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A BIOSYNTHETIC CONTROL ON STRUCTURES SERVING AS LIGANDS FOR SELECTINS: THE PRECURSOR STRUCTURES, 3-SIALYL/SULFO Galß1,3/4GlcNAcß-O-R, WHICH ARE HIGH AFFINITY SUBSTRATES FOR α1,3/4-L-FUCOSYLTRANSFERASES, EXHIBIT THE PHENOMENON OF SUBSTRATE INHIBITION

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The present study reports a control on the biosynthesis of fucosylated structures, serving as ligands for selectins by demonstrating the potential of 3-sialyl or 3-sulfo Galß1,3/4GlcNAcß-containing glycoconjugates as high affinity substrates for α1,3/4-L-fucosyltransferases and as substrate inhibitors at higher concentrations. The synthetic sulfated saccharides and the triantennary sialoglycopeptide from fetuin were potent competitive inhibitors of the transfer of fucose to non-anionic saccharide acceptors and the corresponding triantennary asialoglycopeptide respectively catalyzed by a partially purified α1,3/4-L-fucosyltransferase preparation from Colo 205 (specific activity:transfer of 113.1 nmol Fuc to 2'-FucosylLacNAc per h per mg protein); Ki for the inhibitions by triantennary sialoglycopeptide, 3-SulfoGalß1,3GlcNAcß-O-Allyl and a copolymer from 3-SulfoGalß1,3GlcNAcß-O-Allyl and acrylamide were 51.9 μM, 500 μM and 67.0 μM, respectively. Further, the α1,3-specific anionic acceptor, 3'-SulfoLacNAc, also inhibited the α1,4- activity; Km for the α1,4-specific acceptor, 2-methylGalß1,3GlcNAcß-O-Bn increased from 0.40 mM to 1.35 mM in presence of 3.0 mM 3'-sulfoLacNAc, whereas Ki for the mutual inhibition of α1,3-activity by the former was found to be high (3.64 mM). Furthermore, the phenomenon of substrate inhibition, serving as acceptors at lower concentrations and as inhibitors at higher concentrations, was exhibited by the anionic acceptors; the Hill plots gave the Ki values 342.7 μM, 13.03 mM and 13.36 mM respectively for fetuin triantennary sialo glycopeptide, 3'-sulfoLacNAc and 3-sulfoGalß1,3GlcNAcß-O-Allyl.

Abbreviations: FT, Fucosyltransferase; SGGA, 3-SulfoGalß1, 3GlcNAcß-O-Allyl; MGGB, 2-MethylGalß1, 3GlcNAcß-O-Bn; Bn, Benzyl.

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The fucosylated carbohydrate structures, Lewis x, sialyl Lewis x, sialyl Lewis a, difucosyl sialyl Lewis x and VIM-2 have been shown as potential ligands for E- and P-selectins (1-3). A major endothelial glycoprotein containing sulfate, sialic acid and fucose was found to interact specifically with L-selectin (4). The oligosaccharides, 3-sulfoGalβ1,3/4(Fucα1,4/3)GlcNAcβ1,3Gal, from ovarian cystadenocarcinoma glycoprotein were identified as powerful ligands for E-selectin (5) and further studies showed the sulfated Lewis a tetra and pentasaccharides as the most potent E-selectin ligands (6). Expression of sialyl Lewis a especially in colon carcinoma may facilitate tumor cell attachment to E-selectin and thus contribute to early adhesion events leading to tumor cell extravasation (7). The selectins, in general, because of their ability to recognize fucosylated carbohydrate structures expressed by tumor cells may function in the spread of malignancies (8). Our laboratory was the first one in reporting the chemical synthesis of E selectin ligand, 3'-Sulfo Lewis x as well as in demonstrating the high affinity of the precursor structures 3-SulfoGalβ1,3/4GlcNAcβ- as acceptors for ovarian cancer α1,3/4-fucosyltransferases (9). The present paper reports a biosynthetic control on selectin-ligands resulting from the influence of sulfate or sialyl group at C-3 of Gal moiety in Galβ1,3/4GlcNAc- on the activities of α1,3/4-L-fucosyltransferases, using Colo 205 as the enzyme source.

EXPERIMENTAL PROCEDURES

Cell Culture: The Colo 205 cells were grown in 250 ml plastic T-flasks in RPMI 1640 supplemented with 5% fetal bovine calf serum (GIBCO), 5% Nu serum (Collaborative Research) and 2 mM glutamine in a humidified atmosphere of 95% air and 5% CO2 (pH 7.0) at 37°C. Cells were subcultured with 0.05% Trypsin/0.53 mM EDTA (GIBCO). For experimental use, cells were pelleted at 1500 rpm for 5 min., washed twice with PBS and stored frozen.

