ENDO-β-GALACTOSIDASE OF ESCHERICIA FREUNDII

HYDROLYSIS OF PIG COLONIC MUCIN AND MILK OLIGOSACCHARIDES BY ENDOGLYCOSIDIC ACTION

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The purified keratansulfate degrading enzyme from Eschericia freundii could hydrolyze desialyzed pig colonic mucin and milk oligosaccharides. Desialyzed pig colonic mucin was digested to produce GlcNAcβ(1→3)Gal, GlcNAc-6Sβ(1→3)Gal and resistant polymer. Lacto-N-tetraose and lacto-N-tetraitol were hydrolyzed endoglycosidically to release glucose and sorbitol, respectively. Therefore, this enzyme was found to be an endo-β-galactosidase of rather wide specificity.

To study the structure of glycoproteins, endoglycosidases which specifically hydrolyze sugar portion of glycoprotein are required, as well as exoglycosidases. Recently, endo-β-N-acetylglucosaminidases that can release oligosaccharides from di-N-acetylchitobiosyl-asparagine linked glycoprotein and glycopeptide were found, and these potential utility in clarifing the structure and function of oligosaccharides associated with glycoprotein has been shown (1-4). In the case of another group of glycoproteins, such as sulfated glycoprotein and blood group substances, in which carbohydrates are linked O-glycosidically to protein, it has yet not been shown that the enzymes can hydrolyze them by endoglycosidic action. In this communication, we will present the observation that keratansulfate degrading enzyme from Eschericia freundii can hydrolyze a sulfated glycoprotein from pig colonic mucin and small oligosaccharides such as lacto-N-tetraose and lacto-N-tetraitol as an endo-β-galactosidase.

Abbreviations: lacto-N-tetraose, Galβ(1→3)GlcNAcβ(1→3)Galβ(1→4)Glc
lacto-N-tetraitol, Galβ(1→3)GlcNAcβ(1→3)Galβ(1→4)Sorbitol
GlcNAc-6S, N-acetylglucosamine-6-O-sulfate

MATERIALS AND METHODS

Pig colonic mucin (5) and desialyzed pig colonic mucin were kindly donated by Dr. S. Inoue and Miss M. Miyawaki, of this laboratory. Desialyzed mucin was prepared by mild acid hydrolysis of pig colonic mucin in 0.1 N HCl, 80°, 1 hr, and lyophylized after dialysis against water. In this treatment 95% of sialic acid, 77% of fucose and a small amount of galactose were removed. Lacto-N-tetraose and [^{3}H]-lacto-N-tetraitol (prepared by NaB^3H_4reduction) were kindly donated by Dr. A. Kobata, Kobe University. The strain of Eschericia freundii (6) was kindly donated by Dr. M. Kitamikado, Kyushu University.

Endo-β-galactosidase (4400-fold purification from the culture filtrate of E. freundii cultured in the presence of whale nasal cartilage keratan-sulfate, with 45% recovery) was obtained according the method described by M. Kitamikado (7) with some modification (8). Purified endo-β-galactosidase was free from exo-β-galactosidase, exo-β-N-acetylglucos-aminidase and keratosulfatase.

One enzyme unit is defined as the amount of enzyme required to release 1 μmole of reducing sugar (as galactose) from the substrate per min. under following assay condition. The incubation mixture consisted of 50 μl of enzyme solution and 50 μl of 0.1 M Na-acetate buffer, pH 5.8, containing 150 μg whale nasal cartilage keratansulfate. After incubation for 30 min. at 37°, reducing power produced was measured by the method of Park and Johnson (9).

Paper chromatography was carried out on Toyo No.51A filter paper with descending in solvent 1 (butanol:acetic acid:water 50:15:35), solvent 2 (butanol:pyridine:water 5:3:2) and solvent 3 (ethylacetate:pyridine:water 12:5:4). Sugars were detected with $AgNO_3$-NaOH (10).

Radioactivity of paper chromatograms was determined in a dioxan based scintillation fluid containing 0.7% 2,5-diphenyloxazole (PPO), 0.03% p-bis[2-(5-phenyloxazoil)]benzene (POPOP), and 10% naphthalene, with an

Aloka LSC-502 liquid scintillation spectrometer, after cutting the paper with 1 cm width.

