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RELEASE OF OLIGOSACCHARIDES POSSESSING REDUCING-END N-ACETYLGALACTOSAMINE FROM MUCUS GLYCOPROTEIN IN STREPTOMYCES SP. OH-11242 CULTURE MEDIUM THROUGH ACTION OF ENDO-TYPE GLYCOSIDASE

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A crude enzyme preparation from a culture medium of Streptomyces sp. OH-11242 contained endo-α-N-acetylgalactosaminidase activity. The activity could be induced by the addition of purified porcine gastric mucin to the culture medium. Oligosaccharides corresponding to approximately 2-14 glucose units were detected in the culture medium and also in an incubated reaction mixture of crude enzyme preparation and mucus glycoprotein. The resulting product with N-acetylgalactosamine at the reducing terminal implied the presence of a new type of endo-glycosidase liberating not only Galβ1-3GalNAc but also other larger oligosaccharides by hydrolysis of the O-glycosidic linkage between GalNAc and Ser (Thr). © 1988 Academic Press, Inc.

Mucus glycoproteins, or mucins, are important components which protect tissues and cells from various attacking agents(1,2). Oligosaccharides in mucus glycoproteins are bound to serine (or threonine) by their reducing terminal α-N-acetylgalactosamine residues(1,3,4,5). Although the linkage is cleaved chemically by weak alkalinity through the well known β-elimination reaction, enzymatical cleavage of the linkage should greatly expedite structural studies on the protein part of mucus glycoprotein and investigation of the nature and functions of cell surface O-linked complex carbohydrates. An endo-α-N-acetylgalactosaminidase termed O-glycanase from Diplococcus pneumoniae is known to hydrolyze this linkage(7,8). This enzyme with strict substrate specificity hydrolytically cleaves the α-N-acetylgalactosaminyl linkage of only Galβ1-3GalNAcα1-Ser (or Thr) in both glycopeptides and glycoproteins and liberates the disaccharide moiety. Due to its narrow substrate specificity, application of the enzyme to analysis of natural substances is restricted.

The present report demonstrates for the first time the presence of a new type of endoglycosidase in a culture medium of a strain of Streptomyces which can cleave the innermost α-N-acetylgalactosaminyl linkage and liberate oligosaccharides larger than the disaccharide from porcine gastric mucus glycoprotein.

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MATERIALS AND METHODS

Chemicals---The following materials were purchased from the sources indicated: Sephadex G-10, Pharmacia; Bio-Gel P-4 (minus 400 mesh), Bio-Rad; sodium cyanoborohydride, Aldrich; 2-aminopyridine, Wako Pure Chemical Industries; porcine gastric mucin (Type II) and pepsin, Sigma Chemical Co.. Glucose oligomers used as the standard for thin-layer chromatography (TLC), Bio-Gel P-4 chromatography and Sephadex G-25 chromatography were prepared by acid hydrolysis of dextran (9).

Growth of Streptomyces sp. OH-11242--- Streptomyces sp. OH-11242, isolated from a land soil was used to produce the glycosidases described below. Spores and mycelia of strain OH-11242 grown on an agar slant were inoculated into a 500-ml Sakaguchi flask containing 100 ml of seed medium that was consisted of 2 % glucose, 0.5 % peptone, 0.5 % meat extract, 0.3 % dry yeast cells, 0.5 % NaCl, and 0.3 % $CaCO_3$, pH 7.0. This was followed by incubation at 27°C for two days. The seed culture (4 ml) thus obtained was transferred to 500-ml Sakaguchi flasks each containing 100 ml of production medium consisting of that was 0.26 % ammonium sulfate, 0.24 % KH_2PO_4, 0.43 % K_2HPO_4, 0.10 % $MgSO_4 \cdot 7H_2O$, 0.1 % (v/v) trace salt solution and 0.5 g porcine gastric mucin, pH 7.0. The trace metal solution, pH 7.0, adjusted by NaOH, consisted of 0.64 % $CuSO_4 \cdot 5H_2O$, 0.11 % $FeSO_4 \cdot 7H_2O$, 0.79 % $MnCl_2 \cdot 4H_2O$, 0.15 % $ZnSO_4 \cdot 7H_2O$ and distilled water. The flasks were incubated at 27°C for 3 days with reciprocal shaking (120 strokes/min).