Enzyme Preparation: A pool of Colo 205 cell pellets (1.0 x 10^9 cells) was suspended in 20 ml ice cold 50 mM Tris buffered saline (pH 7.0) containing 2% Triton X-100, and homogenized by Dounce all glass hand-operated grinder. The 20,000g supernatant after dialysis against three changes of one liter each of 25 mM Tris-HCl, pH 7.0, containing 35 mM $MgCl_2$, 10 mM NaN_3 and 1 mM ATP at 4°C was applied to bovine IgG glycopep-Sepharose column (30 ml in bed volume) (9,10), which had been equilibrated with the same buffer. After washing with 100 ml of the equilibration-buffer, elution of the column was done with 100 ml of 1.0 M NaCl in the above buffer. The NaCl eluate was concentrated to ~2.0 ml by ultrafiltration and then dialyzed against three changes of 250 ml of the above buffer at 4°C and stored at 4°C. This preparation had a specific activity of 113.1 (nmol Fuc transferred to 2'-FucosylLacNAc per h per mg protein) with a recovery of 73.4%. Under these conditions, no loss of FT activities was seen for at least two months.

Assay for Fucosyltransferases: The incubation conditions and quantitation of [^{14}C]Fuc-containing products resulting from the various acceptors by Dowex-1-Cl method were followed as described earlier (9). Protein was measured by the BCA method (Pierce Chemical Co.).

Glycopeptides: The diantennary glycopeptide was prepared from bovine IgG (Calbiochem) by pronase digestion, gel filtration and Con A-Sepharose chromatography as described earlier (10). A similar procedure was followed to obtain from fetuin (Sigma), the triantennary sialoglycopeptide, which did not bind to Con A-Sepharose. The asialo glycopeptide was made by heating the triantennary sialoglycopeptide at 80°C in 0.1 N HCl for 1 h and chromatography of the neutralized solution after concentration to 1.0 ml on a Biogel P2 column (1.0 x 116.0 cm) to remove sialic acid.

Synthetic Sulfated Copolymer: The copolymer from 3-SulfoGalß1,3GlcNAcß-O-Allyl and acrylamide was synthesized by following the procedure of Horejsi et al. (11). This preparation contained ~1.0 µmol of the sugar unit per mg weight and was similar in molecular size to dextran of average molecular weight 39,200, as evident from column chromatography on Biogel P60.

RESULTS AND DISCUSSION

The purified enzyme (Table I) acted well on 2'-methyl LacNAcß-O-Bn, 3'-sulfo LacNAc and also on the α1,4 acceptor, 2-methyl Galß1,3GlcNAcß-

TABLE I

Activity of the Partially Purified
α1,3/4-L-Fucosyltransferase from Colo 205 Cells

Acceptor	α-L-Fucosyltransferase Activity	
	Transfer of [^{14}C] Fuc Catalyzed by 1 µg protein (CPM x 10^{-3})	Expressed as percent of the activity towards 2'-Fucosyl LacNAcß-O-Bn
2'-Fucosyl LacNAcß-O-Bn (3mM)	19.37	100
2'-Methyl LacNAcß-O-Bn (3mM)	15.39	79.5
3'-Sialyl LacNAc (3mM)	4.97	25.7
3'-Sulfo LacNAc (3mM)	16.32	84.3
2-Methyl Galß1,3GlcNAcß-O-Bn (3mM)	22.54	116.4
Galß-O-Bn (3mM)	0	0
Fetuin triantennary sialoglycopeptide:		
10 µg	25.47	
200 µg (0.40mM)	26.81	
Fetuin triantennary asialoglycopeptide:		
10 µg	11.39	
200 µg (0.48mM)	109.37	
Bovine IgG diantennary glycopeptide:		
10 µg	5.97	
200 µg (0.56mM)	107.50	

0-Bn exhibiting 79.5%, 84.3% and 116.4% activities respectively with respect to its activity towards 2'-fucosyl LacNAcß-0-Bn. Considerable activity was also shown by this enzyme on 3'-sialyl LacNAc (25.7%). Assay with ß-benzyl galactoside showed the absence of α1,2-FT activity in this enzyme preparation.

The unique interaction of fetuin sialo glycopeptide with Colo 205 α1,3/4-L-FT: The incorporation of [^{14}C] Fuc into fetuin asialo glycopeptide, and bovine IgG glycopeptide at high concentration was several fold more than at the low concentration (Table I). On the contrary, fetuin sialo glycopeptide at both concentrations exhibited nearly the same extent of acceptor activity. This observation suggested that the enzyme was probably inhibited by this sialoglycopeptide at high concentration, namely substrate inhibition.

