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Regioselective Synthesis of α-L-Fucosyl-containing Benzyl Disaccharides by use of α-L-Fucosidases of *Aspergillus niger*

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The activity of fucosidase shows different value depending on disease, eg) fucosidosis and I cell disease are characterized by the absence or deficiences of α-fucosidase, and sera of ovarian cancer patients exhibited a statistically significant deficiency of α-L-fucosidase activity (Zielke *et al.*, 1972; Kress *et al.*, 1980; Barlow *et al.*, 1981). For the purpose of diagnosis of these disease easily, the manual of test can be developed by preparing kits of hydrophobic-binding substrate of fucosidase that bind C18-column.

Key words : Regioselective synthesis, Fucosidase, *Aspergillus niger*, Fucosyl containg benzyldisaccharides, PNP-fucopyranoside

INTRODUCTION

The α-L-fucosyl group is found in glycoprotein or glycolipids (Spiro, 1970), (1-2)-linked or (1-3), (1-4) or (1-6) linked to D-glycosyl residue.

Recently, Svenson and Katasumi Ajisaka reported an enzymic synthesis of methyl α-L-fucopyranosyl-β-D-galactopyranosides by a transglycosylation of the L-fucosyl residue of *para* nitrophenyl α-L-fucopyranoside to methyl β-D-galactopyranoside and N-acetylglucosamine with the aid of an α-L-fucosidase from porcine liver and microorganism. Moreover, an enzyme obtained from microorganism is desirable for a preparative scale synthesis of oligosaccharides, as a large amount of the enzyme can be obtained easily. We report herein the regioselective synthesis of disaccharides containing a (1-3) linked α-L-fucosyl group to N-acetylbenzyl glucosamine by using of α-L-fucosidase. These α-L-fucosyl-containing disaccharides are important not only starting materials for the synthesis of high molecular weight oligosaccharides as components of glycoproteins or glycolipids, but also can use for developing clinical test.

MATERIALS AND METHODS

Material

Q-Sepharose and Bio-gel P2 were purchased from Pharmacia-LKB and Bio-Rad respectively. As a source of *aspergillus niger* culture broth, "Rhozyme" was purchased from Gencor.

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Semipurification of α-L-fucosidase

Rhozyme (2.8 g) was dissolved in 20 mM potassium phosphate buffer (280 ml, pH 7.0) and dialyzed extensively against the same buffer (pH 7.0) during 48hrs. The dialyzed Rhozyme was counted the protein amounts and fucosidase activity,and then the Rhozyme solution was applied onto a Q-sepharose column (2,6× 30 cm) which eluted with a salt gradient (Grove & Serif, 1981) from 0 to 0.5 M NaCl in 20 mM potassium phosphate buffer (pH 7.2, 2L in total), flow rate (5 ml/15 min., 1 drop/3 sec).

Fractions containing α-L-fucosidase activity were pooled from fraction No.168~222, and concentrated 360 ml to 5 ml by a membrane filtration with Amicon PM30.

α-L-fucosidase activity measured with PNP-α-L-fucoside as a substrate was 0.115 unit/ml.

Fucosidase activity;

The activity was determined with PNP-fucoside as modified A'Daniello method (Skood, 1975; D'Aniello, 1982; Weston *et al.*, 1992). For enzyme assay, each volumes of semipurified fucosidase fraction from Rhozyme are diluted to 100 μl,of 5 mM/ml PNP-fucoside dissolved in 0.3 M sodium acetate buffer pH 5,0. The same substrate mixture was incubated in a shaking water bath at 37°C for 1hr. The enzyme reaction was terminated by adding 1 ml of 0.2 M Na_2CO_3. Absorbance of para-nitrophenol (PNP) was read on a Beckman spectrophotometer at a wave length of 400