Hexose was determined by the anthrone reaction (11), reducing sugar was by the method of Park and Johnson (8).

RESULTS AND DISCUSSION

Pig Colonic Mucin Intact and desialyzed pig colonic mucin were incubated with and without the enzyme, and reducing power liberated was determined. As indicated in Fig.1, intact mucin was not hydrolyzed by this enzyme. In the case of the desialyzed mucin, 56 µg reducing sugar as galactose per mg mucin was liberated; thus 27.7% of galactose residue of desialyzed mucin was hydrolyzed.

The reaction mixture was separated through a column of Sephadex G-50 to polymer and small oligosaccharides (Fig.2). The oligosaccharide

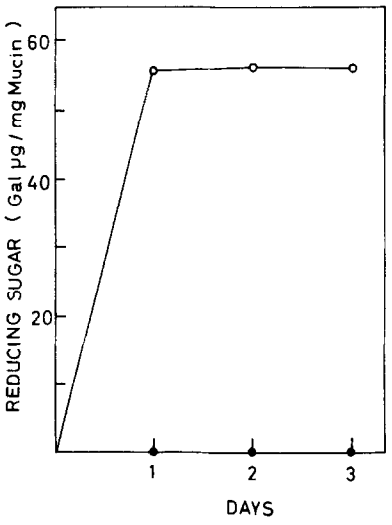

Fig.1 Hydrolysis of pig colonic mucin by endo-β-galactosidase. The intact (—●—) or desialyzed (—○—) pig colonic mucin (20 mg) were dissolved in 200 µl of 0.1 M Na-acetate buffer, pH 5.8, and 100 µl (0.5 units) of purified enzyme were added, respectively. Incubation was carried out at 37° in the presence of toluene, and at 24 hr intervals a portion (10 µl) of incubation mixture was removed for the measurement of reducing power. In the control experiment, the enzyme and substrate were incubated separately, and mixed only before assaying for reducing power.

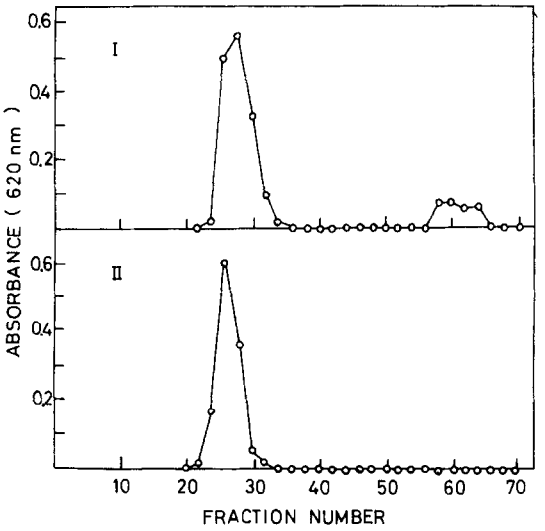

Fig.2 Gel filtration of enzymatic digest of desialyzed mucin on Sephadex G-50.
Desialized mucin (20 mg) was digested for 3 days in the condition indicated in Fig.1. The digest was applied to a column (1 x 130 cm) equilibrated with 0.2 M NaCl, and eluted with same solution (I). Fractions of 1 ml were collected, and assayed for hexose. The elution profile of an identically treated sample of desialized mucin but without added enzyme, is shown (II).

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fraction was subjected to paper chromatography in solvent 1 and 2, and two oligosaccharides were observed. One had the same mobility as GlcNAcβ(1→3)Gal (R_{gal} 0.62 in solvent 1) and the other had the same mobility as GlcNAc-6Sβ(1→3)Gal (R_{gal} 0.26 in solvent 1) that were the digestion products of bovine corneal keratansulfate by endo-β-galactosidase (8).

When the polymer fraction was applied on the column of Sephadex G-200, it was eluted in the void volume with no difference between the digest and the control without enzyme.