High performance liquid chromatography---To obtain the pyridylamino (PA) derivative by reversed-phase column chromatography, a Jasco TRI ROTAR chromatograph provided with a Jasco VL-614 injector and Hitachi F-1000 fluorescence spectrophotometer was used. PA-sugars were separated by a reversed-phase column (4.6 X 250 mm, Ultrasphere-ODS, 5 μm, Beckman Instruments, Inc.) using a 0.25 M sodium citrate buffer, pH 4.0, containing 1 % acetonitrile, at a flow rate of 0.5 ml/min. For the gel filtration of PA-oligosaccharide, a Hitachi 655A-12 liquid chromatograph equipped with a Rheodyne model 7125 injector and a Jasco FP-210 spectrofluorometer was used. PA-sugars were separated on a TSK-GEL G2000PW column (10 μm, 7.5 X 600 mm, Toyo Soda Manufacturing Co., Ltd.), using a 20 mM ammonium acetate buffer, pH 7.5, at a flow rate of 0.5 ml/min. For detection of PA-sugar, an excitation wavelength of 320 nm and emission wavelength of 400 nm were used. All chromatography was performed at room temperature.

Preparation of oligosaccharides from the culture medium---The culture fluid was dialyzed against distilled water and the dialyzate concentrated by a rotary evaporator. The soluble part of the dialyzable material in 0.1 M acetic acid was applied onto Sephadex G-25 column (2.3 x 100 cm) equilibrated with 0.1 M acetic acid. The fractions corresponding to 6-14 glucose oligomers (from 261 ml to 291 ml) were collected and further analysis of the oligosaccharide fraction on TLC was carried out according to the method of Holmes et al.(10).

Analysis of the reducing terminal sugar of oligosaccharide by pyridylamination and HPLC --- Oligosaccharide components were reductively aminated with 2-aminopyridine by sodium cyanoborohydride. The pyridylamino derivatives of oligosaccharides thus prepared were purified by gel filtration on a Sephadex G-10 column (11). The reducing end sugar of the purified oligosaccharide by TLC was determined as follows. Pyridylaminated oligosaccharide was hydrolyzed with 6 N HCl at 100°C for 6 h in an evacuated sealed tube and the resultant pyridylamino monosaccharides were analyzed by HPLC after their amino groups had been reacetylated as described previously (11). To determine the carbohydrate composition of the oligosaccharide, monosaccharides in its hydrolyzate were separated from the pyridylaminated reducing end, GalNAc, by a Dowex 50W x 2 (H^+ form) and then coupled with 2-aminopyridine. The resultant pyridylaminated monosaccharide mixture was analyzed by HPLC as above.

Preparation of purified porcine gastric mucin--- 100 g of porcine gastric mucin were made up to a final volume of 500 ml by the addition of 0.001 % NaN_3 and the pH was adjusted to 2.5 with conc HCl. One gram of

pepsin was added to this suspension followed by incubation at 37 °C with slow shaking. After 24 h and 48 h, an additional 1 g of pepsin was added, and digestion was continued up to 72 h. At the end of incubation, the reaction mixture was neutralized by adding NaOH followed by centrifugation at 10,000 rpm for 30 min. To remove the mucopolysaccharide, 5 g of cetyl pyridinium chloride (CPC) were dissolved in the reaction mixture which had been maintained overnight at room temperature. The insoluble complex formed with CPC was removed by centrifugation and the clear supernatant thus obtained was throughly dialyzed against tap water. The solution remaining in the dialysis bag was then subjected to ethanol fractionation. After removing the insoluble material formed at a 33 % (v/v) concentration of ethanol, ethanol was added to a concentration of 50 %. The precipitate produced during overnight at 4 °C, was collected by centrifugation at 10,000 rpm for 60 min. The resultant colorless powder, weighing 31 g after lyophilization, was used as the source of purified mucin for subsequent experiments.

Preparation of crude enzyme from culture fluid --- The culture fluid of OH-11242 was concentrated by lyophilization and, following its dialysis, the precipitate thus produced was removed by centrifugation at 10,000 rpm for 20 min. The supernatant was brought to 80% saturation by adding solid ammonium sulfate with stirring and the resulting precipitate was collected by centrifugation. The precipitate was dissolved in a minimum amount of water and dialyzed against distilled water. The crude enzyme preparation could be stored at 4 °C for at least several weeks without loss of endo-glycosidase activity. The enzyme preparation contained no protease activity. It showed β-N-acetyl glucosaminidase, α-N-acetyl galactosaminidase, α-galactosidase, β-galactosidase and α-fucosidase activity toward p-nitrophenyl derivatives and purified porcine gastric mucin.

