Partial purification and characterization of an endo- α -*N*-acetylgalactosaminidase from the culture medium of *Streptomyces* sp. OH-11242

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For the purification of a new type of endo- α -*N*-acetylgalactosaminidase from the culture medium of *Streptomyces* sp. OH-11242 (endo-GalNAc-ase-S) [Iwase, Ishii, Ishihara, Tanaka, Omura & Hotta (1988) *Biochem. Biophys. Res. Commun.* **151**, 422–428], a method for assaying enzyme activity was established. Using purified pig gastric mucus glycoprotein (PGM) as the substrate, oligosaccharides liberated from PGM were pyridylaminated, and the reducing terminal sugars of oligosaccharides larger than Gal β 1-3GalNAc were analysed by h.p.l.c. The crude enzyme of endo-GalNAc-ase-S was prepared as an 80% (w/v) ammonium sulphate precipitate from the concentrated culture medium. The enzyme was partially purified by gel chromatofocusing and subsequent DEAE-Toyopearl chromatography. Endo-enzyme activity eluted around pI 4.8 on a gel chromatofocusing column and eluted with 0.19–0.25 M-NaCl on a DEAE-Toyopearl column. In the enzyme fraction obtained, no exo-glycosidases or proteases could be detected. The molecular mass of the enzyme was estimated as 105 kDa by gel filtration, and the optimum pH was 5.5. Endo-GalNAc-ase-S hydrolysed the O-glycosidic linkage between GalNAc and Ser (Thr) in ³H-labelled and unlabelled asialofetuin, liberating both the disaccharide (Gal β 1-3GalNAc) and the tetrasaccharide [Gal β 1-3(Gal β 1-4GlcNAc β 1-6)GalNAc]. When endo- α -*N*-acetylgalactosaminidase from *Alcaligenes* sp. (endo-GalNAc-ase-A) was incubated with ³H-labelled and unlabelled asialofetuin, only the disaccharide (Gal β 1-3GalNAc) was liberated.

INTRODUCTION

Mucus glycoproteins are major glycoproteins in mucus secretions of epithelial cells in the salivary, gastrointestinal, respiratory and urogenital tracts [1–4]. They are important components that protect tissues and cells from various agents [2,5]. The major role of the oligosaccharide in mucus glycoproteins is that of protecting glycoproteins from degradation. Recently, such mucin-type oligosaccharides were found in hepatoma ascite cell membranes and human gastric cancer cells, suggesting possible relationships between oligosaccharide chains and cancerous cells [6,7].

To investigate the oligosaccharide constituent of mucin-type glycoproteins, the O-glycosidic linkage was cleaved chemically through weak alkalinity obtained in β -elimination reactions. The enzymic cleavage of linkages should be very useful for elucidating the features and functions of O-linked complex carbohydrates. Endo- α -*N*-acetylgalactosaminidase (EC 3.2.1.97, endo-GalNAc-ase), i.e. ‘O-Glycanase’ from *Diplococcus pneumoniae* (endo-GalNAc-ase-D [8–10]) and endo- α -*N*-acetylgalactosaminidase from *Alcaligenes* sp. (endo-GalNAc-ase-A) [11], which are both commercially available, hydrolytically cleave α -*N*-acetylgalactosaminyl linkages of only Gal β 1-3GalNAc α 1-Ser (or Thr) in glycopeptides and glycoproteins to liberate the disaccharide moiety.

A new glycosidase, endo- α -*N*-acetylgalactosaminidase from *Streptomyces* sp. (endo-GalNAc-ase-S), was found to be capable of liberating not only Gal β 1-3GalNAc but other larger oligosaccharides as well through the hydrolysis of the O-glycosidic linkage between GalNAc and Ser (Thr) residues [12–14a].

To assay the activity of endo-GalNAc-ase-D or -A, asialofetuin was used as the substrate and the enzyme activity was expressed in terms of the released disaccharide (Gal β 1-3GalNAc) [15]. However, by this assay method measurement of endo-GalNAc-ase-S activity was not possible, because liberation of oligosaccharides larger than the disaccharide could not be confirmed.

The authors established a reliable method for assaying the activity of endo-GalNAc-ase-S activity. The reaction mixture containing PGM was incubated, and oligosaccharides liberated from PGM used as the substrate were subjected to pyridylation. The reducing-terminal sugars of the oligosaccharides larger than Gal β 1-3GalNAc were analysed by h.p.l.c. By this method of enzyme purification, the enzyme was partially purified and characterized. To examine the substrate specificity of endo-GalNAc-ase-S, asialofetuin, the oligosaccharide structures of which had already been determined, was used as the substrate. The action of this enzyme on asialofetuin was compared with that of endo-GalNAc-ase-A.

EXPERIMENTAL

Materials

The following materials were purchased from the sources indicated. NaB³H₄ was from NEN Research Products (du Pont, Boston, MA, U.S.A.). Polybuffer exchanger PBE 94, Polybuffer 74 for chromatofocusing and Sephadex G-200 and G-15 were purchased from Pharmacia (Uppsala, Sweden). DEAE-Toyopearl and Toyopearl HW-50S were from Tosoh (Tokyo, Japan). Pig gastric mucin (Type II), pepsin and azocasein were from

Abbreviations used: endo-GalNAc-ase-S, -D and -A; endo- α -*N*-acetylgalactosaminidase from culture media of *Streptomyces* sp. OH-11242, *Diplococcus pneumoniae* and *Alcaligenes* sp. respectively; PGM, pig gastric mucus glycoprotein; CPC, cetylpyridinium chloride; PA, pyridylaminated derivatives (e.g. PA-sugar); PEG, polyethylene glycol; 2-AP, 2-aminopyridine.

