupergit C

One unit of Endo B was mixed under rotation with 1 g of Eupergit C in 0.1 M potassium phosphate buffer pH 7.5 for 72 h at 4°C. The matrix was washed with 500 ml of the same buffer pH 7.5 for 48 h. The gel was washed with 0.1 M sodium phosphate buffer pH 7.5 and equilibrated in 0.2 M sodium phosphate-0.1 M sodium citrate pH 5.0.

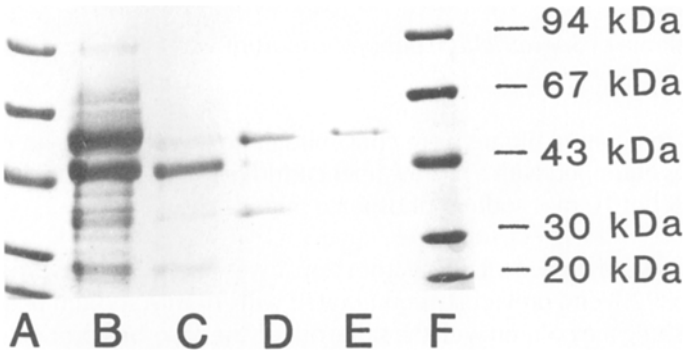

Figure 1. SDS-PAGE under reducing conditions of samples obtained from the individual purification steps of Endo B from *Sporotrichum dimorphosporum*. Lanes A and F: molecular mass standards; lane B: hydroxyapatite-Ultrogel chromatography (step 2); lane C: DEAE Trisacryl M chromatography (step 3); lane D: PBE 94 chromatofocusing (step 4); lane E: affinity chromatography on ε aminocaproyl-*N*-acetylglucosaminylamine-Sepharose 6B (step 5).

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis of Endo B preparations under reducing conditions was performed as described by Laemmli [22], using a 5-15% gradient separation gel. Molecular mass standards were: phosphorylase b (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa). Gels were stained with Coomassie blue G-250.

Results

Purification

The endoglycosidase activities were detected all along the purification procedure by using glycopeptides containing *N*-acetyllactosamine type glycan: $(Gal)_2(GlcNAc)_2(Man)_3(GlcNAc)_2$ or oligomannoside type glycan: $(Man)_6(GlcNAc)_2$ as substrates. As shown in Table 1, the purification rates of both enzyme activities were very similar throughout purification.

In the final purification step (Step 5), the two activities were isolated with a yield of about 17-18% and the purification factor was 450 for the activity towards $(Gal)_2(GlcNAc)_2(Man)_3(GlcNAc)_2$-Asn and 480 for the activity towards $(Man)_6(GlcNAc)_2$ Asn. The ratio of specific activities against oligomannoside type substrate and *N*-acetyllactosamine type substrate was constant all along the purification scheme: the value was 2.5±2.0. These results suggest that both activities are located on the same protein.

Table 2. Properties of Endo B isolated from *Sporotrichum dimorphosporum*.
A: With $(Man)_{8-9}(GlcNAc)_2Asn$ as substrate. B : With $(Gal)_2(GlcNAc)_2(Man)_3(GlcNAc)_2Asn$ as substrate.

Properties of Endo B	A	B
Optimal temperature	60°C	60°C
Optimum pH	5.5	4.5
K_M (mM)	0.39	0.62
V_{max} (μmole/min/mg of protein)	0.26	0.16
pI	3.0	3.0
Mol wt.	89,000	89,000

The purified Endo B preparation was free from exoglycosidases such as α-D-neuraminidase, β-D-galactosidase and α-D-mannosidase. However, it still possessed a weak β-*N*-acetylglucosaminidase activity towards *p*-NP-β-GlcNAc but not towards natural substrates. Protease activity could not be detected after incubation times from 1 h to 72 h at 37°C using ^{125}I-labeled bovine serum albumin, ^{125}I-labeled human serotransferrin, ^{125}I-labeled ovalbumin or Azocoll as substrates.

General Properties

The purified enzyme preparation obtained after affinity chromatography on an ε-aminocaproyl-*N*-acetylglucosaminylamine-Sepharose 6B column (Step 5) showed an apparent molecular mass of 89 kDa as determined by gel filtration. In SDS-PAGE under reducing conditions the enzyme was homogeneous and gave a single band at 45 kDa (Fig. 1). The pI was determined as 3.0. With $(Man)_6(GlcNAc)_2$-*N*-^{14}C-acetyl-Asn or $(Gal)_2(GlcNAc)_2(Man)_3(GlcNAc)_2$-*N*-^{14}C-acetyl-Asn as substrates, the enzyme showed maximal activity at 60°C and at pH 5.5 and 4.5 respectively (Table 2). Temperature and pH stability was the same for both activities (data not shown).

