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RELEASE OF T-ANTIGEN, A CARCINOMA MARKER FROM NATIVE HUMAN CELLS, BY ENDO-α-N-ACETYLGALACTOSAMINIDASE OF ALCALIGENES SP.

Jian-Qiang FAN[#], Kazushi KANATANI[*], Nobushi ANDOH[*], Kenji YAMAMOTO, Hidehiko KUMAGAI and Tatsurokuro TOCHIKURA

Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, JAPAN

[*] Japan Immunoresearch Laboratories, 17-5 Sakaemachi, Takasaki 370, JAPAN

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Summary: The endo-α-N-acetylgalactosaminidase from Alcaligenes sp. could release T-antigen (Galβ1→3GalNAc), a carcinoma-associated marker from asialo glycophorin and human cells. The released T-antigen from human asialo erythrocytes was determined by thin layer chromatography and gas liquid chromatography. The released T-antigen from human gastric carcinoma cell Kato III was identified by high performance liquid chromatographies with a reverse-phase column and a size fractionation column. Released T-antigen could be analyzed quantitatively by a sensitive method including pyridylamino derivatization and following high performance liquid chromatography. This suggests that the enzyme is useful for detection and determination of T-antigen from cells.

Glycosidases are considered to be useful tools for elucidation of the structures and functions of cell surface carbohydrates, because they can liberate the sugars or sugar chains from cell surface glycoproteins or glycolipids without strong damage to cells (1). Cell surface carbohydrates are very complex in length and type, and the glycosidases usually show much less activity on native cells than on pure glycoproteins, or do not show the activity at all on cells (2,3).

Previously (4,5), we reported an endo-α-N-acetylgalactosaminidase isolated from an Alcaligenes sp. with high enzyme activity on various glycoproteins or glycopeptides. It releases a disaccharide, Galβ1→3GalNAc, which is contained in most O-glycoside sugar chains as a core structure. This disaccharide is also determined on the cell surface as a Thomsen-Friedenreich an-

[#] To whom correspondence should be addressed.

Abbreviations used: PA, pyridylamino; GalNAc, N-acetylgalactosamine; Gal, galactose; GalN, galactosamine; NeuAc, N-acetylneuraminic acid; PNA, peanut agglutinin; HPLC, high performance liquid chromatography.

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tigen (T-antigen) immunodeterminant group, which has been proposed as a specific carcinoma marker (6), because the T-antigen is found to exist only on the surface of many carcinomas as an unmasked form. Recently, we developed a sensitive method for detection and determination of an O-linked disaccharide, Galβ1→3GalNAc by a combination of endo-α-N-acetylgalactosaminidase and HPLC.[1] Therefore, investigation on the release of T-antigen from native cells by the enzyme is valuable and significant for microanalysis of cell malignant alteration. In this communication, we describe the reaction of releasing T-antigen from human erythrocytes and native human gastric carcinoma cells (Kato III) by endo-α-N-acetylgalactosaminidase of Alcaligenes sp.

MATERIALS AND METHODS

Materials. Glycophorin was prepared from outdated human red cells according to the method of Marchesi et al (7). Peanut agglutinin (PNA) lectin and PNA-agarose were purchased from Hohnen Oil Co. Ltd. A standard disaccharide, Galβ1→3GalNAc, was prepared from asialofetuin by the method will be reported elsewhere (8). Neuraminidase of Arthrobacter ureafaciens was obtained from the Marukin Soy Sauce Co., and endo-α-N-acetylgalactosaminidase of Alcaligenes sp. was purified according to the method reported previously (9). Kato III human gastric cancer cells were cultured on RPMI-1640 (Mazleton Biologics. Inc. KS., USA) supplemented with 10% fetal bovine serum (Microbiol. Assoc., Md., USA), under 5% CO_2 at 37°C for 3 days. Other reagents were from commercially available products.

Enzymic Reaction. Before use, the endo-α-N-acetylgalactosaminidase and neuraminidase were dialyzed against 10mM phosphate-buffered saline (pH6.0). For releasing disaccharide, the standard enzymic reaction was carried out in 1ml of the saline described above containing 15% fresh human red cells, 100mU neuraminidase and 11.4mU endo-α-N-acetylgalactosaminidase, or 0.5ml of the saline containing 3.6 x 10^7 Kato III cells and 11.4mU endo-α-N-acetylgalactosaminidase at 37°C. After approximate time, the supernatant of reaction mixture was obtained by centrifugation at 1000 rpm for 5min.