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Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of the highest analytical grade available. Endo-GalNAc-ase-A (EC 3.2.1.97) was purchased from Seikagaku Kogyo (Tokyo, Japan). Galactose oxidase from *Doctyllum dendroides* (EC 1.1.3.9) was purchased from Funakoshi (Tokyo, Japan). Catalase extracted from *Aspergillus niger* (EC 1.11.1.6) was from Serva (Heidelberg, Germany). Asialofetuin was prepared by mild acid hydrolysis (0.1 M-HCl, 80 °C, 60 min) of fetuin obtained from GIBCO (New York, NY, U.S.A.). BCA Protein Assay reagent was purchased from Pierce (Rockford, IL, U.S.A.).

Growth of *Streptomyces* sp. OH-11242

Streptomyces sp. OH-11242, isolated from soil, was used to produce glycosidases for subsequent experiments. Spores and mycelia of strain OH-11242, grown on an agar slant, were inoculated into a 500 ml culture flask containing 100 ml of seed medium consisting of 2 % (w/v) glucose, 0.5 % (w/v) peptone, 0.5 % (v/v) meat extract, 0.3 % (w/v) dry yeast cells, 0.5 % (w/v) NaCl, and 0.3 % (w/v) CaCO₃, pH 7.0. This system was incubated with shaking at 27 °C for 2 days. The seed culture (4 ml) obtained was transferred to a 500 ml culture flask. Each flask contained 100 ml of production medium consisting of 0.26 % (w/v) ammonium sulphate, 0.24 % KH₂PO₄, 0.43 % (w/v) K₂HPO₄, 0.10 % (w/v) MgSO₄·7H₂O, 0.1 % (v/v) trace metal solution, and 0.5 g of PGM, pH 7.0. A trace metal solution, adjusted to pH 7.0 by NaOH, consisted of 0.64 % (w/v) CuSO₄·5H₂O, 0.11 % (w/v) FeSO₄·7H₂O, 0.79 % (w/v) MnCl₂·4H₂O, 0.15 % (w/v) ZnSO₄·7H₂O and distilled water. The flasks were incubated at 27 °C for 3 days with reciprocal shaking (120 strokes/min).

Preparation of PGM

Pig gastric mucin (100 g) was made up of a final volume of 500 ml by adding 0.001 % (w/v) NaN₃. The pH was adjusted to 2.5 with conc. HCl. Pepsin (1 g) was added to the suspension, followed by incubation at 37 °C with slow shaking. After both 24 h and 48 h, 1 g of pepsin was added, and digestion was continued up to 72 h. The reaction mixture was neutralized by 1 M-NaOH and was then centrifuged at 9500 *g* for 30 min. To remove the mucopolysaccharide, 5 g of cetylpyridinium chloride (CPC) was dissolved in the reaction mixture that had been left overnight at room temperature. The insoluble complex formed with CPC was removed by centrifugation at 8000 *g* for 60 min and the clear supernatant thus obtained was thoroughly dialysed against tap water. The solution that remained in the dialysis bag was subjected to ethanol fractionation. After removal of the insoluble material formed at a 33 % (v/v) concentration of ethanol, ethanol was added to a concentration of 50 % (v/v). The precipitate produced overnight at 4 °C was collected by centrifugation at 9500 *g* for 60 min. The resultant colourless powder, weighing 31 g after freeze-drying, was used as the source of PGM for subsequent experiments.

Conditions for h.p.l.c. analysis

Pyridylaminated (PA) derivatives were separated as reported previously [16,17] using a reversed-phase column (Ultrasphere-ODS, 2 × 250 mm × 4.6 mm internal diameter; Beckman) with 0.125 M-sodium citrate buffer, pH 4.0, containing 0.75 % (v/v) acetonitrile, at a flow rate of 0.5 ml/min. To detect PA-sugars, a Jasco FP-210 spectrofluorimeter was used at an excitation wavelength of 320 nm and an emission wavelength of 400 nm. Approx. 10 pmol of the sample for each peak was injected. PA-sugars were also separated on a TSK-GEL G2000PW column (7.5 mm internal diameter × 600 mm;

Fig. 1. Method for detecting reducing-terminal sugars of oligosaccharides liberated from PGM by endo-GalNAc-ase-S

H.p.l.c. profile of the pyridylaminated derivatives of oligosaccharides from PGM digested with endo-GalNAc-ase-S. PA-sugars were separated on a TSK-gel G2000PW column. The PA-oligosaccharide fraction which were larger than Galβ1-3GalNAc-PA, was collected (shown by a bar), and concentrated to dryness. Arrows indicate the positions of elution from the column of the following standard pyridylamino derivatives: 1, Galβ1-3GalNAc-PA; 2, GlcNAc-PA and GalNAc-PA; 3, Gal-PA and Glc-PA; 4, 2-aminopyridine (2-AP). (b) H.p.l.c. profile of reducing-terminal sugars of oligosaccharides liberated from PGM through enzymic action. Oligosaccharide-PA was hydrolysed with 4 M-HCl at 100 °C for 6 h in a sealed tube and the PA-monosaccharides obtained were re-acetylated and chromatographed on an Ultrasphere ODS column. Elution positions of standard PA-sugars are indicated by arrows.