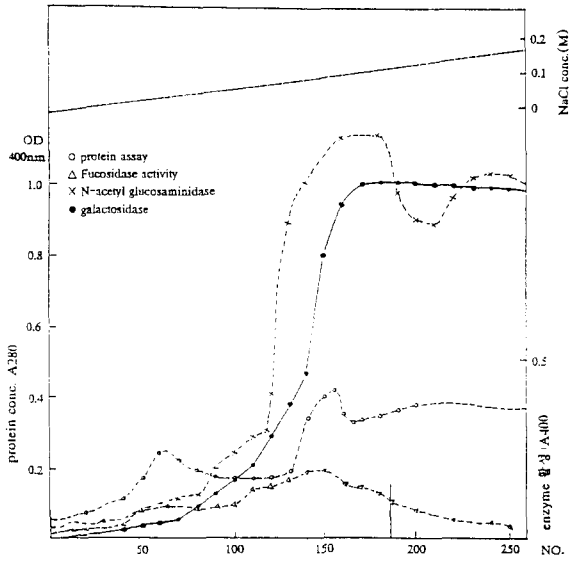


Fig. 1. Amount of protein and enzyme activity. The curves are values followed of tube No. that depend on salt gradient. ○: protein assay, △: fucosidase activity, ×: N-acetyl glucosaminidase, ●: galactosidase

nm. Galactosidase activity; substrate- PNP-β-D-galactoside, N-acetylglucosaminidase activity; substrate PNP-β-D-N- acetylglucosaminide, manosidase activity ; substrate PNP-β-D-manoside, the manual is same above.

Protein Assay

It's determined by Bio Rad protein assay reagent at A 595 nm.

Synthesis of α-L-fucosyl 1-3-N-acetylglucosamine;

N-acetyl glucosamine 125 mg and PNP-α-L-fucopyranoside 25 mg were dissolved in 0.1 M acetate buffer (pH 5.0, 2.1 ml) and N,N-dimethyl formamide (250 μl). The semipurified α-L-fucosidase solution (600 μl, 0.07 unit) was added to the substrate solution, and the mixture was incubated for 13 hrs. at 37°C (Svensson & Thiem, 1990; Yazawa *et al.*, 1986). The reaction mixture was heated in a boiling water bath for 10 min. (15,000 rpm, 30 min.),the denatured enzyme was centrifuged off, and the supernatant was applied onto Biogel P_2 (200-400 mesh; 1.2×80 cm), and the reaction mixture was eluted with 0.1 M pyridine acetate pH 5.5 (BeMiller,1980) . The eluant was collected from fraction No.43~49 (7.9 ml) (flow rate 1.13/15 min.), and yielded 220 μg/ml from fucose standard curve (Fig 2 ,3).

Synthesis of α-L-fucosyl 1-3-N-acetyl-β-O-benzyl-glucosamine;

N-acetyl-β-O-benzylglucosamine 110 mg and PNP-α-L-fucopyranoside 25 mg were dissolved in 0.1 M acetate buffer (pH 5.0, 2.1 ml) and N,N-dimethyl formamide (250 ml). The semipurified α-L-fucosidase solution (600 ml, 0.07 unit) was added to the substrate solution, and then treated the same manual above. (Svensson & Thiem, 1990; Yazawa *et al.*, 1986).

The eluant was collected from fraction No.40-60 (flow rate 1.13/15 min.) (Fig. 4).

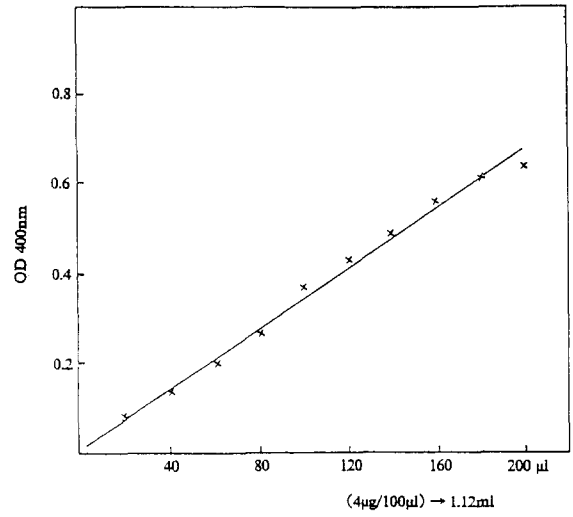


Fig. 2. Fucose standard curve. Fucose (40 μg/ml) of each volumes are counted by 6-deoxy hexose test at 400 nm.