Determination of Disaccharide by Pyridylaminated Method. For determination of the released disaccharide, a pyridylaminated method was used according to the method of Kondo et al (10). To the supernatant of reaction mixture, 9-volumes of ethanol were added. After kept at -20°C for 3hr, the fraction containing the disaccharide was obtained by centrifugation at 3000 rpm for 5min, followed by evaporation to dryness. This procedure was repeated several times to remove impurities (proteins, salts etc.) as much as possible. The dried sample was mixed with 10μl of 2-aminopyridine (2.76g/ml acetic acid) and heated at 90°C for 60min, followed by the addition of 10μl of 20% dimethylamine-borane in acetic acid at 80°C for 50min. To remove the excess reagent, the reaction mixture was diluted with 0.3ml of water and applied to a Sephadex G-10 column (1 x 50cm) equilibrated with 10mM ammonium acetate buffer (pH6.0). The fractions containing Pa-disaccharide were combined for determination with HPLCs.

HPLC Analysis. A liquid chromatograph (Hitachi model L-6200) equipped with a fluorescence spectrophotometer (Hitachi model F-1050) and a Rheodyne model 7125 injector was used. The pyridylamino-derivatives were separated using a reversed-phase column (4.6 x 250mm, Ultrasphere-ODS, 5μm, Beckman Instruments,

[1] Details will be reported in elsewhere by J.-Q. Fan et al.

Inc.) with 25mM citrate buffer (pH5.4), containing 1.0% acetonitrile, or a size-fractionation column (4.6 x 250mm, TSK-GEL Amido-80, Tosoh Co.) with 3% acetic acid-triethylamine (pH7.3), containing 80% acetonitrile, at a flow rate of 1.0ml/min. For detection, an excitation wavelength of 310nm and an emission wavelength of 380nm were used. The temperature of the column was kept at 40°C for ODS and 70°C for Amido-80, respectively.

Thin Layer Chromatography (TLC). Thin layer chromatography was performed with the following solvent: Isopropyl alcohol-pyridine-H_2O-acetic acid (8:8:4:1). The spots were detected by a diphenylamine-aniline reagent (11).

Gas Liquid Chromatography. After hydrolysis with 4M trifluoroacetic acid at 100°C for 4h, the monosaccharides were determined as the trimethylsilyl derivatives by a gas liquid chromatography spectrometer with a glass column (3 x 2000mm) of 3% SE-52 on chromosorb W (80-100 mesh). A flame ionization detector was used for the analysis.

RESULTS AND DISCUSSION

Release of Disaccharide, Galβ1→3GalNAc from Asialo Glycophorin. Glycophorin is a major glycoprotein on human erythrocyte membrane, and contains 60% carbohydrate and 40% protein (12). The carbohydrates were determined as 15 O-linked sugar chains, a tetrasaccharide of NeuAcα2→3Galβ1→3(NeuAcα2→6) GalNAc, and 1 N-linked sugar chain (13). The Alcaligenes endo-α-N-acetylgalactosaminidase could act on the O-linked sugar chains of glycophorin, after sialic acid release (5). We showed here that after treatment with endo-α-N-acetylgalactosaminidase, the asialo glycophorin could not bind to a PNA-agarose column (Fig. 1), which was reported to selectively recognize the disaccharide, Galβ1→3GalNAc (14). This indicates that most of the disaccharides contained in asialo glycophorin were released. This was also confirmed by comparing the amount of released disaccharide and total disaccharide in asialo glycophorin, which was determined by gas chromatography, after acid hydrolysis (data not shown). The Michaelis constant (Km) for asialo glycophorin was observed to be 0.32mM. This value is 10-times lower, in comparison with those

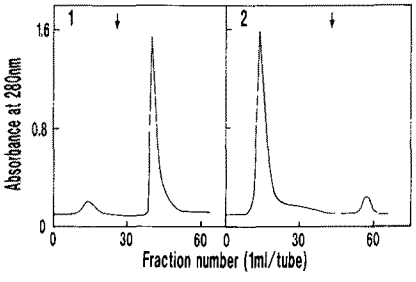

Fig. 1. Affinity Chromatography of Asialo Glycophorin on PNA-agarose Column. Sixteen mg of asialo glycophorin before (1) and after (2) treatment with 50mU endo-α-N-acetylgalactosaminidase in 1ml of 10mM citrate buffer (pH4.5) at 37°C for 12hr was applied to a column of PNA-agarose (1 x 15 cm) equilibrated with 50mM potassium-phosphate buffer (pH6.0). After washing with the same buffer, the absorbed asialo glycophorin was eluted with the buffer containing 0.1M galactose. The arrows indicate the position of buffer change.

