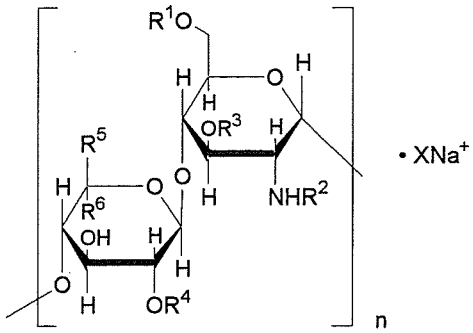

R^1, R^3, R^4= SO_3^{2-} or H

R^2= SO_3^{2-} or CH_3CO

R^5= CO_2^-, R^6= H

or

R^5= H, R^6= CO_2^-

n = 4-21

図1 パルナパリンナトリウムの構造

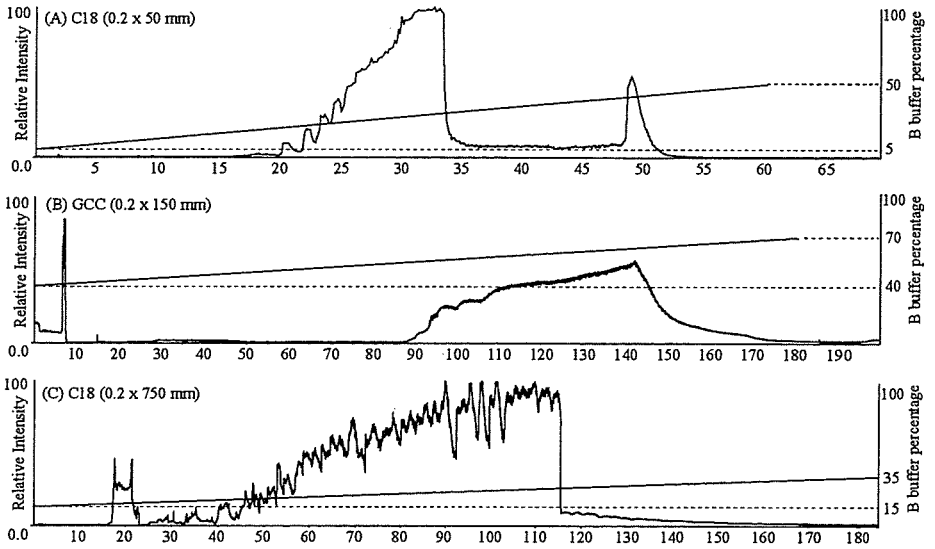

図2 バルナバリンナトリウムのnanoLC/ESI-TOFMSにより得られたトータルイオンクロマトグラム (TIC) の比較. (A) C18カラム (0.2 x 50 mm), グラジュエント条件：5-50% B (60 分間), (B) グラファイトカーボンカラム (GCC, 0.2 x 150 mm), グラジュエント条件：40-70% B (180 分間), (C) C18 カラム (0.2 x 750 mm), グラジュエント条件：15-35% B (180 分間).

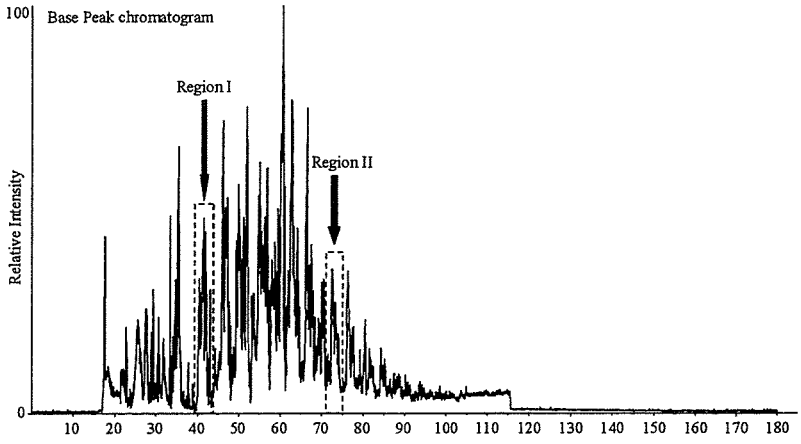

図3 パルナパリンナトリウムのC18 (0.2 x 750 mm) カラムを用いたnanoLC/ESI-TOFMS により得られたベースピーククロマトグラム.