Tosoh), using 0.02 M-ammonium acetate buffer, pH 7.5, at a flow rate of 0.5 ml/min.

Assays of enzyme activity

Assay of endo-GalNAc-ase-S activity. For the determination of endo-GalNAc-ase-S activity, 1 mg of PGM was incubated with 50 μl of enzyme solution and an appropriate amount of buffer, in a total volume of 200 μl. After incubation at 37 °C for 17 h, the reaction mixture was boiled at 100 °C for 10 min and precipitated by ethanol [final concentration 80 % (v/v)]. Following centrifugation at 6000 *g* for 5 min, the supernatant was used for the following experiment. A portion of the solution was analysed for oligosaccharide content by t.l.c. The other oligosaccharides in the solution were pyridylaminated as described previously [16] and chromatographed on a gel-filtration column (TSK-GEL G2000PW) to obtain oligosaccharides larger than Galβ1-3GalNAc. The fraction of oligosaccharide-PAs which were larger than Galβ1-3GalNAc-PA was collected (shown by a bar in Fig. 1a) and concentrated to dryness. To determine the reducing-terminal sugars of oligosaccharides liberated from PGM through enzyme reactions, oligosaccharide-PA was hydrolysed with 4 M-HCl at 100 °C for 6 h in a sealed tube, and the pyridylaminated monosaccharides produced were chromatographed on a reversed-phase column following re-acetylation of the amino groups, as previously described [12] (Fig. 1b). One unit of endo-GalNAc-ase-S activity is defined as the amount of enzyme which can liberate 1 μmol of oligosaccharides with *N*-acetylgalactosamine at the reducing terminal in 1 min under standard conditions using PGM as a substrate. The amount of oligosaccharide liberated from PGM was calculated based on the

Table 1. Purification of endo-GalNAc-ase-S from *Streptomyces* sp. OH-11242

Step	Volume (ml)	Total protein (mg)	Total activity (units)	$10^{-3} \times$ Specific activity (units/mg)	Purification (fold)	Recovery (%)
Culture fluid	467	131	0.026	0.198	1	100
Ammonium sulphate precipitation	10	46.7	0.024	0.514	2.6	92
Gel chromatofocusing	8.0	1.49	0.016	10.7	54.1	62
DEAE-Toyopearl	6.4	0.22	0.0037	16.8	84.9	14

peak height of the h.p.l.c. profile that reflects the amounts of its reducing terminal GalNAc-PA.

Assay of the activity of the other enzyme. Exoglycosidase activity was assayed using appropriate *p*-nitrophenyl glycosides (*p*-nitrophenyl β -Gal, β -GlcNAc, α -Gal, α -GalNAc, α -Fuc). The reaction mixture was incubated at 37 °C for 1 h, and the absorbance was measured at 400 nm. One unit of exoglycosidase activity is defined as that amount of enzyme that can liberate 1 μ mol of *p*-nitrophenol in 1 min from the *p*-nitrophenyl glycoside. Protease activity was determined by incubating azocasein (12.5 mg) with the enzyme preparation in 200 μ l of 0.1 M-Tris/HCl buffer, pH 7.6, at 37 °C for 17 h. The reaction was terminated by the addition of 3.2 ml of 5% trichloroacetic acid, after which the absorbance of the supernatant was measured at 440 nm.

Analytical methods

Neutral sugars were assayed by the phenol/H₂SO₄ reaction with galactose as a standard [18]. Sialic acid was determined through a modified Warren method, using *N*-acetylneuraminic acid as a standard [19]. The protein content was determined by using the Pierce BCA Protein Assay reagent and BSA as a standard [20].

Preparation of crude enzyme from the culture fluid

The culture fluid of *Streptomyces* sp. OH-11242 was dialysed against distilled water. The dialysate was brought to 80% (w/v) saturation by adding solid ammonium sulphate with stirring. The precipitate that formed was collected by centrifugation at 9500 *g* for 20 min, dissolved in a minimum amount of water and dialysed against distilled water.

Gel chromatofocusing

The crude enzyme preparation was dialysed against 0.025 M-imidazole/HCl buffer, pH 7.4, (starting buffer for gel chromatofocusing). A PBE 74 column (1.0 cm $\times$ 25.5 cm) was equilibrated with 0.025 M-imidazole/HCl buffer, pH 7.4, and 10 ml of crude enzyme preparation was applied on to the column, which was subsequently eluted with polybuffer 74/HCl, pH 4.0, in degassed distilled water to generate a linear pH gradient from 7.4 to 4.0. A 5 ml volume of eluant was obtained. The enzyme activity in each eluted fraction was measured as described above. The enzyme fractions were concentrated to about one-tenth of the original volume by dialysis against polyethylene glycol (PEG) 20000.