Enzymatic confirmation of the linkage α1,3/4-L-Fuc in the products arising from fetuin sialo glycopeptide and its corresponding asialo glycopeptide: [^{14}C] Fucosylated products from both sialo glycopeptide and asialo glycopeptide were isolated by separate chromatography of the reaction mixtures on Biogel P6 column (12). The [^{14}C] Fucosylated product from fetuin sialo glycopeptide was desialylated by Vibrio cholerae neuraminidase and the resulting glycopeptide was isolated by Biogel P6 chromatography. This asialo [^{14}C] glycopeptide and [^{14}C] fucosylated product from fetuin asialo glycopeptide B were treated separately with α-L-fucosidase (Almond Meal; Oxford Glycosystems) specific for α1→3/4 linkage and also with α-L-fucosidase from Earthworm and Leech (both enzymes act on Fucα1,2Gal linkage very efficiently) (13). The release of [^{14}C]Fuc was quantitated by thin layer chromatography (12); 87.1% and 88.7% of [^{14}C] Fuc from VCN treated [^{14}C] fucosylated fetuin glycopeptide and from [^{14}C] fucosylated fetuin asialo glycopeptide respectively were released by the action of 20 μ units of α-L-fucosidase from Almond Meal. The [^{14}C] fucosylated fetuin glycopeptides (1/4 of the amount used in the above experiment) were also treated separately with α-L-fucosidase from Earthworm (120 μ units) and Leech (70 μ units) (13); the unit of activity of these enzymes was based on the hydrolysis of 2'-Fucosyl lactose and was equivalent to that defined for Almond Meal Fucosidase. These treatments did not result in any significant release of fucose.

Inhibition Due to Acceptor Competition:

(i) Competition between fetuin sialo and asialo glycopeptides: Fig. 1A shows the incorporation of [^{14}C] Fuc into fetuin sialo glycopeptide both in presence and absence of fetuin asialoglycopeptide and also the incorporation of [^{14}C] Fuc into the competitive acceptor,

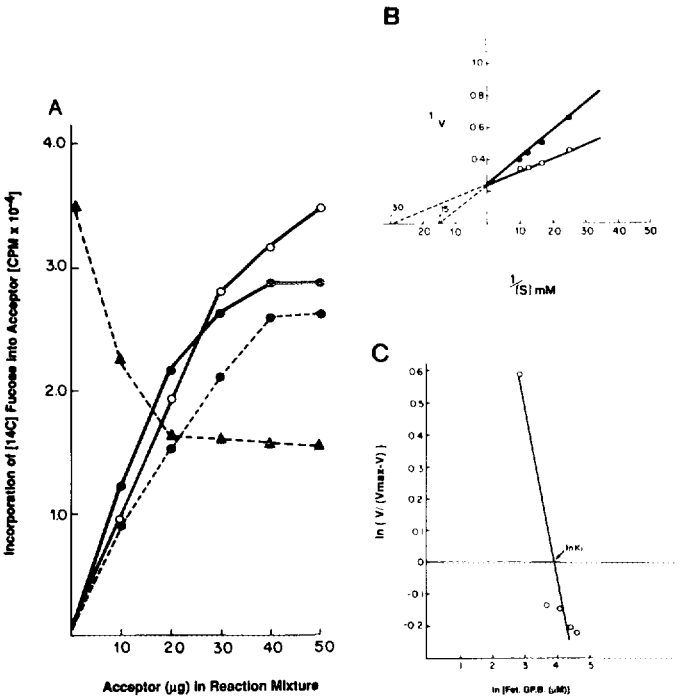

Fig. 1. The acceptor activity of fetuin asialo glycopeptide in presence of increasing concentration of fetuin sialo glycopeptide.

A determination of Km (fetuin sialo glycopeptide in presence and absence of fetuin asialo glycopeptide) and Ki (fetuin sialo glycopeptide as the inhibitor) were made.

A. Incorporation of [^{14}C] Fuc into
(a) Fetuin asialo glycopeptide [○—○—○],
(b) Fetuin sialo glycopeptide [●—●—●],
(c) Fetuin sialo glycopeptide in presence of 50 μg fetuin asialo glycopeptide [●···●···●] and
(d) Fetuin asialo glycopeptide in presence of increasing concentration of fetuin sialo glycopeptide [▲···▲···▲].

B. Lineweaver-Burke plot of [^{14}C] Fuc incorporation into fetuin sialo glycopeptide in presence [●—●—●] and absence [○—○—○] of fetuin asialo glycopeptide.