Method of enzyme assay---To determine endo-glycosidase activity, 100 mg of a purified porcine gastric mucin were incubated with 5 ml of crude enzyme preparation in 1 ml of 0.5 M citrate phosphate buffer, pH 5.0, at 37 °C for 24 h. The reaction mixture was boiled at 100 °C for 5 min and dialyzed against distilled water. The dialyzate was concentrated by a rotary evaporator and the low-molecular-weight products were analyzed by Bio-Gel P-4 chromatography, TLC and HPLC. Bio-Gel P-4 (minus 400 mesh) chromatography was conducted using a water-jacketed thick-wall column (1.6 x 100 cm) prewarmed at 55 °C, and equilibrated with deaerated distilled water. The analytical methods of TLC and HPLC are described above.

RESULTS AND DISCUSSION

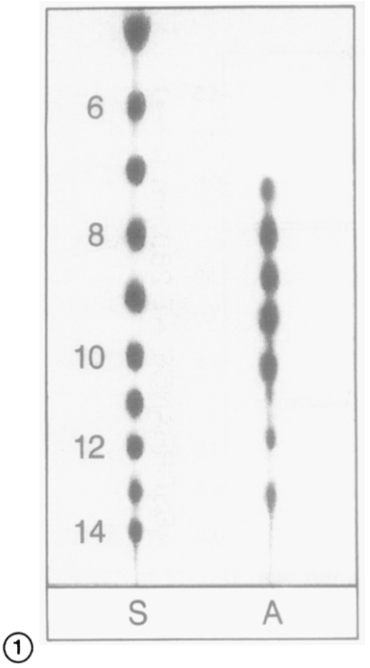
A partially purified preparation of endo-glycosidase was obtained from the culture fluid of Streptomyces sp. OH-11242 as described in "Materials and Methods". This endo-glycosidase preparation is considered to cleaved the peptide carbohydrate linkage in porcine gastric mucin (abbreviated as PGM) as follows.

(oligosaccharide)-GalNAc-Ser or Thr(peptide)

$$\longrightarrow \text{(oligosaccharide)-GalNAc + Ser or Thr(peptide)}$$

This conclusion is based on the following experimental facts.

Presence of oligosaccharides possessing the reducing-end GalNAc residue in the culture fluid of OH-11242---The size of oligosaccharides prepared from the culture fluid of OH-11242 corresponded to 7-14 glucose oligomers on TLC (Fig.1). Analysis of the reducing end sugar of the oligosaccharides



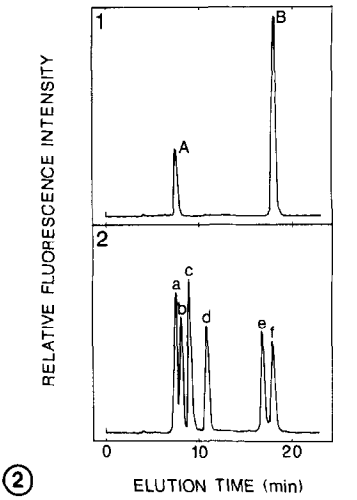


Fig. 1. Thin-layer chromatogram of oligosaccharides in the culture fluid of OH-11242. They were purified by Sephadex G-25 column chromatography, and applied on to a silica gel 60 plate. The plate was developed at room temperature for 20 h using the following solvent system: n-propanol/acetic acid/water(3:3:2,v/v). Oligosaccharides were visualized with orcinol-H_2SO_4 reagent. S, mixture of standard glucose oligomers (vertical numbers indicate glucose units); A, oligosaccharides prepared from the culture fluid of OH-11242.

Fig. 2. HPLC profile of the reducing end sugar prepared from the pyridylamino derivative of oligosaccharides in the culture fluid of OH-11242. PA-sugars were separated on a reversed-phase column as described in "Materials and Methods". (1) PA-sugars derived from PA-oligosaccharides. (2) Standard PA-sugars prepared from commercial source; a,PA-Gal ; b,PA-Glc ; c,PA-Man and PA-Xyl ; d,PA-Fuc ; e,PA-GlcNAc ; f,PA-GalNAc.

was carried out by HPLC as described in "Materials and Methods". The recovery of reducing end sugars as PA-sugars from the PA-oligosaccharide during the treatment was about 80 %. As evident from Fig.2, peaks A and B derived from PA-oligosaccharide corresponded to standard PA-Gal and PA-GalNAc, respectively, and no other PA-sugars could be detected. Thus, the reducing end sugar of oligosaccharides in the culture fluid of OH-11242 was concluded to be Gal or GalNAc. The estimated relative content of PA-Gal and PA-GalNAc was 20 % and 80 %, respectively. Actually, these oligosaccharides may be considered to be produced from PGM by the action of endo-α-N-acetylgalactosaminidase and/or endo-β-galactosidase in the culture fluid of OH-11242.