DEAE-Toyopearl chromatography

A portion of an enzyme fraction eluted from gel chromatofocusing was dialysed against 0.005 M-phosphate buffer, pH 7.0, (starting buffer for DEAE-Toyopearl chromatography). A DEAE-Toyopearl column (1.0 cm $\times$ 25.5 cm) was equilibrated with 0.005 M-phosphate buffer, pH 7.0, and the enzyme fraction eluted from the gel chromatofocusing column

was introduced. The enzymes were eluted with a linear gradient of 0–0.35 M-NaCl and collected in 5 ml fractions. The DEAE-Toyopearl used here was a weak anion exchanger; DEAE-Toyopearl 650 was derived by introducing diethylaminoethyl groups into Toyopearl HW65 of large pore size [21]. Some of the pooled solution was concentrated to 1.5 ml by dialysis against PEG 20000. The purification process and its success are detailed in Table 1.

Preparation of ³H-labelled glycoprotein

Asialofetuin and PGM were labelled by galactose oxidase followed by NaB³H₄ reduction according to a modified method of Morell *et al.* [22]. The reaction mixture after labelling was adjusted to pH 5 and purified by gel filtration on a Sephacryl S-400 column.

Assays of optimum pH or molecular mass for endo-GalNAc-ase-S

To determine the optimum pH for endo-GalNAc-ase-S, [³H]PGM (1 $\times$ 10⁵ d.p.m.) was incubated with 20 μ l of enzyme solution and an appropriate amount of buffer in a total volume of 80 μ l. After incubation at 37 °C for 17 h, the reaction mixture was added to PGM (400 mg), in a total volume of 120 ml, and precipitated with ethanol [final concentration 80% (v/v)]. After centrifugation at 6000 *g* for 5 min, the supernatant was used for the following experiment. A portion of the solution (400 μ l) was assayed for radioactivity by the liquid scintillation method.

Substrate specificity of endo-GalNAc-ase-S

The action of endo-GalNAc-ase-S on glycoprotein was compared with that of endo-GalNAc-ase-A. Using unlabelled asialofetuin as the substrate, the reaction products were pyridylaminated, further fractionated on a Sephadex G-10 column and then analysed by h.p.l.c. Using [³H]asialofetuin as the substrate, the reaction products were detected by radioactivity following fractionation on a Sephadex G-15 column and were analysed by t.l.c.

RESULTS

Assay method for endo-GalNAc-ase-S activity

The crude enzyme preparation from the culture medium of *Streptomyces* sp. OH-11242 contained endo- α -N-acetyl-galactosaminidase, endo- β -N-acetylglucosaminidase, α -N-acetyl-galactosaminidase, β -N-acetylglucosaminidase, α -fucosidase, α -galactosidase, β -galactosidase, α -mannosidase and β -mannosidase (Table 2). To isolate endo-GalNAc-ase-S from the glycosidase mixture and to discriminate endo-GalNAc-ase-S from known endo-GalNAc-ase which can release only Gal β 1-3GalNAc, a new assay method for the enzyme activity should be established. To detect broad substrate specificity of endo-GalNAc-ase-S, the confirmation of the liberation from the

Table 2. Exoglycosidase and protease activities in each enzyme preparation

Abbreviation: N.D., not detectable under standard conditions.

Step	Enzyme activity (units/mg)							Protease (A_{440})
	α -GalNAc-ase	β -GlcNAc-ase	α -Fuc-ase	α -Gal-ase	β -Gal-ase	α -Man-ase	β -Man-ase	
Culture fluid	14.2	13.8	10.4	18.4	10.5	2.18	0.306	0.31
Ammonium sulphate precipitation	35.7	34.8	25.3	44.7	25.3	4.40	0.792	0.29
Gel chromatofocusing	16.6	316	140	66.2	N.D.	N.D.	N.D.	0.10
DEAE-Toyopearl	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Fig. 2. Gel chromatofocusing of endo-GalNAc-ase-S

The crude enzyme preparation was applied onto a polybuffer exchanger (PBE 74) column (1.0 cm $\times$ 25.5 cm) and eluted as described in the Experimental section. Fractions were assayed for endo-GalNAc-ase-S (hatched area), β -*N*-acetylglucosaminidase ($\circ$), α -*N*-acetylgalactosaminidase ($\square$), α -fucosidase ($\triangle$), β -galactosidase ($\blacktriangle$), and pH (-----). Endo-GalNAc-ase-S activity, shown by peak height in reversed-phase h.p.l.c., reflects the amounts of reducing-terminal GalNAc-PA of oligosaccharides liberated from PGM by the enzyme (hatched area).

substrate of larger oligosaccharides than Gal β 1-3GalNAc with *N*-acetylgalactosamine at the reducing terminal was carried out by their pyridylamination in conjunction with h.p.l.c. analysis. As shown in Fig. 1, our new method was shown to be sufficiently sensitive and quantitative to detect rapidly the reducing-terminal sugars of oligosaccharides larger than Gal β 1-3GalNAc.