Kinetic parameters vary with substrates. With $(Man)_6(GlcNAc)_2$-Asn as substrate the K_M value was 0.62 mM and the V_{max} was 0.28 μmol/min/mg of protein, with $(Man)_{8-9}(GlcNAc)_2$-Asn the K_M value was 0.39 mM and V_{max} was 0.26 μmol/min/mg of protein. In the case of *N*-acetyllactosamine type activity, by using the mixture of desialylated glycopeptides isolated from human serotransferrin which contained 85% of bi-antennary and 15% of tri-antennary glycans [5, 6], the K_M value was 0.92 mM and the V_{max} was 0.05 μmol/min/mg of protein. The K_M and V_{max} values were 0.62 mM and 0.16 μmol/min/mg of protein respectively when pure bi-antennary glycopeptides were used as substrates.

The enzyme activities were stable to repeated freeze-thawing as well as to freeze-drying (residual activities after each treatment: 85%) and could be stored at 4°C for up to one year

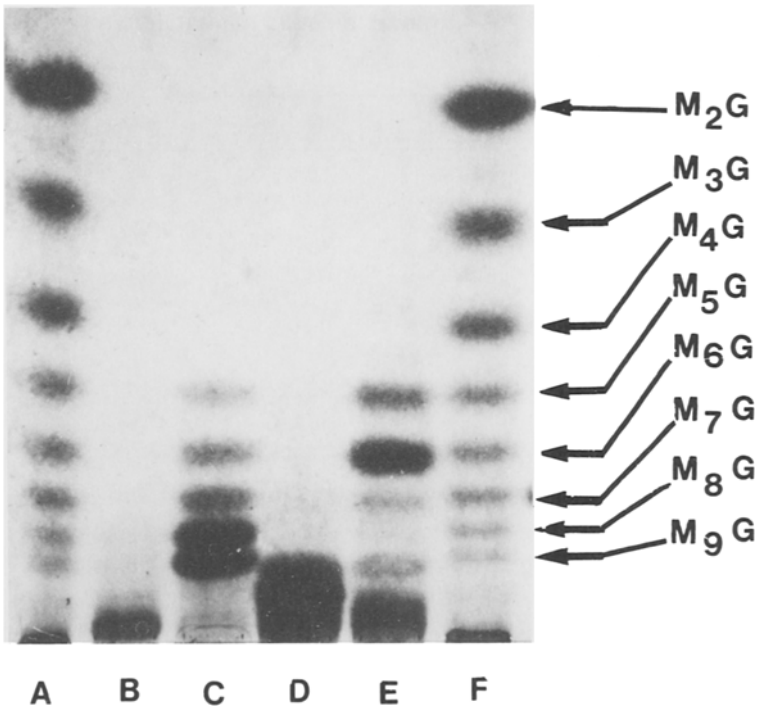

Figure 2. TLC analysis of bovine lactotransferrin and ovalbumin glycopeptide mixtures hydrolyzed by the Endo B from *Sporotrichum dimorphosporum*. Lanes A and F: standard mannosides isolated from the urine of patients with Mannosidosis. Lane B: mixture of bovine lactotransferrin glycopeptides. Lane C: hydrolysate of bovine lactotransferrin glycopeptides. Lane D: mixture of ovalbumin glycopeptides. Lane E: hydrolysate of ovalbumin glycopeptides. M_2G to M_9G [$(Man)_2GlcNAc$ to $(Man)_9GlcNAc$].

without any loss of activity. No loss of activity could be detected during the immobilization process on Eupergit C and after 40 days of storage at room temperature or at 40°C in the presence of substrate. The stability at 60°C for the immobilized enzyme was the same as in the soluble form (incubation times did not exceed three days). In its soluble form, the Endo B was inactive in the presence of 0.01% of SDS (final concentration), while in the same condition the immobilized enzyme activity was not modified and 58% and 26% of residual activities were recovered respectively with 0.05% and 0.1% of SDS. In the presence of 1.6 M and 4 M urea, 88% and 25% of activities were obtained, respectively. A 1.5-fold increased activity was observed when assays were carried out with buffer containing 0.01% to 3% Triton X-100.