Detection of endo-α-N-acetylgalactosaminidase activity in the culture fluid of OH-11242 ---PGM was treated with a crude enzyme preparation and oligosaccharide thus produced was analyzed by Bio-Gel P-4 column chromatography as described in "Materials and Methods". Fig.3-1 shows a

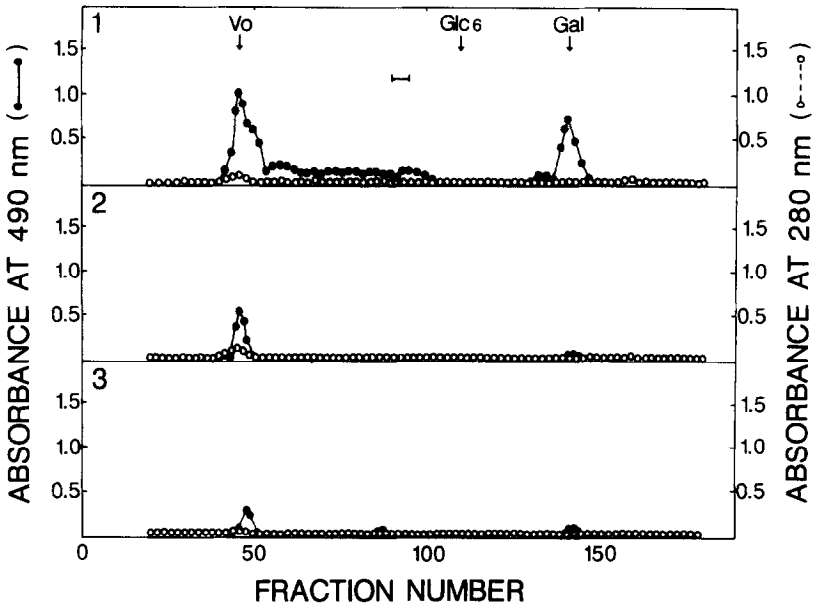


Fig. 3. Bio-Gel P-4 column chromatography of oligosaccharides produced by action of the crude enzyme on PGM. The column (1.6 x 100 cm) was eluted under the conditions described in "Materials and Methods" and 1.2 ml fractions were collected.

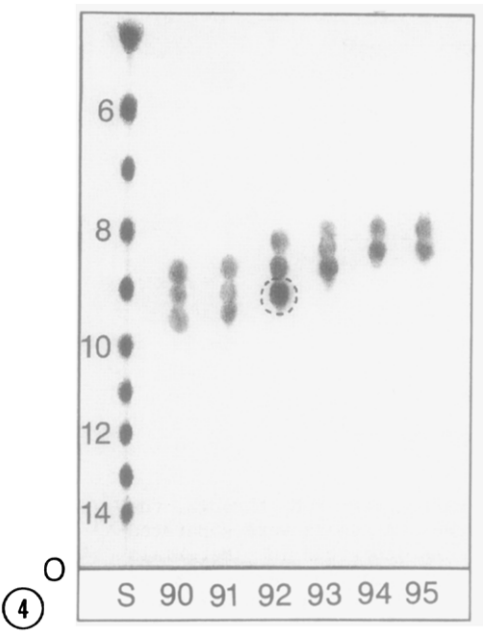
1 The oligosaccharide from PGM treated with endoglycosidase as described in "Materials and Methods".

2 The oligosaccharide from PGM treated with the boiled enzyme.

3 The oligosaccharide from incubated endo-glycosidase without PGM.

The fractions were analyzed for ultraviolet absorbance at 280 nm and by phenol-sulfuric acid reaction for hexose. Void volume (Vo) and elution positions of standard malto-oligosaccharide are indicated by arrows. The solid bar indicates pooled fractions, used for subsequent experiments.

typical elution profile of oligosaccharides of various sizes, liberated along with monosaccharide. Oligosaccharides larger than maltohexaose were particularly abundant. When PGM was incubated with the boiled enzyme, a very small peak appeared at the void volume position as shown in Fig. 3-2. This peak may possibly have arisen from PGM that leaked during dialysis. A negligible small peak at the void volume position (Fig. 3-3) was detected in the incubation mixture of the enzyme itself. The oligosaccharides fractionated on Bio-Gel P-4 column chromatography (Fig.3-1) were further analyzed by thin-layer chromatography (Fig. 4). An oligosaccharide enclosed by a dotted line on the chromatogram was extracted with distilled water. Analysis of the reducing end sugar and sugar composition of the oligosaccharide component was conducted as described in "Materials and Methods". The reducing end sugar of the oligosaccharide was determined as GalNAc (Fig.5); the oligosaccharide contained GalNAc, GlcNAc, Gal and Fuc in the ratio, 1:3:3:2. It is thus evident that the crude enzyme preparation contains an endo-α-N-acetylgalactosaminidase capable of liberating a nona-saccharide with N-acetylgalactosamine at the reducing terminal from PGM. An oligosaccharide structure having the same sugar composition as the