Purification of endo-GalNAc-ase-S

Gel chromatofocusing was carried out on the crude enzyme preparation and enzyme activity was detected in the pH regions of 5.2 and 4.7–4.9. The chromatographic profile of activity is shown in Fig. 2. Endo-GalNAc-ase-S and exoglycosidase activities in the eluted fractions were measured as described in the Experimental section. Endo-GalNAc-ase-S activity was detected as a major peak at pI 4.8, and weak activity was found around pI 5.2. Fractions with enzyme activity were pooled, the pH adjusted to 7.0 with 0.2 M-phosphate buffer, pH 7.0, and a solution of the enzyme was concentrated to about one-tenth of the original volume by dialysis against PEG 20000. The con-

centrated enzyme showed no loss of activity during at least 6 months of storage. Following elution after gel chromatofocusing, pooled fractions possessing enzyme activity were applied on to a DEAE-Toyopearl column. Fig. 3 shows the elution profile of the endo-enzyme. The enzyme bound fairly strongly to the column and eluted around 0.19–0.25 M-NaCl (Fig. 3). From this purification step, endo-GalNAc-ase-S was completely separated from exoglycosidase and other contaminating proteins (Table 2). The fractions having enzyme activity were pooled and made into a solution that was concentrated to 1.5 ml by dialysis against PEG 20000. An enzyme partially purified by gel chromatofocusing and a DEAE-Toyopearl column was incubated with asialofetuin or PGM. The product that was obtained with *N*-acetylgalactosamine at the reducing terminal was Gal β 1-3GalNAc, in addition to other larger oligosaccharides. With *p*-nitrophenyl glycoside as the substrate, the partially purified enzyme was found to contain no exoglycosidase. It liberated no [3 H]galactose from either [3 H]PGM or [3 H]asialofetuin and liberated no galactose from unlabelled asialofetuin as the substrate in an experiment for examining the properties and substrate specificity of endo-GalNAc-ase-S. The result indicated that the purified endo-GalNAc-ase-S was absolutely free from β -galactosidase, acting toward not only synthetic substrate but also natural substrate. The results of endo-GalNAc-ase-S purification are summarized in Table 1. The enzyme was purified 85-fold from culture medium with a recovery of about 14%.

Properties of endo-GalNAc-ase-S

The activity of the enzyme with [3 H]PGM as the substrate at a different buffer pH is shown in Fig. 4. Optimum pH was approx. 5.5. About 20–70% inhibition of activity was observed on using Tris/maleate or Tris/HCl buffer instead of Mes or Hepes, and this may possibly have been due to the Tris buffer.

The molecular mass of the enzyme was determined by gel filtration on Sephadex G-200 by the method of Andrews [24]. The apparent molecular mass was shown to be 105 kDa, based on examination of a series of standard proteins whose positions were determined from absorbance at 280 nm.

Substrate specificity of endo-GalNAc-ase-S

For more precise determination of enzyme specificity, 3 H-labelled or unlabelled desialylated fetuin was used as the substrate, because fetuin contains sugar chains such as trisaccharide (NeuAc α 2-3Gal β 1-3GalNAc), tetrasaccharide [NeuAc α 2-3Gal β 1-3(NeuAc α 2-6)GalNAc], and hexasaccharide [NeuAc α 2-3Gal β 1-3(NeuAc α 2-3Gal β 1-4 GlcNAc β 1-6)GalNAc] [25,26].

Using [3 H]asialofetuin as the substrate. To determine the substrate specificity of endo-GalNAc-ase-S, its action on glycoprotein was compared with that of endo-GalNAc-ase-A or alkaline sodium borohydride treatment (β -elimination reaction and simultaneous reduction of the carbohydrate chain). Using

Fig. 3. DEAE-Toyopearl chromatography of endo-GalNAc-ase-S

The enzyme solution was applied on to a DEAE-Toyopearl column (1.0 cm $\times$ 25.5 cm) and eluted as described in the Experimental section. Fractions were assayed for endo-GalNAc-ase-S (hatched area), β -N-acetylglucosaminidase ($\circ$), α -N-acetylgalactosaminidase ($\square$) and α -fucosidase ($\triangle$) activity. NaCl concentration in the gradient is indicated by ----. Endo-GalNAc-ase-S activity was the same as shown in Fig. 2 (hatched area).

Fig. 4. pH optimum of endo-GalNAc-ase-S

The enzyme reaction was conducted in a reaction mixture consisting of [3 H]PGM as the substrate, using 0.1 M-citrate ($\square$), AcOH/NaOH ($\bullet$), Mes ($\blacksquare$), Hepes ($\circ$), Tris/maleate ($\triangle$) or Tris/HCl ($\blacktriangle$) buffer of various pH values.

[3 H]asialofetuin, reaction products were detected by their radioactivity following fractionation on a Sephadex G-15 column and analysis by t.l.c.