C. Hill plot of [^{14}C] Fuc incorporation into fetuin asialo glycopeptide in presence of the competitive inhibitor, fetuin sialo glycopeptide.

namely fetuin asialo glycopeptide. The incorporation of [^{14}C] Fuc into the asialo glycopeptide has not reached maximum at 50 μg level whereas that into sialoglycopeptide reached the plateau by 40 μg level. This finding is consistent with the data in Table I, where these glycopeptides were tested for their acceptor ability at 10 μg and 200 μg levels. Lineweaver-Burke plot of the above data [Fig. 1B] shows that both curves intercept the Y axis at the same point and the intercept on

the X-axis is considerably decreased in presence of asialoglycopeptide due to the increase in the slope of the curve. Km for fetuin sialoglycopeptide (approximate molecular weight 5000) in the absence of asialo glycopeptide (approximate molecular weight 4100) was 33 μM and Km in presence of 0.125 mM asialo glycopeptide was 67 μM. The inhibition of incorporation of [^{14}C] Fuc into asialo glycopeptide by varying the concentration of the sialo glycopeptide was examined by the Hill plot (14), ln of fetuin sialoglycopeptide in μM versus ln $\{V/_{(Vmax-V)}\}$. From the incercept of the curve on the X-axis (Fig. 1C), which represents ln Ki, the Ki was found to be 51.9 μM.

(ii) Competition between MGGB and SGGA: Fig. 2A shows the decrease in the incorporation of [^{14}C] Fuc into SGGA of increasing concentrations brought about by 3.0 mM MGGB and also the mutual drop in the transfer of [^{14}C] Fuc into MGGB with the increasing concentration of SGGA. Lineweaver-Burke plot (Fig. 2B) showed the Km for SGGA as 101 μM and 435 μM in absence and presence of MGGB; Ki for SGGA as the inhibitor was determined by Lineweaver-Burke plot as 500 μM (Fig. 2C).

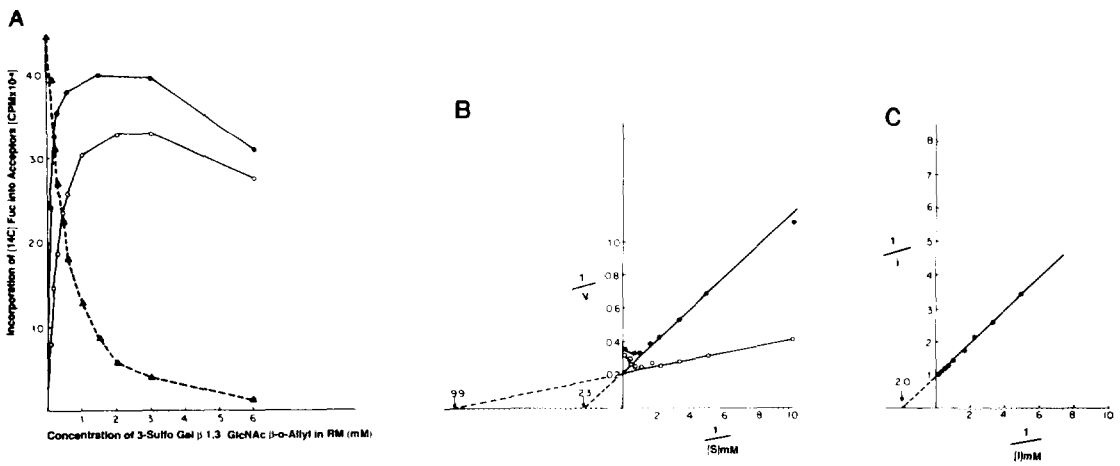

Fig. 2. The acceptor activity of MGGB in presence of increasing concentrations of SGGA.
A determination of Km for SGGA in presence and absence of MGGB and Ki for SGGA as the inhibitor was made.
A. Incorporation of [^{14}C]Fuc into SGGA ●—●—●.
SGGA in presence of MGGB (3.0 mM) ○—○—○.
MGGB in presence of increasing concentrations of SGGA ▲---▲---▲.
B. Lineweaver-Burke plot of [^{14}C] Fuc incorporation into SGGA in presence ●—●—● and absence ○—○—○ of MGGB.
C. Lineweaver-Burke plot of [^{14}C] Fuc incorporation into MGGB in presence of increasing concentrations of SGGA.

(iii) Inhibition by the Copolymer (approximate molecular weight 40,000) from SGGA and Acrylamide: The incorporation of [^{14}C] Fuc into MGGB steadily decreased when the concentration of the copolymer was increased; the inhibition of the activity reached 87% (Fig. 3A) at 200 μM level of the copolymer; Ki as determined by Lineweaver-Burke plot was 67 μM (Fig. 3B).