Substrate Specificity of Endo B

The specificity of the Endo B towards oligomannoside type glycans from ovalbumin and from bovine lactotransferrin and towards hybrid type glycans from ovalbumin was determined.

Table 3. Hydrolysis of ^{13}C-acetylated glycopeptides by endo-β-*N*-acetylglucosaminidase B isolated from *Sporotricum dimorphosporum*.

Substrates	Hydrolysis (%)
Bovine lactotransferrin glycopeptides	90
Ovalbumin glycopeptides mixture	60
Fractionated ovalbumin glycopeptides:	
$(Gal)_1(GlcNAc)_3(Man)_5(GlcNAc)_2Asn$ [GP-I][a]	0
$(Gal)_1(GlcNAc)_3(Man)_4(GlcNAc)_2Asn$ [GP-II A]	0
$(GlcNAc)_3(Man)_5(GlcNAc)_2Asn$ [GP-II B]	0
$(GlcNAc)_2(Man)_5(GlcNAc)_2Asn$ [GP-III A]	90
$(Man)_7(GlcNAc)_2Asn$ [GP-III B]	90
$(GlcNAc)_3(Man)_4(GlcNAc)_2Asn$ [GP-III C]	0
$(Man)_6(GlcNAc)_2Asn$ [GP-IV]	95
$(Man)_5(GlcNAc)_2Asn$ [GP-V]	92
$(Man)_4(GlcNAc)_2Asn$ [GP-VI]	95

[a] The ovalbumin glycopeptide nomenclature used by Kobata and co-workers is shown in the square brackets.

Hydrolysis of a Mixture of Bovine Lactotransferrin Glycopeptides: The mixture of glycopeptides obtained from pronase digestion of bovine lactotransferrin was hydrolyzed by the Endo B to an extent of 90%. As shown in Fig. 2 (lanes B and C), five oligosaccharides were released and co-migrated in TLC analysis with the following standard oligosaccharides: $(Man)_9GlcNAc$, $(Man)_8GlcNAc$, $(Man)_7GlcNAc$, $(Man)_6GlcNAc$ and $(Man)_5GlcNAc$ isolated from urine of patients with Mannosidosis. The $(Man)_9GlcNAc$ and $(Man)_8GlcNAc$ oligosaccharides, the structures of which have been previously determined by methylation and ^{1}H-NMR analysis [23], were the most important components. When Endo B hydrolysis was performed on the mixture of desialylated glycopeptides, enzyme activity reached 100%, indicating that the 10% of glycopeptides not hydrolyzed in the previous experiment possess mono- or di-sialylated bi-antennary glycans [14, 15], since these glycans are only slightly or not hydrolyzed by Endo B [8]. This result was confirmed by the observation that the glycopeptide fraction eluted from a Con A-Sepharose column with a 200 mM α-methyl glucoside solution and which contained only glycopeptides with oligomannoside type glycans was completely hydrolyzed by the enzyme under the same conditions.

Table 4. Relative sugar composition of ethanolic precipitates of ovalbumin fractions F1 to F3 and bovine lactotransferrin before and after hydrolysis by the endo-β-*N*-acetylglucosaminidase B.

	Gal	Man	GlcNAc
Ovalbumin F1	0.1	0.54	1
Ovalbumin F1 + Endo B	0.11	0.53	1
Ovalbumin F2	0.10	0.98	1
Ovalbumin F2 + Endo B	0.10	0.97	1
Ovalbumin F3	traces	2.02	1
Ovalbumin F3 + Endo B	traces	2.03	1
Bovine lactotransferrin	1	12.47	3.23
Bovine lactotransferrin + Endo B	1	8.13	2.49

Hydrolysis of Glycopeptides Isolated from Ovalbumin: As shown in Table 3, the mixture of *N*-^{14}C-acetylated ovalbumin glycopeptides was hydrolyzed by the Endo B to an extent of 60%. This hydrolysis did not change even after 24 h incubation and addition of fresh enzyme after 16 h of incubation. Fig. 2 (lanes D and E) shows the TLC pattern of the released products. In comparison with standard oligosaccharides from Mannosidosis urine, three oligosaccharides co-migrating with $(Man)_6GlcNAc$, $(Man)_6GlcNAc$, and $(Man)_7GlcNAc$ were detected. As shown in Fig. 2 (lane E), some products still remained at the starting line of the chromatogram. These results indicate that the Endo B did not cleave all of the ovalbumin glycopeptides, thus explaining why the hydrolysis obtained with labeled glycopeptides never exceeded 60%.