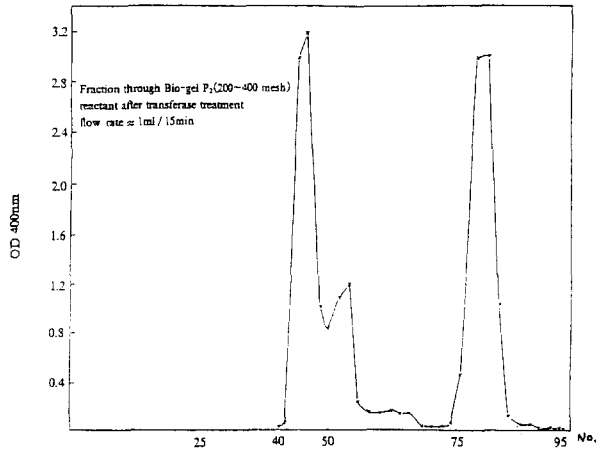


Fig. 3. Fucose amount of fucoside on each fraction. The fractions through Bio-gel P2 (200~400 mesh) of reactant (containing Fuc-GlcNac) after transferase treatment (flow rate≈1 ml/15 min.)

Assay of carbohydrate contents and purification;

The carbohydrate contents of each fraction was measured (Dubois *et al.*, 1956; Clamp *et al.*, 1967)) with thioglycolic acid-H_2SO_4 reagent and UV-detection (248 nm). Fractions containing the benzyl-disaccharide were applied on C18 Sep-Pack (elute with methanol), and silica gel (chloroform-methyl alchol -water, 13 : 6 : 1, RF; 0.49) after concentration.

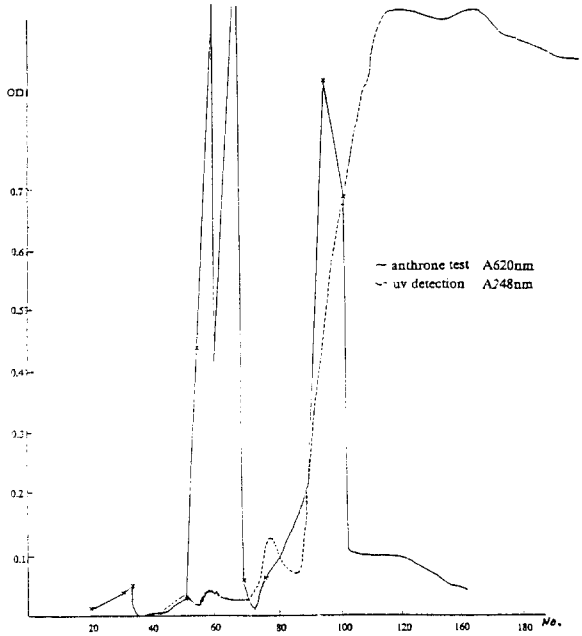


Fig. 4. Benzyl fucoside 's counted on each fraction by Anthrone test and UV detection. —: Anthrone test at 620 nm, ---: UV detection at 248 nm. The fractions through Bio-gel of reactant after fucosidase reaction.

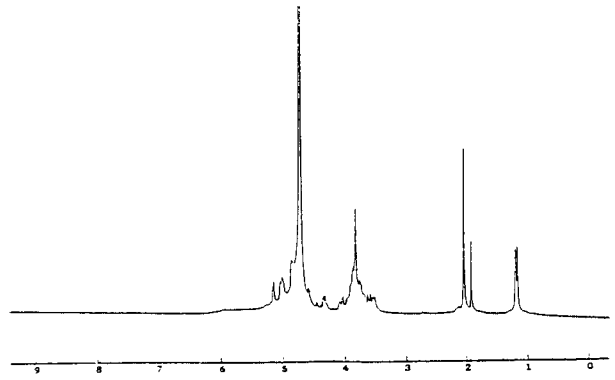


Fig. 5. ^{1}H-nmr (Fuc-GlcNac) 200 MHz.