Competition Between α1,3- and α1,4-specific Acceptors for Colo 205 enzyme: Fig. 4 reports the competitive inhibition of α1,4-L-FT activity by 3'-sulfo LacNAc, a specific acceptor for α1,3-L-FT. The incorporation of [^{14}C] Fuc into 2-methylGalβ1,3GlcNAcβ-O-Bn both in presence and absence of 3'-sulfo LacNAc (Fig. 4A) shows the inhibition of [^{14}C] Fuc incorporation in presence of 3'-sulfo LacNAc. Lineweaver-Burke plot of the above data (Fig. 4B) shows that both curves intercept the Y-axis at the same point and the intercept on the X-axis is considerably decreased in presence of 3'-sulfo LacNAc due to the increase in the slope of the curve. Km for 2-methylGalβ1,3GlcNAcβ-O-Bn in the absence of 3-sulfo LacNAc was 0.397 mM and in presence of 3'-sulfo LacNAc was 1.351 mM. Ki for the inhibition of [^{14}C] Fuc incorporation into 3'-sulfo LacNAc by varying the concentration of 2-methylGalβ1,3GlcNAcβ-O-Bn was calculated by Hill plot (13) (Fig. 4C) as 3.64 mM.

Substrate inhibition of Colo 205 α1,3-L-FT by fetuin sialo glycopeptide: The acceptor activity (Fig. 5A) showed maximum at 20 μg

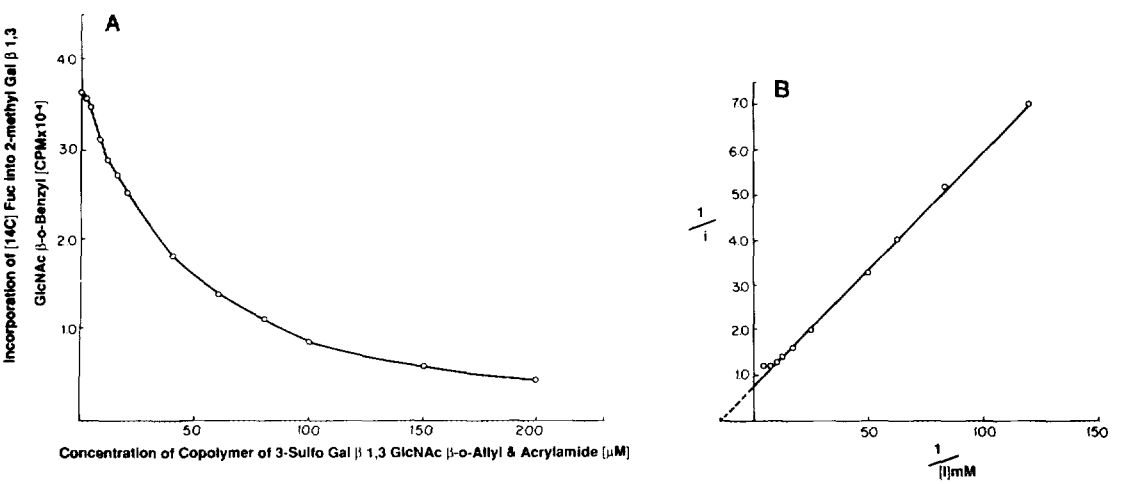

Fig. 3. The acceptor activity of MGGB in presence of increasing concentrations of the copolymer from SGGA and acrylamide.
A. Incorporation of [^{14}C] Fuc into MGGB in presence of increasing concentrations of the copolymer.
B. Lineweaver-Burke plot of [^{14}C] Fuc incorporation into MGGB in presence of increasing concentrations of the copolymer.