In order to study in more detail the action of the Endo B towards ovalbumin glycans, glycoasparagines with known structures fractionated on Dowex 50-X2 column were used. The hydrolysis rates were determined by using labeled substrates (Table 3). Endo B hydrolyzes the following structures: $(Man)_7(GlcNAc)_2Asn$ (90%), $(Man)_6(GlcNAc)_2Asn$ (95%), $(Man)_5(GlcNAc)_2Asn$ (92%), $(Man)_4(GlcNAc)_2Asn$ (90%). Glycoasparagines of the hybrid type, such as GP-I, GP-IIA, GP-IIB and GP-IIIC were not hydrolyzed, but the $(GlcNAc)_2(Man)_5(GlcNAc)_4Asn$ glycoasparagine corresponding to GP-IIIA was hydrolyzed to an extent of 90%.

Hydrolysis of Glycoproteins: The Endo B activity was tested on each fraction (F-1 to F-3) of ovalbumin obtained by chromatography on Con A-Sepharose column as described in the Materials and Methods section. As shown in Table 4, the sugar composition of the ethanol

precipitates did not change after 24 h of incubation with the enzyme preparation. No sugar was detected in the supernatants either by TLC or GLC analysis . These results clearly demonstrate that the Endo B is inactive toward native ovalbumin glycoprotein. In contrast, under the same conditions, the native bovine lactotransferrin was deglycosylated to an extent of 40% by the Endo B, as determined by GLC.

Evidence that Two Catalytic Sites of Endo-β-N-Acetylglucosaminidase B Act on Both Oligomannoside and N-Acetyllactosamine Type Substrates: In order to determine whether the hydrolysis of the substrates of the oligomannoside and *N*-acetyllactosamine type is caused by a single or by two distinct enzymes, a cross inhibition experiment was performed by using a mixture of $(Man)_{8-9}(GlcNAc)_2$-*N*-^{14}C-acetyl-Asn and $(Gal)_2(GlcNAc)_{2(}(Man)_3$-$(GlcNAc)_2$-*N*-^{14}C-acetyl-Asn as substrate, in different ratios. The comparison of theoretical values calculated according to Segel's method [30] with the experimental results (Table 5) shows that the hydrolysis rates (V values) did not decrease as would be the case when the two substrates compete for a single catalytic site but resulted from the sum of the V values for each substrate, indicating therefore the presence of two different catalytic sites.

Discussion

The activity of endo-β-*N*-acetylglucosaminidases towards oligomannoside, *N*-acetyllactosamine and hybrid type structures depends on the origin of the enzymes. In fact, among microbial enzymes, the Endo H from *Streptomyces plicatus* [7, 24-26] and the Endo F from *Flavobacterium meningosepticum* [27] cleave both oligomannoside and hybrid type glycans. The Endo C_{II} from *Clostridium perfringens* [7, 28] hydrolyzes all of the oligomannoside and only some hybrid type glycans, and some *N*-acetyllactosamine type glycans are hydrolyzed only by Endo D from *Diplococcus pneumoniae* [7, 29]. In Table 6, we compare Endo B activity to Endo H and Endo C_{II} activities towards oligomannoside and hybrid type glycans. Glycopeptides with oligomannoside type glycans containing from four to nine mannose residues were hydrolyzed by both enzymes. Endo C_{II} and Endo B cleave only one type of hybrid structure containing $(Man)_5(GlcNAc)_4$ as present in the ovalbumin glycopeptide (GP-IIIA, while Endo H has a broader substrate specificity toward hybrid type structures, since all of the ovalbumin glycopeptides were hydrolyzed by this enzyme. Therefore, on the basis of their substrate specificity, the endo-β-*N*-acetylglucosaminidases isolated until now can be classified into three groups. The first group consists of enzymes with an Endo H-like activity and hydrolyzing all of the oligomannoside and hybrid type structures present in ovalbumin. The second group is composed of the enzymes carrying an Endo C_{II}-like activity, i.e. enzymes which are inactive on ovalbumin hybrid type glycans, particularly those possessing non-reducing galactose terminal residues. The third group is constituted of Endo D-like enzymes which hydrolyze only the *N*-acetyllactosamine type structures. On the basis of its substrate specificity toward oligomannoside, hybrid and *N*-acetyllactosamine type structures, Endo B from *Basidiomyces sporotrichum dimorphosporum* could be classified as an Endo C_{II} and Endo D type of enzyme.