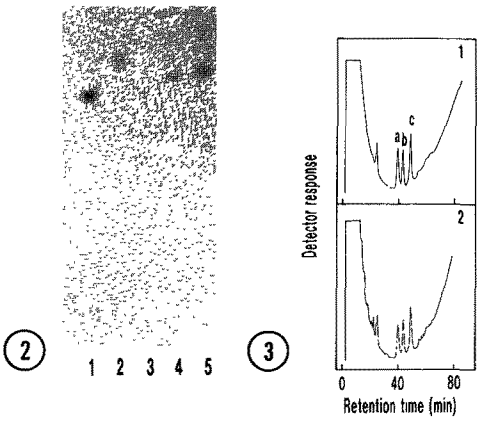

Fig. 2. Thin Layer Chromatography of Released Disaccharide of Human Erythrocytes.
The enzymic reaction and purification procedures were carried out as described under MATERIALS AND METHODS. Lane 1, Gal; lane 2, GalNAc; lane 3, from reaction mixture without the enzyme; lane 4, from reaction mixture with the enzyme; and lane 5, standard disaccharide, Galβ1→3GalNAc.

Fig. 3. Gas Liquid Chromatograms of Hydrolyzates of Oligosaccharide Released from Human Erythrocytes and Standard Disaccharide.
The temperature program used was 170°C for 4min, followed by a temperature increase at a rate of 0.5°C/min to 210°C and maintaining the temperature at 210°C for 1min. a, assigned to GalN, and b, c , assigned to Gal were conformed by another respective run of Gal and GalN at the same condition.
1, standard disaccharide; 2, oligosaccharide released from human erythrocytes by endo-α-N-acetylgalactosaminidase.

for asialofetuin (3.7mM) and asialo κ-casein glycopeptide (3.2mM) (5), which shows that the Alcaligenes endo-α-N-acetylgalactosaminidase has higher affinity to asialo glycophorin.

Release of T-antigen from Human Erythrocytes. Since the enzyme could release the disaccharide from asialo glycophorin with high potency, its action on human erythrocytes was tested further.

The hemagglutinating activities of PNA, a T-antigen recognizing lectin, for native human blood cells, neuraminidase treated blood cells, and neuraminidase and endo-α-N-acetylgalactosaminidase treated blood cells were compared. Since T-antigen on native erythrocytes was masked by two sialic acids, the native cells could not be agglutinated by PNA at any concentration of PNA. The neuraminidase treated cells were strongly agglutinated, and by subsequent treatment with endo-α-N-acetylgalactosaminidase the agglutination was mitigated but could not be dissolved. This suggests that the endo-α-N-acetylgalactosaminidase partially liberated the T-antigen from human erythrocytes under this reaction condition.

To confirm the T-antigen release, the following procedures were carried out. The enzymic reaction mixture which was obtained by the method described under MATERIALS AND METHODS was subjected to thin layer chromatography. As

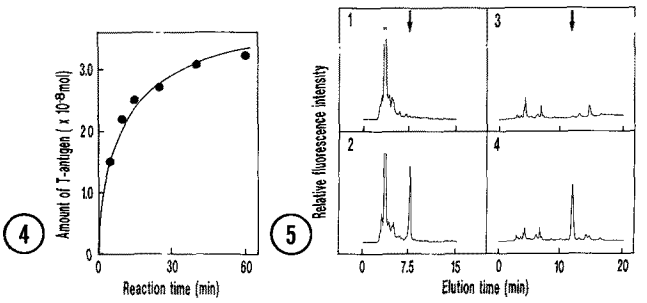

Fig. 4. Release of T-antigen from Human Erythrocytes by Endo-α-N-acetylgalactosaminidase.
The enzyme reactions were carried out as described under MATERIALS AND METHODS. The released T-antigen was determined on reverse-phase HPLC as its pyridylamino-derivative.