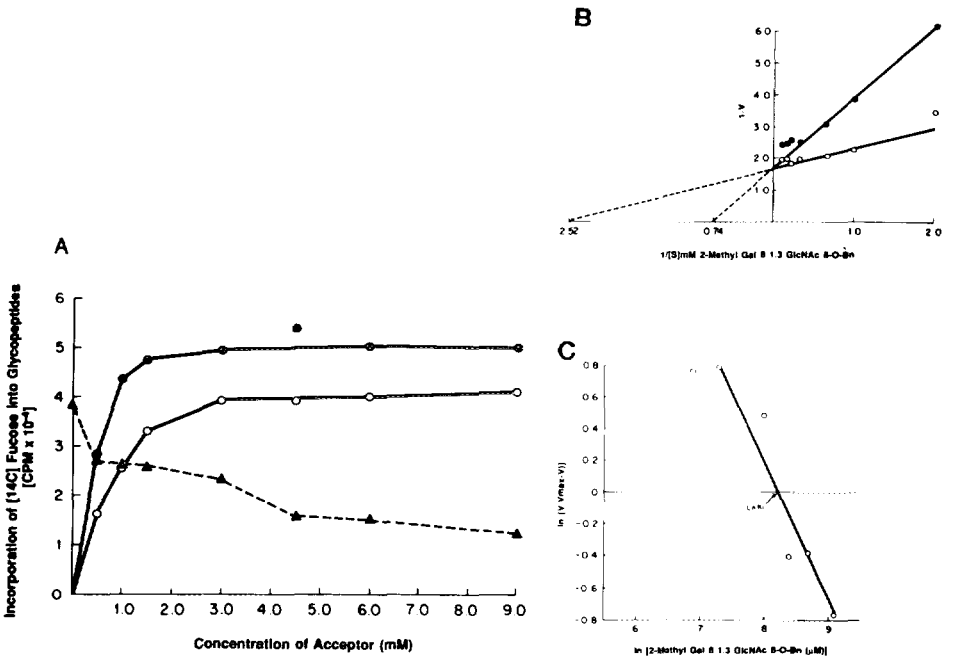

Fig. 4. Inhibition of Colo 205 α1,4-L-fucosyltransferase activity by 3'-sulfo LacNAc, a specific acceptor for α1,3-L-fucosyltransferase.

A determination of Km (2-methyl Galß1,3GlcNAcß-O-Bn in presence and absence of 3'-sulfo LacNAc) and Ki (2-methyl Galß1,3GlcNAcß-O-Bn as the inhibitor) were made.

A. Incorporation of [^{14}C] Fuc into 2-methyl Galß1,3GlcNAcß-O-Bn in presence [○—○—○] and absence [●—●—●] of 3'-sulfo LacNAc (3mM). [▲···▲···▲] Incorporation of [^{14}C] Fuc into 3'-sulfo LacNAc.

B. Lineweaver-Burke plot of [^{14}C] Fuc incorporation into 2-methyl Galß1,3GlcNAcß-O-Bn in presence [●—●—●] and absence [○—○—○] of 3'-sulfo LacNAc.

C. Hill plot of [^{14}C] Fuc incorporation into 3'-sulfo LacNAc in presence of the competitive inhibitor, 2-methyl Galß1,3GlcNAcß-O-Bn.

level, forming a plateau between 40-80 μg and then was dropping steadily to 20.9% of the maximum activity at 320 μg. Lineweaver-Burke plot of this data (Fig. 5B) showed that the resulting curve has an upward deflection at high substrate concentration. This pattern has been shown by others to be indicative of substrate inhibition (14,16). Hill plot (14) of the above data was made (Fig. 5C) and the X-axis intercepts of the curves represented the natural logarithm of Km and Ki. Km and Ki thus obtained were 23.2 μM and 347.2 μM respectively. This Km value (23.2 μM) was reasonably close to the Km value obtained earlier by Lineweaver-Burke plot (Fig. 1B; 33.0 μM).

Substrate inhibition of Colo 205 α1,3-L-FT by 3'-sulfo LacNAc: Maximum activity was seen (Fig. 6A) at 1 mM level of 3'-sulfo LacNAc,