The oligomannoside and *N*-acetyllactosamine activities cannot be separated and have the same ratio all along the purification procedure. Both activities possess the same temperature and pH stability, the same molecular mass and the same pI. These results suggest that they

Table 5. Hydrolysis of mixtures of *N*-[^{14}C]acetylated oligomannoside and *N*-acetyllactosamine type substrates by endo-β-*N*-acetylglucosaminidase B.
A = $(Man)_{8-9}(GlcNAc)_2Asn$; B = $(Gal)_2(GlcNAc)_2(Man)_3(GlcNAc)_2Asn$; V^A = hydrolysis rate of A ; V^B = hydrolysis rate of B ; Vmix = Hydrolysis rate value of the mixture of A and B ; $V^A max$ = Maximal hydrolysis rate of A ; $V^B max$ = Maximal hydrolysis rate of B ; $K_M{}^A$ = Michaelis constant for A ; $K_M{}^B$ = Michaelis constant for B.

Substrate (nmol)		Substrate ratio	Hydrolysis rate (nmol/min)					
			Experimental			Theoretical [30]		
						2 catalytic sites	1 catalytic site	
[A]	[B]	α = [A]/[B]	V^A	V^B	Vmix	Vmix = V^A+V^B	$\frac{K_M{}^A}{\alpha K_M{}^B}$	= $\frac{Vmax^A - Vmix}{Vmix - Vmax^B}$
12	0	-	0.50	-	-	-		-
0	120	-	-	1.48	-	-		-
12	120	0.1	-	-	1.92	1.98		0.17
18	0	-	0.75	-	-	-		-
0	60	-	-	0.80	-	-		-
18	60	0.3	-	-	1.46	1.55		0.19
60	0	-	1.95	-	-	-		-
0	60	-	-	0.80	-	-		-
60	60	1.0	-	-	2.73	2.75		0.22

are located on a single protein. In addition the cross inhibition between $(Man)_{8-9}(GlcNAc)_2$-Asn and $(Gal)_2(GlcNAc)_2(Man)_3(GlcNAc)_2$-Asn indicates the occurrence of two catalytic sites.

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Table 6. Comparison of Endo H, Endo C_{II} and Endo B activities towards glycopeptides with oligomannosidic and hybrid type glycans.
R = GlcNAcβ1-4GlcNAcβ1-*N*Asn. + : hydrolyzed. - : not hydrolyzed.

Substrates	Endo H [17-20]	Endo C [17, 22]	Endo B
Oligomannoside glycans			
Manα1 6 Manβ1-4-R	-	-	+
Manα1 6 Manβ1-4R 3 Manα1	+	-	+
GP-VI Manα1-3Manα1 6 Manβ1-4-R 3 Manα1	+	+	+
GP-V Manα1 6 Manα1 3 | Manα1 6 Manβ1-4-R 3 Manα1	+	+	+
GP-IV Manα1 6 Manα1 3 | Manα1 6 Manβ1-4-R 3 Manα1-2Manα1	+	+	+
GP-IIIB Manα1-2Manα1 6 Manα1 3 | Manα1 6 Manβ1-4-R 3 Manα1-2Manα1	+	+	+

Table 6 (cont.).

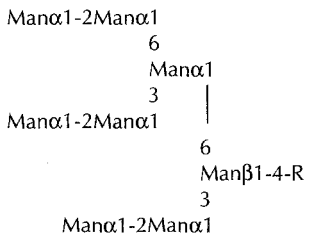
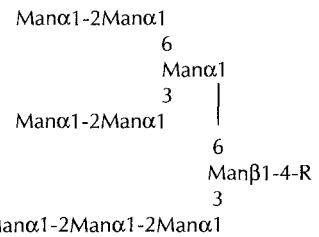
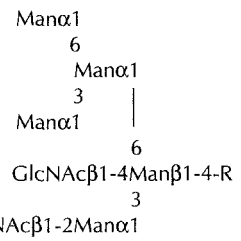
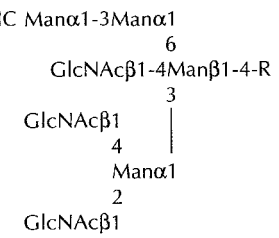
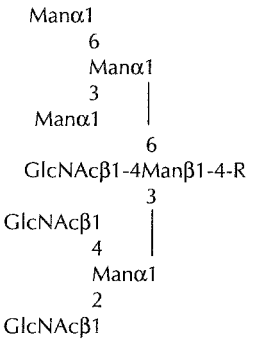
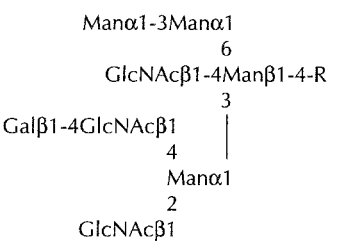
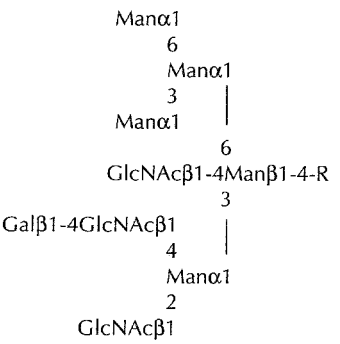
Substrates	Endo H [17-20]	Endo C [17, 22]	Endo B
	+	+	+
	+	+	+
Hybrid glycans			
GP-IIIA 	+	+	+
GP-IIIC 	+	-	-