Elution profiles from the Sephadex G-15 column are shown in Fig. 5. In a control experiment, [3 H]asialofetuin as the substrate was incubated at 37 $^{\circ}$ C for 17 h and applied on to the Sephadex G-15 column. Because a [3 H]asialofetuin peak was observed for fractions 65–80 and production of any other low-molecular-mass material could not be detected, the chromatogram was thus used as the control. For cleavage of O-linked oligosaccharides from asialofetuin, [3 H]asialofetuin was treated with alkaline sodium borohydride and applied to the Sephadex G-15 column. Two peaks for fractions 83–100 and fractions 101–130 were observed

Fig. 5. Sephadex G-15 column chromatography of [3 H]asialofetuin and degradation products

The column (0.75 cm $\times$ 57 cm) was eluted with 40 mM-phosphate buffer, pH 7.0, and 500 μ l fractions were collected. Product radioactivity was determined by the liquid scintillation method. The excluded volume was determined by eluting Dextran T-500 (V_0). The column volume was calibrated by eluting galactose (V_i). Elution positions of V_0 and V_i are indicated by arrows. (a) [3 H]Asialofetuin; (b) product obtained by alkaline sodium borohydride treatment of [3 H]asialofetuin; (c) endo-GalNAc-ase-A digest of [3 H]asialofetuin; and (d) endo-GalNAc-ase-S digest of [3 H]asialofetuin.

and corresponded to tetrasaccharide [Gal β 1-3(Gal β 1-4GlcNAc β 1-6)GalNAcH $_2$] and disaccharide (Gal β 1-3GalNAcH $_2$) (Fig. 5b). The structure of fetuin oligosaccharide has been reported [25]. When [3 H]asialofetuin was incubated with endo-GalNAc-ase-A at 37 $^{\circ}$ C for 17 h, two peaks for fractions 101–130 and fractions 131–152 (Fig. 5c) were noted which corresponded to Gal β 1-3GalNAc, based on the substrate specificity of endo-GalNAc-ase-A and monosaccharide. The amount of Gal β 1-3GalNAc liberated from [3 H]asialofetuin by endo-GalNAc-ase-A was about 85% of the disaccharides (Gal β 1-3GalNAcH $_2$) liberated from [3 H]asialofetuin by alkaline sodium borohydride treatment. The peak from fraction 131 to fraction 152 was galactose released from [3 H]asialofetuin, through cleavage by exo-galactosidase in endo-GalNAc-ase-A (Fig. 5c). When endo-GalNAc-ase-S was incubated with [3 H]asialofetuin at 37 $^{\circ}$ C for 17 h, two peaks appeared around fractions 83–104 and fractions 105–130 and corresponded to tetrasaccharide [Gal β 1-3(Gal β 1-4GlcNAc β 1-6)GalNAc] and disaccharide (Gal β 1-3GalNAc) (Fig. 5d). The amounts of tetrasaccharide [Gal β 1-3(Gal β 1-4GlcNAc β 1-6)GalNAc] and disaccharide (Gal β 1-3GalNAc) liberated from [3 H]asialofetuin by endo-GalNAc-ase-S were about 70% of the tetrasaccharide and 20% of the disaccharide liberated from [3 H]asialofetuin by

Fig. 6. T.l.c. profiles of oligosaccharides liberated from [^3H]asialofetuin by endo-GalNAc-ase-S, -A or alkaline sodium borohydride treatment

Degradation products from [^3H]asialofetuin were separated by Sephadex G-15 column chromatography and applied to a silica gel 60 plate. The plate was developed at room temperature twice, 7 h each, using the butanol/acetic acid/water (3:3:1, by vol.) solvent system. Radioactivity on the silica gel plate was determined after slicing the plate into 5 mm-wide segments. Each slice was saturated with water before measuring radioactivity. (a) Oligosaccharide from [^3H]asialofetuin treated with alkaline sodium borohydride; (b) oligosaccharide from [^3H]asialofetuin treated with endo-GalNAc-ase-A; (c) oligosaccharides from [^3H]asialofetuin treated with endo-GalNAc-ase-S; and (d) standard oligosaccharide, Gal β 1-3GalNAc $_2$.

alkaline sodium borohydride treatment. Therefore, the relative content of tetrasaccharide to disaccharide released from asialofetuin by the enzyme treatment was slightly different from that obtained by alkaline sodium borohydride treatment.

For further examination, oligosaccharides liberated from [^3H]asialofetuin (from fraction 83 to fraction 130 in Fig. 5) were analysed by t.l.c. after being reduced by sodium borohydride and desalted (Fig. 6). As shown in Fig. 6, when [^3H]asialofetuin was treated with alkaline sodium borohydride two radioactive peaks appeared, one at 6.0–6.5 cm and the other at 8.0–9.5 cm from the origin (Fig. 6a). When [^3H]asialofetuin was incubated with endo-GalNAc-ase-A, one peak appeared at 8.0–9.5 cm from the origin, corresponding to a disaccharide, Gal β 1-3GalNAc $_2$. When [^3H]asialofetuin was incubated with endo-GalNAc-ase-S, two peaks, one at 6.0–6.5 cm and the other at 8.0–9.5 cm from the origin, could be seen (Fig. 6c). The standard oligosaccharide peak, Gal β 1-3GalNAc $_2$, was seen at 8.0–9.0 cm from the origin (Fig. 6d). Endo-GalNAc-ase-S would thus appear to hydrolyse the O-glycosidic linkages between GalNAc and Ser (Thr) in [^3H]asialofetuin, to liberate a disaccharide (Gal β 1-3GalNAc) and a tetrasaccharide [Gal β 1-3(Gal β 1-4GlcNAc β 1-6)GalNAc]. Therefore, a new enzyme with broad substrate specificity differing from that of endo-GalNAc-ase-A or -D was obtained in the present study through the partial purification of endo-GalNAc-ase-S.