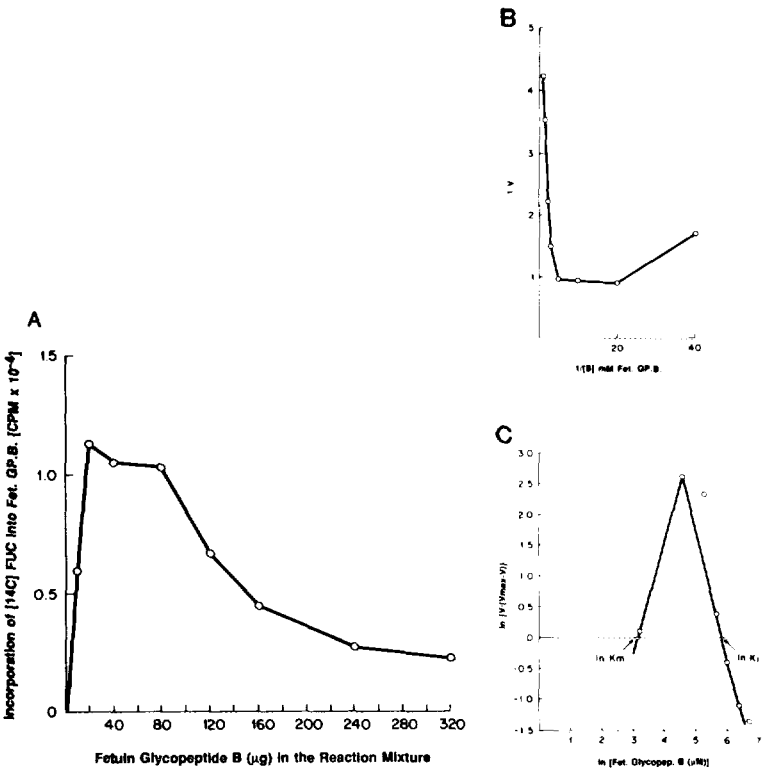

Fig. 5. Substrate inhibition of Colo 205 α1,3-L-fucosyltransferase by fetuin sialo glycopeptide.
A. Incorporation of [^{14}C] Fuc into fetuin sialo glycopeptide of various concentrations.
B. Lineweaver-Burke plot of the acceptor activity of fetuin sialo glycopeptide against its concentration.
C. Hill plot of the acceptor activity of fetuin sialo glycopeptide for a determination of Km and Ki.

then dropped steadily with increasing concentration of 3'-sulfo LacNAc and reached 46.8% of the maximum activity at 12.0 mM level. Lineweaver-Burke plot (Fig. 6B) showed that the resulting curve has an upward deflection at high substrate concentration, as noticed above in the case of fetuin sialo glycopeptide B (Fig. 5B), exhibiting the pattern of substrate inhibition. The above data was subjected to Hill plot (14) and the Ki calculated from the intercept of the curve on the X-axis was 13.03 mM.

Substrate inhbition of Colo 205 α1,4-L-FT activity by SGGA: The maximum activity noticed at 0.5 mM level of SGGA, dropped steadily to about 50% at 15.0 mM level (Fig. 7A); Km and Ki values were determined by Hill plot (14) (Fig. 7B) as 77.5 μM and 13.36 mM respectively.

Rubbo et al. (14) analyzed the substrate inhibition of xanthine oxidase by the Hill plot and came up with 7.3 μM and 360 μM as the Km

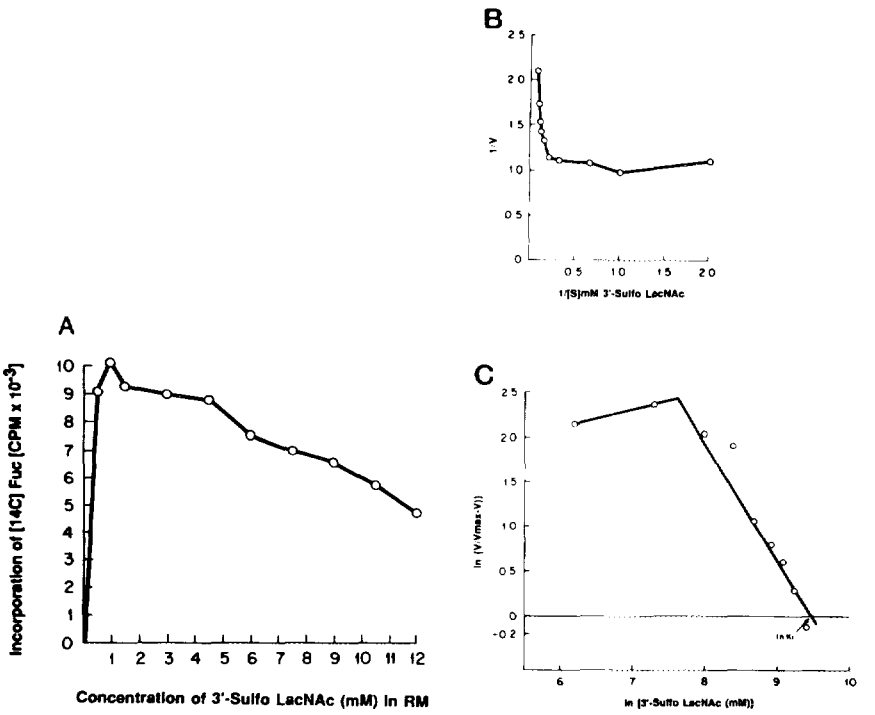

Fig. 6. Substrate inhibition of Colo 205 α1,3-L-fucosyltransferase by 3'-sulfo LacNAc.
A. Incorporation of [^{14}C] Fuc into 3'-sulfo LacNAc of various concentrations.
B. Lineweaver-Burke plot of the acceptor activity of 3'-sulfo LacNAc against its concentration.
C. Hill plot of the acceptor activity of 3'-sulfo LacNAc for Ki determination.

and Ki values respectively. We found the Km and Ki values for fetuin sialo glycopeptide as acceptor and inhibitor by the method of Hill plot as 23.3 μM and 347.2 μM, respectively. The Ki value for the substrate inhibition of Colo 205 enzyme by 3'-sulfo LacNAc and 3-sulfoGalß1,3GlcNAcß-0-Allyl were found to be 13.03 mM and 13.36 mM respectively. The above data would indicate that the natural acceptor, namely, fetuin sialo glycopeptide, is a very efficient substrate inhibitor of Colo 205 α1,3/4-L-FT.