Table 6 (cont.).

Substrates	Endo H [17-20]	Endo C [17, 22]	Endo B
GP-IIB 	+	-	-
GP-II A 	+	-	-
GP-I 	+	-	-

References

1 Tarentino AL, Trimble RB, Maley F (1961) Methods Enzymol 50:574-80.
2 Nishigaki M, Muramatsu T, Kobata A (1974) Biochem Biophys Res Commun 59:638-45.
3 Chien SF, Weinburg R, Li SC, Li YT (1977) Biochem Biophys Res Commun 76:317-23.
4 Tarentino AL, Maley F (1978) Methods Enzymol 50:580-84.
5 Kobata A (1978) Methods Enzymol 50:567-74.
6 Muramatsu T (1978) Methods Enzymol 50:555-79.
7 Kobata A (1979) Anal Biochem 100:1-14.
8 Bouquelet S, Strecker G, Montreuil J, Spik G (1980) Biochimie 62:43-49.
9 Shepherd V, Montgomery R (1978) Carbohydr Res 61:147-57.
10 Huang CG, Mayer HE, Montgomery R (1970) Carbohydr Res 13:127-37.
11 Spik G, Bayard B, Fournet B, Strecker G, Bouquelet S, Montreuil J (1975) FEBS Lett 50:296-99.
12 Spik G, Debruyne V, Montreuil J, van Halbeek H, Vliegenthart JFG (1985) FEBS Lett 183:65-69.
13 Chéron A, Mazurier J, Fournet B (1977) CR Acad Sci, Paris, série D 284:585-88.
14 Spik G, Coddeville B, Legrand D, Mazurier J, Leger D, Goavec M, Montreuil J (1985) in Proteins of Iron Storage and Transport, eds. Spik G, Montreuil J, Crichton RR; Mazurier J, Elsevier, Amsterdam, 47-51.
15 Spik G, Coddeville B, Montreuil J (1988) Biochimie 70:1459-69.
16 Koide N, Muramatsu T (1975) Biochem Biophys Res Commun 66:411-16.
17 Corfield AP, Beau JM, Schauer R (1978) Hoppe Seylers Z Physiol Chem 359:1325-42.
18 Morell AG, van Den Hamer CJA, Scheinberg IH, Ashwell GJ (1966) J Biol Chem 241:3745-49.
19 Chavira R, Jr, Burnett TJ, Hageman JH (1984) Anal Biochem 136:446-50.
20 Salacinski PRP, McLean C, Sykes SEC, Lemen-Jones VV; Lowry PJ (1981) Anal Biochem 117:136-46.
21 Zanetta JP, Breckenridge WC, Wincendon G (1972) J Chromatogr 69:291-304.
22 Laemmli UK (1970) Nature 227:680-85.
23 van Halbeek H, Dorland L, Vliegenthart JFG, Spik G, Chéron A, Montreuil J (1981) Biochim Biophys Acta 675:293-96.
24 Trimble RB, Tarentino AL, Plummer TH Jr, Maley F (1978) J Biol Chem 253:4508-11.
25 Tai T, Yamashita K, Ito S, Kobata A (1977) J Biol Chem 252:6687-94.
26 Yamashita K, Tachibana Y, Kobata A (1978) J Biol Chem 253:3862-69.
27 Helder JH, Alexander S (1982) Proc Nat Acad Sci USA 79:4540-4.
28 Ito J, Muramatsu T, Kobata A (1975) Arch Biochem Biophys 171:78-86.
29 Koide N, Muramatsu T (1974) J Biol Chem 249:4897-904.
30 Segel IH (1974) Enzymes Kinetics, Wiley, New York p. 113-18.