Using unlabelled asialofetuin as the substrate. To confirm the results obtained with [^3H]asialofetuin as the substrate, an unlabelled substrate was used following the method described in the Experimental section. After incubating asialofetuin with endo-GalNAc-ase-S or -A, the liberated oligosaccharides were pyridylaminated and their chromatographic profiles are shown in Fig. 7. With asialofetuin as the substrate, endo-GalNAc-ase-

Fig. 7. Comparison of oligosaccharides liberated from asialofetuin by endo-GalNAc-ase-S or -A

Asialofetuin was incubated with endo-GalNAc-ase-S or -A and the liberated oligosaccharides were pyridylaminated and chromatographed by gel filtration on a Sephadex G-10 column (results not shown). The fractions 79–90 from the Sephadex G-10 column were collected, and PA-sugars in the solution of the fractions were separated on an Ultrasphere ODS column. (a) H.p.l.c. profile of fractions 79–90 from Sephadex G-10 for the oligosaccharide liberated by endo-GalNAc-ase-S. The peak (elution time about 34 min) contained the disaccharide, Gal β 1-3GalNAc. The proposed structure for the peak (about 44 min) was Gal β 1-3(Gal β 1-4GlcNAc β 1-6)GalNAc-PA. (b) H.p.l.c. profile of fractions 79–90 from Sephadex G-10 for the oligosaccharide liberated by endo-GalNAc-ase-A. The peak (about 34 min) contained the disaccharide, Gal β 1-3GalNAc.

S liberated not only disaccharide (Gal β 1-3GalNAc) but tetrasaccharide [Gal β 1-3(Gal β 1-4GlcNAc β 1-6)GalNAc] as well. Asialofetuin incubated with endo-GalNAc-ase-A liberated only the disaccharide (Gal β 1-3GalNAc). The same results were obtained upon using [^3H]asialofetuin as the substrate.

DISCUSSION

Endo- α -N-acetylgalactosaminidase was first detected in 1972 in the culture medium of *Clostridium perfringens* [27]. Similar enzymes have subsequently been obtained from the culture fluid of *Diplococcus pneumoniae* and *Alcaligenes* sp. [8–11] and purified, and have been found to possess strict substrate specificity. They selectively liberate only the disaccharide, Gal β 1-3GalNAc, from various glycopeptides and glycoproteins containing O-glycosidic linkage oligosaccharides. Because these enzymes liberated only Gal β 1-3GalNAc from O-glycoside-type glycopeptides and glycoproteins, various studies have been conducted on them in view of their very narrow substrate specificity. Although enzymes possessing such specificity are useful for the clarification of the structures and functions of carbohydrate chains in glycoproteins, enzymes of broader substrate specificity are desired.

In 1988, oligosaccharides consisting of around two to ten

sugars with N-acetylgalactosamine at the reducing terminal were detected in the culture fluid of *Streptomyces* sp. OH-11242 incubated with mucin as the sole carbon source, thus suggesting the induction of endo-glycosidase [12]. The substrate specificity differs from that of endo-GalNAc-ases already reported and may possibly liberate oligosaccharides larger than Gal β 1-3GalNAc from PGM [12–14]. The Morgan–Elson method [15] for detecting N-acetyl amino sugars was used to measure endo-GalNAc-ase-D, -A activity; however, the detection of endo-GalNAc-ase-S activity was not possible. Therefore, as a means for doing this, as well as to purify the enzyme, an assay method in which pyridylation and h.p.l.c. are conducted in conjunction was established in the present study. It was found to be adequately sensitive (with high resolution) for studying oligosaccharides and monosaccharides without isotope labelling. Pyridylation could be used for measuring enzyme activity, with PA-oligosaccharide as the acceptor of glycosyltransferase or the substrate of glycosidase [28–31]. The PA-oligosaccharide and PA-monosaccharide thus obtained were analysed by h.p.l.c. for the measurement of enzyme activity. The application of pyridylation for assay purposes, in which an oligosaccharide is liberated by action on carbohydrate–protein linkages such as those in endo-GalNAc-ase-S, is presented in this paper for the first time. Compared with the Elson–Morgan method [15], ours is more reliable and more sensitive for assaying endo-GalNAc-ase-S activity, and thus may facilitate the discovery of new enzymes related to glycoproteins. A crude enzyme preparation obtained by 80 % (w/v) ammonium sulphate precipitation of culture fluid was first subjected to gel chromatofocusing. From this, the recovery of endo-GalNAc-ase-S and removal of the main exoglycosidase were conducted efficiently, and the apparent isoelectric point of the former was determined. The results for this presented in Fig. 2 show at least two peaks of enzyme activity. One major isoelectric point of endo-GalNAc-ase-S was at pI 4.8, while weaker activity was apparent at pI 5.2. The components of endo-GalNAc-ase-S giving rise to these two isoelectric points could not be identified in this study. The isoelectric point of the main component of endo-GalNAc-ase-S, pI 4.8, was essentially the same as that reported for endo-GalNAc-ase-A and that of the minor component of endo-GalNAc-ase-D [8–11]. A fraction having the activity of endo-GalNAc-ase-S was applied on to a DEAE-Toyopearl column, and elution was efficiently carried out since endo-GalNAc-ase-S separated completely from exo-glycosidase. Endo-GalNAc-ase-S eluted from DEAE-Toyopearl at around 0.19–0.25 M-NaCl, and endo-GalNAc-ase-D and -A were eluted from DEAE-Sephadex A-25 at around 0.12–0.13 M-NaCl and from DEAE-Sephadex A-50 at 0.45 M-NaCl [8,11].