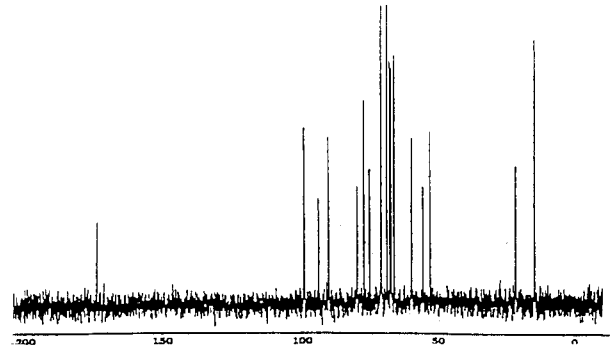


Fig. 6. ^{13}C-nmr (Fuc-GlcNac) 100 MHz.

Analytical procedure

^{1}H and ^{13}C-nmr spectra were recorded with BRUKER spectrometer operating at 200 and 100 MHz respectively. In ^{13}C-nmr spectra, chemical shifts were refered to tetra methyl silane added as internal reference (TMS). This data described had been measured for solutions in D_2O.

RESULTS AND DISCUSSION

The α-D-fucosidase from Rhozyme was purified on sepharose column according to report Katsumi *et al.* (Hakomori *et al.*, 1984). As a source of Aspergillus Niger culture broth, "Rhozyme" which is purchased from Genecor Inc. (CA, USA). The collected fractions contained β-D-N-acetylglucosaminidase activity, 0.115 unit/mg; β-D-galactosidase activity, 0.588 unit/mg; and β-D-mannosidase activity, 0.710 unit/mg together with α-L-fucosidase activity, 0.018 unit/mg. this semi-purified enzyme preparation was used in the following experiments without further purification (Fig. 1).

Incubation of PNP-α-L-fucopyranoside and N-acetyl-β-O-benzylglucosamine in the presence of semi-purified α-L-fucosidase was examined by chemical assay and nmr spectra, (Fig. 7, 8) (Coxon, 1965; Bock *et al.*, 1985; Koerner *et al.*, 1987) after purified on silica gel and C18 sep-pack.

The ^{1}H and ^{13}C-nmr spectra of disaccharide and aromatic ring were recorded and analyzed (Fig. 7, 8 Table 1). The data were in agreement with those of β-O-benzylglycoside of α-3-L-fucopyranosyl-N-acetylglucosamine and α-L-fucopyranosyl 1-3-N-acetylglucosamine (Fig. 5, 6, Table 1), as reported by Baumann *et al.*.

In the ^{1}H-nmr spectra of α-L-fucosyl containing oligosaccharides, H-5 of the α-L-fucosyl residue is known to exhibit chemical shifts characteristic for the linkage of the fucosyl group. The chemical shift of H-5 for the present disacchsride was at 4.35 (Ajisaka *et al.*, 1992), and that for the corresponding benzylglycosides was reported at 4.37. Therefore, the disaccharide was identified as β-O-benzylglycoside of α-3-L-fucopyranosyl-N-acetylglucosamine. The lower-field shifts of the C-3 signals indicated that the L-fucosyl group is bound to O-3 of the N-acetylglucosamino-benzyl derivatives.

In the ^{13}C-nmr spectrum of C-1 of the α-L-fucopyranosyl residue resonated at δ-98.67, and the spectrum of N-acetylglucosamino benzoate residue also showed a lower field shift of 79.29. These lower field shifts indicated a binding of the fucosyl group to O-3 of the N-acetyl glucosamino benzoate residue. The α-L-fucosidase from *aspergillus niger* used in the present study may be the same one as that used by Bahl. Benzyl group containing disaccharide, (Fig. 9) components of glycoconjugate, were synthesized easily and regioselectively by selection of the appropriate

Table I. ^{1}H and ^{13}C-n.m.r data (δ values) of (1-3) linked benzyl α-L-fucosyl disaccharides and α-L-fucosyl disaccharide