In their study on the substrate inhibition of Xanthine oxidase, Rubbo et al. (14) pointed out that the symmetry of the Hill plots with slopes of +1 and -1 would indicate no involvement of cooperativity or allosteric effects in substrate inhibition. The symmetry of the Hill plots for both fetuin sialo glycopeptide B and SGGA would strongly be indicative of the absence of cooperativity or allosteric effects in their inhibition. One mechanism for substrate inhibition suggested by Cleland (15) was the increased ionic strength at high substrate

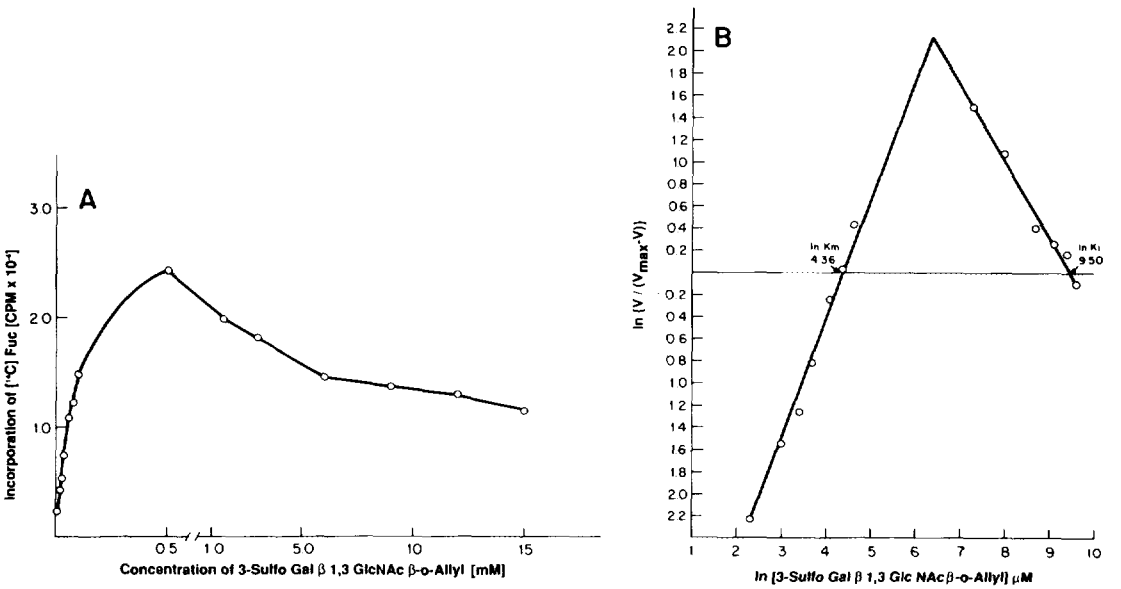

Fig. 7. Substrate inhibition of Colo 205 α1,4-L-fucosyltransferase by SGGA.
A. Incorporation of [^{14}C] Fuc into SGGA of various concentrations.
B. Hill plot of the acceptor activity of SGGA for a determination of Km and Ki.

concentration. This mechanism can be ruled out because the substrate inhibition occurs in all cases (fetuin sialo glycopeptide: 3-8 mM; 3'-sulfo LacNAc: 6-12 mM and SGGA: 0.5-15 mM) at low concentrations making insignificant contribution to the ionic strength of the reaction mixture. Satischandran and Markham (16) attributed the potent substrate inhibition of E. coli adenosine-5'-phosphosulfate (APS) kinase by APS to the formation of a dead-end E - APS complex. Furman et al. (17), in their substrate inhibition study of human immunodeficiency virus type 1 reverse transcriptase suggested that a secondary low affinity binding site for substrate may exist in the Michaelis complex either within or near the catalytic site. The substrate inhibition shown by fetuin sialo glycopeptide and by 3'-sulfo LacNAc could probably be explained by the mechanism proposed by Furman et al. for reverse transcriptase (17), due to the fact that Colo 205 enzyme catalyzing both α1,3 and α1,4-L-fucose transfer may have two binding sites, one being near or within the catalytic site.

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