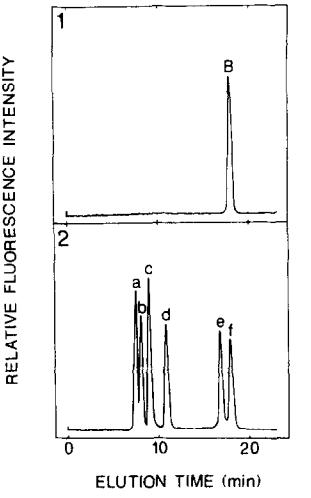


Fig. 4. Thin-layer chromatogram of released oligosaccharides from PGM by OH-11242 crude enzyme. Each fraction on Bio-Gel P-4 column chromatography (position indicated by the solid bar in Fig.3-1) was concentrated separately by a rotary evaporator. Developmental conditions for TLC were the same as those described in Fig.1. S, mixture of standard glucose oligomers (vertical numbers indicate glucose units). The numbers under the origin (O in the figure) are fraction numbers on the Bio-Gel P-4 column chromatography. The oligosaccharide extracted from spot indicated by the dotted circle was used for subsequent experiments.

Fig. 5. HPLC profile of PA-sugar derived from pyridylaminated oligosaccharide. Conditions for HPLC analyses were the same as those in Fig. 2. (1) PA-sugar derived from the reducing terminal of the oligosaccharide purified by TLC (Fig. 4). (2) Standard PA-sugars derived from a commercial source (Fig. 2).

above has recently been reported for gastric mucin from porcine and human (12,13).

Comparison of oligosaccharides released from PGM by the action of OH-11242 enzyme and commercial O-glycanase (endo-α-N-acetylgalactosaminidase from Diplococcus pneumoniae)---When PGM was digested with the OH-11242 enzyme preparation, oligosaccharides corresponding to malto-hexaose were observed to be liberated on a Bio-Gel P-4 column (Fig. 3-1). HPLC analysis of the pyridylamino derivative of the oligosaccharides also showed oligosaccharides with molecular weight at least higher than that of maltopentaose to be present(Fig.6).

On digesting PGM with O-glycanase, the pyridylamino derivative of the liberated oligosaccharide appeared as a single peak on HPLC. Its enzyme specificity and elution position on HPLC clearly indicated this component to be the disaccharide, Galβ1-3GalNAc.

In summary, it is clear that the crude enzyme preparation from Streptomyces sp. OH-11242 culture medium contains a new type of

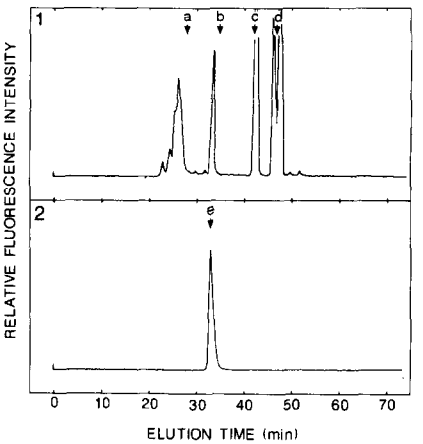


Fig. 6. Comparison of oligosaccharides released from PGM through the action of OH-11242 crude enzyme and O-Glycanase. PA-sugars were separated on a TSK-GEL G2000PW column under the conditions described in "Materials and Methods". (1) HPLC profile of the pyridylamino derivative of oligosaccharide from PGM digested with the OH-11242 enzyme preparation. (2) HPLC profile of the pyridylamino derivative of oligosaccharide from PGM digested with O-Glycanase. Elution positions of standard PA-sugars are indicated by arrows; a,PA-Glc_5; b,PA-Glc_2; c,PA-GalNAc; d,PA-Gal; e,PA-Galβ1-3GalNAc.

endo-glycosidase capable of hydrolyzing the GalNAc-Ser(Thr) linkage, resulting in the liberation of a oligosaccharide larger than disaccharide. Purification of the endoglycosidase and clarification of its properties such as pH optimum and exact substrate specificity are now being pursued.

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