Residue or group.	*Ref. PPM(δ)		**Exp. PPM(δ)
^{1}H_n.m.r	H-1	5.06	4.95
Fuc.	H-2	3.60	3.57
	H-3	3.75	3.66
	H-4	3.84	3.84
	H-5	4.35	4.37
	H-6	1.18	1.19
GlcNAc	H-1	5.15	4.99
or BZ.	H-2	4.04	4.01
	H-3	3.68	3.65
	H-4	3.53	3.53
	H-5	3.88	3.89
	H-6	3.82	3.83
NH CO CH_3		2.04	1.97
C_6H_5-			7.52-7.43(m.5H,Ar.)
^{13}C-n.m.r	C-1	98.72	98.67
Fuc.	C-2	67.03	66.91
	C-3	68.59	67.58
	C-4	70.85	70.80
	C-5	65.92	65.85
	C-6	14.18	14.14
GlcNAc	C-1	90.06	98.79
or BZ.	C-2	52.65	54.25
	C-3	77.08	79.29
	C-4	67.55	68.49
	C-5	70.75	74.85
	C-6	59.61	59.79
-NH CO CH_3		20.98	21.13
C_6H_5-			70.46

*Ref: fucose-N-acetyiglucosamine
*Exp: fucose-N-acetyl-benzyl-glucosamine

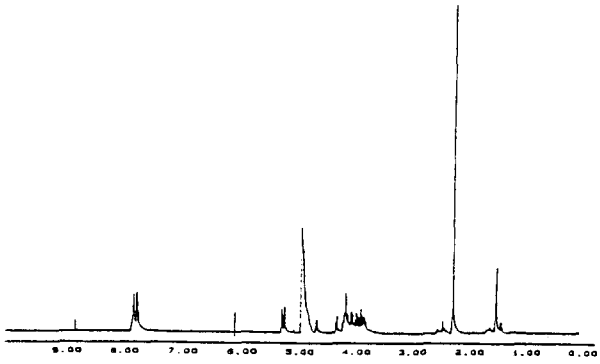


Fig. 7. ^{1}H-nmr (Benzyl glycoside of Fuc-GlcNac) 200 MHz.

enzyme.

CONCLUSION

We found fucosyltransferase activity of α-L-fucosidase can transfer fucosyl group to N-acetylglucosamine with hydrophobic group.

The measurement of fucosidase activity on fucoside bond by binding hydrophobic group on column

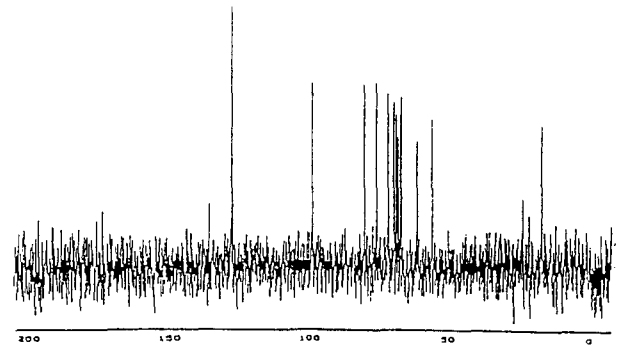
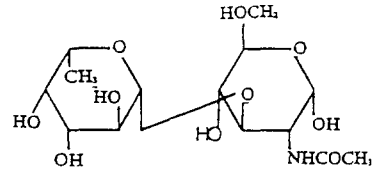
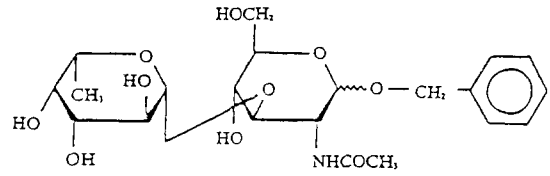


Fig. 8. ^{13}C-nmr (Benzyl glycoside of Fuc-GlcNac) 100 MHz.



α-L-fucopyranosyl 1-3 .N-acetylglucosamine



β-O-benzylglucoside of α-3-L-fucopyranosyl-N-acetylglucosamine

Fig. 9. structures of Fuc-GlcNac and Benzyl glycoside of Fuc-GlcNac. Structures of products synthesized from tranferase activity of α-L-fucosidase of *Aspergillus niger.*

showes posibility to make convenient diagnosis kit caused by fucosidase activity, such as ovarian cancer, I cell disease and fucosidosis etc.. For the practical use of the kit, the conditions for the fucosidase activity and yield on the column are to be investigated.

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