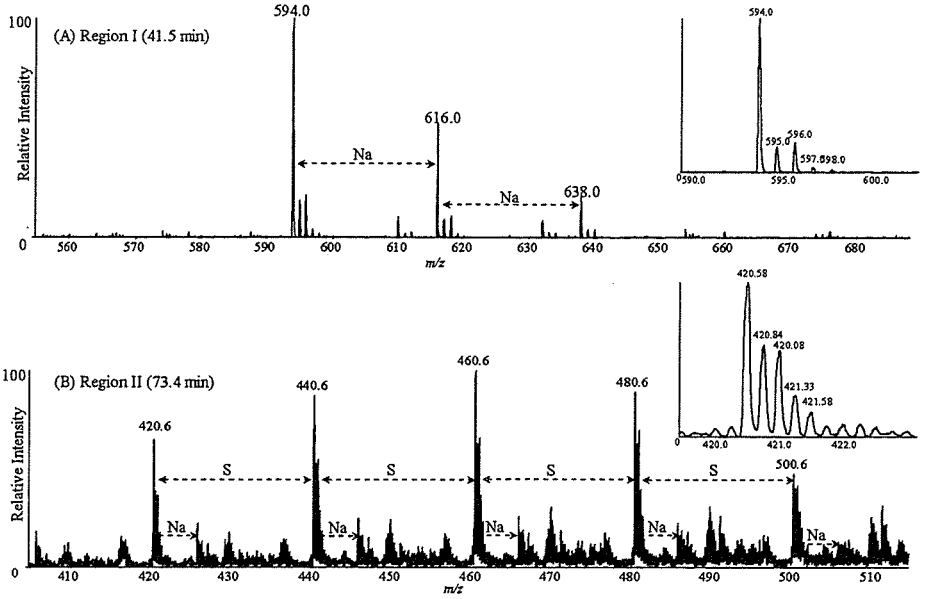

図4 GAG 糖鎖の解析. (A) 41.5 分付近に検出されたイオンのマススペクトル. (B) 73.4 分付近に検出されたイオンのマススペクトル. Na : ナトリウム, S : 硫酸基.

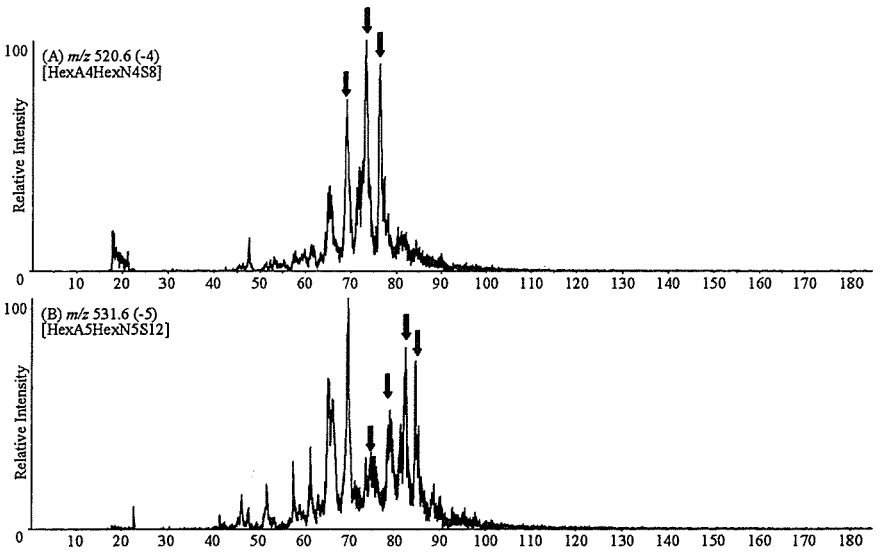

図5 エクストラクテッドイオンクロマトグラム (EIC) による異性体の解析．(A) *m/z* 520.6 の EIC, (B) *m/z* 531.6 のEIC．矢印で示したピークは 同位体が検出されたピーク．HexA：グルクロン酸またはイズロン酸，HexN：グルコサミン，S：硫酸基．