Fig. 5. Determination of Released T-antigen of Kato III on HPLCs.
Enzymic release and purification were performed according to the method as shown in the text except that 5 x 10^7 cells were used. The released T-antigen was pyridylaminated and determined on a size-fractionation HPLC (1, 2) and a reverse-phase HPLC (3, 4). 1 and 3, reaction mixture without the enzyme; 2 and 4, reaction mixture with the enzyme. The arrows show the position of PA-disaccharide elution.

shown in Fig. 2, a carbohydrate spot at the Rf value similar to that of the standard disaccharide, Galβ1→3GalNAc was found in the reaction mixture incubated with endo-α-N-acetylgalactosaminidase (lane 4), but not in the reaction mixture without the enzyme (lane 3). This spot could be detected by Morgan-Elson reagent (15) (data not shown), which indicated that the carbohydrate has an N-acetyl amino sugar at the reducing end. The components of carbohydrate released from erythrocyte were also determined by gas liquid chromatography (Fig. 3). The profile of gas liquid chromatography was found to be very similar to that of standard disaccharide, which demonstrates that the released carbohydrate contains galactose and N-acetylgalactosamine (the ratio of this two sugars was determined as 1:1.16). These findings indicate that the endo-α-N-acetylgalactosaminidase could release T-antigen from erythrocytes after neuraminidase treatment. Fig. 4 shows the time course of the T-

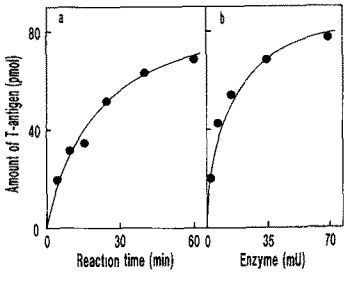

Fig. 6. Release of T-antigen from Kato III by Endo-α-N-acetylgalactosaminidase.
All experimental procedures were done as indicated under MATERIALS AND METHODS except: a, 14mU enzyme was used; and b, the reaction time was 30min.

antigen release by endo-α-N-acetylgalactosaminidase. After 1-hr reaction, approximately 30nmol of disaccharide was liberated, which suggests that the enzyme acts not only on glycoprotein but also on erythrocytes with high potency.

Release of T-antigen from Native Human Gastric Cancer Cell Kato III. Since the T-antigen was found to be present on many carcinomas, such as lymphoma, breast carcinoma and gastric cancer (16-18), and are scarcely expressed on normal human cells (19), it was proposed that it is useful as a carcinoma-associated marker. Therefore, if the endo-α-N-acetylgalactosaminidase could release the T-antigen, the enzyme could be considered to be useful for judgment of emerging clinical cancer, because the enzymic released disaccharide (an epitope of T-antigen) has an aldehyde residue in the reducing end and is detectable quantitatively in a pico-level assay.

Kato III, human gastric carcinoma cell line which was reported to contain peanut agglutinins receptor, T-antigen (18), was used in this investigation. The carbohydrates produced in the enzymic reaction mixture were derived with pyridylamino reagent by the method described in MATERIALS AND METHODS. As shown in Fig. 5, after treatment with the enzyme, a new PA-derivative of carbohydrate was appeared. This PA-derivative was found to be eluted at the same position as the pyridylamino-disaccharide standard on reverse-phase HPLC and size fractionation HPLC. Since Tomiya et al proposed that these two HPLCs could be used for structural analysis of the released oligosaccharides (20), we decided that this PA-carbohydrate was a PA-disaccharide. This means that the endo-α-N-acetylgalactosaminidase can liberate the T-antigen from native Kato III cells. The amounts of PA-disaccharide increased with the reaction time and the increase of the enzyme amounts as shown in Fig. 6. This indicated that the enzyme could be used to detect the T-antigen at the pmol-level.

Compared with erythrocytes, released T-antigen of Kato III was found much less (approximately 0.5nmol PA-disaccharide was detected from 5×10^7 cells by incubation with 20mU of enzyme at 37°C for 3hr). This may be due to the fact that the cell surface carbohydrates of Kato III were more complex (18), which showed interruption of the enzyme to attack T-antigen. Although the T-antigen could be detected by the lectins recognizing T-antigen, such as PNA and amaranthin, however, the the lectin-interaction found not only with T-disaccharide, but also with other carbohydrates with lower potency (21). Therefore, the endo-α-N-acetylgalactosaminidase of Alcaligenes sp. was considered to be useful for monitoring the amount of T-antigen (Galβ1→3GalNAc) in cells, because this enzyme could liberate the T-antigen from native cells and the released T-antigen could be detected sensitively in a very small scale as described above.

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