These findings suggest that the pig colonic mucin has GlcNAcβ(1→3)Gal and GlcNAc-6Sβ(1→3)Gal structure similar to keratansulfate in the area near the nonreducing terminal and was hydrolyzed by the enzyme to disaccharides and residual polymer.

Lacto-N-Tetraose and Lacto-N-Tetraitol Lacto-N-tetraose was incubated with the purified endo-β-galactosidase, and the rate of hydrolysis was determined using glucose oxidase (12). As indicated in Fig.3, no lag of glucose liberation was observed. Thus it is excluded that concerted actions of exo-β-galactosidase and exo-β-N-acetylglucosaminidase released glucose.

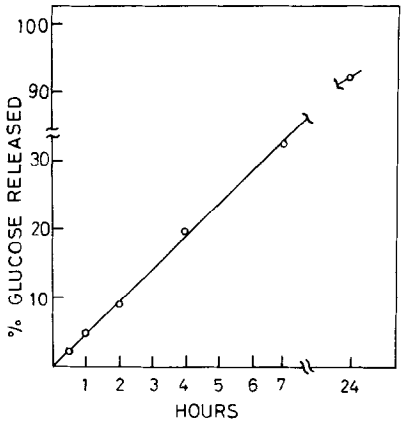

Fig.3 Release of glucose from lacto-N-tetraose by endo-β-galactosidase. The lacto-N-tetraose (170 μg) in 400 μl of 0.1 M Na-acetatebuffer, pH 5.8, was incubated with 100 μl (0.02 units) of purified enzyme at 37°. At the times indicated in the inset, 50 μl of aliquots were withdrawn. After heating for 2 min. at 100° to inactivate endo-β-galactosidase, released glucose was determined by the glucose-oxidase method (12).

[^{3}H]-Lacto-N-tetraitol was incubated with a greater amount of the enzyme at 37° for 20 hrs. When the reaction mixture was subjected to paper chromatography, only [^{3}H]-sorbitol and starting material were observed and no intermediate oligosaccharide alcohol was found (Fig.4).

These indicate the liberation of glucose or sorbitol was directly the result of hydrolysis by endoglycosidase action. In addition, it was found that galactosyl glucose is more rapidly hydrolyzed than galactosyl sorbitol.

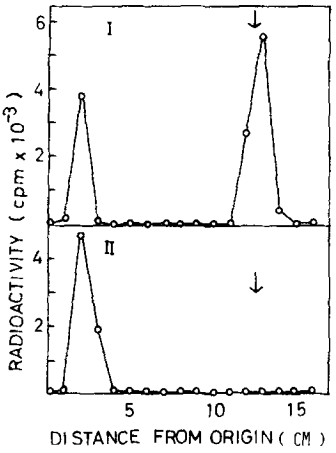

Fig.4 Hydrolysis of [^{3}H]-lacto-N-tetraitol by endo-β-galactosidase. [^{3}H]-lacto-N-teraitol (20 µl, 5 x 10^4 cpm) in 0.1 M Na-acetate buffer, pH 5.8, was incubated with 10 µl (0.2 units) of purified enzyme at 37° for 20 hrs. Incubation mixture with (I) and without (II) enzyme were subjected to paper chromatography in solvent 3, and radioactivity was monitored. The position of the sorbitol is indicated by the arrow.

Therefore, this enzyme catalyze the reaction as follows.

Galβ(1→3)GlcNAcβ(1→3)Galβ(1→4)Glc (or sorbitol) ⟶

Galβ(1→3)GlcNAcβ(1→3)Gal + Glc (or sorbitol)

Endo-β-galactosidase appear to have wide specificity; i.e. the enzyme can act both low molecular substance such as lacto-N-tetraose and high molecular substances such as keratansulfate or pig colonic mucin, both of these substances having the common structure of GlcNAcβ(1→3)Gal. In addition, enzymatic hydrolysis occured in both linkages of galactosyl N-acetylglucosamine (in keratansulfate) and galactosyl glucose (in lacto-N-tetraose).

These results indicate that the endo-β-galactosidase will be useful for studying the structure of glycoproteins and oligosaccharides.

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