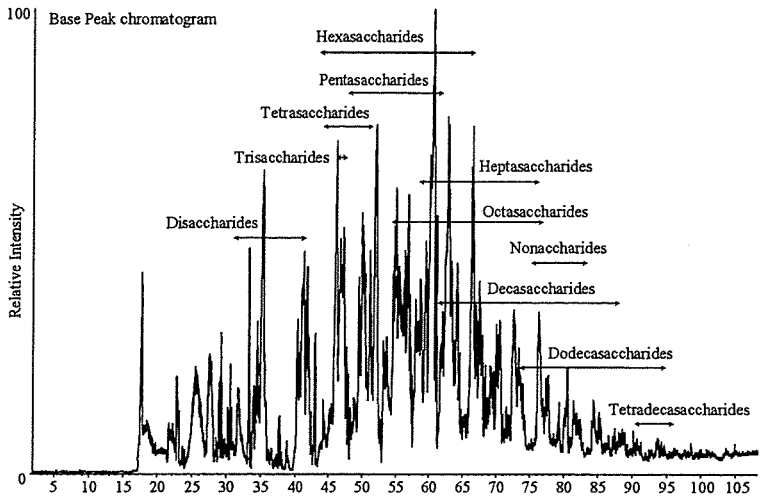

図6 糖鎖の長さと溶出時間の関係．矢印は、各糖鎖が溶出された範囲を表す。クロマトグラムはパルナパリンナトリウムのC18 (0.2 x 750 mm) カラムを用いたnanoLC/ESI-TOFMS により得られたベースピーククロマトグラム (図3 の拡大).