The method for examining the properties of endo-GalNAc-ase-S was found useful for clarifying in a simple manner those of the present enzyme. After the enzyme was incubated with [3 H]PGM, the radioactivity in the supernatant after ethanol precipitation was counted and the optimum pH and molecular mass were measured. This pH, 5.5, was close to the optimum of endo-GalNAc-ase-A but different from that of endo-GalNAc-ase-D, pH 6–pH 8 [8–11]. However, an accurate comparison is not possible because of variation in this parameter, according to the substrate used and the incubation time. The optimum pH of endo-GalNAc-ase-A has been shown to change when there is a different amino acid in the protein portion linked to the oligosaccharide of a dansylated glycopeptide, such as Gal-GalNAc-Ser or Gal-GalNAc-Thr [32]. The molecular mass of endo-GalNAc-ase-S is 105 kDa, which is less than that of endo-GalNAc-ase-A or -D, 160 kDa [8–11]. This may make the enzyme more accessible to the carbohydrate–protein linkage of PGM than endo-GalNAc-ase-A and -D, with consequent release

of oligosaccharide chains that are not liberated by endo-GalNAc-ase-A or -D.

To determine the substrate specificity of endo-GalNAc-ase-S, bovine fetuin, the oligosaccharide structures of which have already been determined, was used as the substrate. The oligosaccharide structures and molar ratios of O-linked carbohydrates of bovine fetuin have been determined precisely. Each molecule has two oligosaccharide chains, and the molar ratios of the disaccharide (Gal β 1-3GalNAc) and tetrasaccharide [Gal β 1-3(Gal β 1-4GlcNAc β 1-6)GalNAc] were estimated as 2.85 and 0.15 respectively [25]. Different amino acids linked to each oligosaccharide of bovine fetuin have been studied and the disaccharide was shown to be linked to serine or threonine, while the tetrasaccharide was linked to serine [26,33]. A comparison was made of the following reaction products: [3 H]asialofetuin treated with alkaline sodium borohydride and [3 H]asialofetuin incubated with endo-GalNAc-ase-A and -S. The incubation of [3 H]asialofetuin with endo-GalNAc-ase-S caused the liberation not only of disaccharide (Gal β 1-3GalNAc) but also tetrasaccharide [Gal β 1-3(Gal β 1-4GlcNAc β 1-6)GalNAc]. Different components were liberated by endo-GalNAc-ase-A, whose substrate specificity has been determined [11]. The amount of tetrasaccharide liberated was larger than that of disaccharide, and the disaccharide has been shown to be present in a greater amount in [3 H]asialofetuin than the tetrasaccharide. Endo-GalNAc-ase-S may perhaps preferentially liberate oligosaccharide linked to serine, rather than to threonine. Endo-GalNAc-ase-S liberates more oligosaccharides that are linked to serine from glycopeptides that were prepared from PGM than those linked to threonine, as indicated by the results of a preliminary experiment (results not shown). Endo-GalNAc-ase-S may recognize not only the glycon of substrates but aglycon amino acids as well. Endo-GalNAc-ase-A liberates oligosaccharide chains from both dansyl (DNS)-Ser-linked and DNS-Thr-linked glycosides more easily from the former than the latter. DNS-Ser-GalNAc-Gal and DNS-Thr-GalNAc-Gal showed K_m values of 0.17 and 1.43 mM respectively. Their optimum pHs also differ [32].

Endo-GalNAc-ase was found to be present in the culture fluid of *Streptomyces* sp. OH-11242 from which both a disaccharide (Gal β 1-3GalNAc) and oligosaccharides of larger size were liberated. The crude enzyme preparation was purified until exoglycosidase could no longer be detected. Parameters such as molecular mass and optimum pH were determined, and substrate specificity was assessed with both 3 H-labelled and unlabelled asialofetuin as substrate. Other properties of endo-GalNAc-ase-S, such as its behaviour toward various compounds, need to be studied in the future and a more precise determination of substrate specificity made. This enzyme should prove useful for clarifying the functions of the oligosaccharide chains of O-glycosidic-linkage glycoproteins and should find application in the biosynthesis of compounds whose molecules are quite large, such as mucin.

We are grateful to Dr. Kazuhiko Ishihara, Dr. Yukinobu Goso and Dr. Susumu Ohara for their valuable comments. We are indebted to Miss Tsukiko Urata for her competent technical assistance. This work was supported in part by a grant for special research from Kitasato University, School of Medicine, No. 83324, by a Grant-in-Aid from the Japanese Ministry of Education, Science and Culture and a grant from the TERUMO Life Science Foundation.

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Received 16 December 1991/5 May 1992; accepted 22 May 1992