表1 バルナパリンナトリウムのnanoLC/ESI-TOFMS(negative ion mode)により得られた主要単糖

Oligosaccharide size	Eluted times (min)	Observed ion (m/z)	charge (negative)	The number of residues	Composition[a] HexA	HexN	HexNAc	Sulfate	Sulfates/unit[b]	Theoretical Mass[c] (Da)	Caluculated Mass[c] (Da)	Error
Disaccharides	31.7,34.5	434.13	1	2	1	1	0	1	1.0	435.07	435.13	-0.06
	34.5,41.1	514.05	1	2	1	1	0	2	2.0	515.02	515.05	-0.03
	34.5,41.1	514.05	1	2	1	1	0	2	2.0	515.02	515.05	-0.03
	40.4,41.3	594.04	1	2	1	1	0	3	3.0	594.98	595.04	-0.06
Trisaccharide	46.7,47.3	424.56	2	3	2	1	0	4	2.7	850.97	851.12	-0.15
Tetrasaccharides	44.2,45.1,49.5,51.2	505.07	2	4	2	2	0	4	2.0	1012.04	1012.14	-0.10
	48.7,49.6,51.2	545.05	2	4	2	2	0	5	2.5	1092.00	1092.10	-0.10
	55.0,56.3	585.03	2	4	2	2	0	6	3.0	1171.95	1172.06	-0.11
Pentasaccharides	48.3,53.2	625.57	2	5	2	3	0	5	2.0	1253.06	1253.14	-0.08
	53.2,58.7	665.60	2	5	2	3	0	6	2.4	1333.02	1333.20	-0.18
	58.7,67.6	705.55	2	5	2	3	0	7	2.8	1412.98	1413.10	-0.12
	62.8	745.52	2	5	2	3	0	8	3.2	1492.94	1493.04	-0.11
	51	633.06	2	5	3	2	0	5	2.0	1268.03	1268.12	-0.09
	55.5	673.04	2	5	3	2	0	6	2.4	1347.98	1348.08	-0.10
	60.3	713.02	2	5	3	2	0	7	2.8	1427.94	1428.04	-0.10
Hexasaccharides	43.6,45.3	694.62	2	6	3	2	1	4	1.3	1391.15	1391.24	-0.09
	48.5,49.4	734.60	2	6	3	2	1	5	1.7	1471.11	1471.20	-0.09
	53	774.58	2	6	3	2	1	6	2.0	1551.06	1551.16	-0.10
	53.3	713.56	2	6	3	3	0	5	1.7	1429.10	1429.12	-0.02
	53.4,58.8	753.57	2	6	3	3	0	6	2.0	1509.05	1509.14	-0.09
	53.8,58.7	793.56	2	6	3	3	0	7	2.3	1589.01	1589.12	-0.11
	61.7,63.3,66.5,67.5	555.39	3	6	3	3	0	8	2.7	1668.97	1669.17	-0.20
Hepnasaccharides	58.7	555.74	3	7	3	4	0	6	1.7	1670.12	1670.22	-0.10
	61.4	582.43	3	7	3	4	0	7	2.0	1750.08	1750.29	-0.21
	61.4	609.07	3	7	3	4	0	8	2.3	1830.04	1830.21	-0.17
	65.1,68.8	635.73	3	7	3	4	0	9	2.6	1909.99	1910.19	-0.20
	68.6	662.38	3	7	3	4	0	10	2.9	1989.95	1990.14	-0.19
	61.8,63.2,67.3	614.07	3	7	4	3	0	8	2.3	1845.00	1845.21	-0.21
	66.8	640.70	3	7	4	3	0	9	2.6	1924.96	1925.10	-0.14
	76.8	667.32	3	7	4	3	0	10	2.9	2004.91	2004.96	-0.05
Octasaccharides	53.1,57.7	628.48	3	8	4	3	1	6	1.5	1888.17	1888.44	-0.28
	57.7	655.10	3	8	4	3	1	7	1.8	1968.12	1968.30	-0.18
	61.7	681.75	3	8	4	3	1	8	2.0	2048.08	2048.25	-0.17
	73.6,75.4	420.61	4	8	4	4	0	4	1.0	1686.24	1686.44	-0.20
	73.6,75.5	440.62	4	8	4	4	0	5	1.3	1766.20	1766.48	-0.28
	73.6,76.5	460.61	4	8	4	4	0	6	1.5	1846.15	1846.44	-0.29
	73.6,76.5	480.61	4	8	4	4	0	7	1.8	1926.11	1926.44	-0.33
	69.2,73.3,76.2	500.58	4	8	4	4	0	8	2.0	2006.07	2006.32	-0.25

	69.0,73.3,76.3	520.58	4	8	4	4	0	9	2.3	2086.02	2086.32	-0.30
	69.0,73.3,76.3	540.56	4	8	4	4	0	10	2.5	2165.98	2166.24	-0.26
	73.3,76.3	560.56	4	8	4	4	0	11	2.8	2245.94	2246.24	-0.30
	73.3,76.3	580.55	4	8	4	4	0	12	3.0	2325.90	2326.20	-0.30
nonasaccharides	75.0,78.1	620.81	4	9	4	5	0	12	2.7	2486.96	2487.24	-0.28
	78.1,81.5	640.83	4	9	4	5	0	13	2.9	2566.92	2567.32	-0.40
	81.5,83.8	660.81	4	9	4	5	0	14	3.1	2646.88	2647.24	-0.36
	73.6,77.2	584.55	4	9	5	4	0	10	2.2	2342.01	2342.20	-0.19
	77.3,80.5	604.55	4	9	5	4	0	11	2.4	2421.97	2422.20	-0.23
	78	624.54	4	9	5	4	0	12	2.7	2501.93	2502.16	-0.23
Decasaccharides	61.6	595.33	4	10	5	4	1	8	1.6	2385.18	2385.32	-0.14
	63.5,65	615.37	4	10	5	4	1	9	1.8	2465.14	2465.48	-0.34
	68.7,72.3	635.35	4	10	5	4	1	10	2.0	2545.09	2545.40	-0.31
	72.3	655.33	4	10	5	4	1	11	2.2	2625.05	2625.32	-0.27
	75.2	675.30	4	10	5	4	1	12	2.4	2705.01	2705.20	-0.19
	75.2,78.5,80.1	604.82	4	10	5	5	0	9	1.8	2423.13	2423.28	-0.15
	75.2,80.5	499.67	5	10	5	5	0	10	2.0	2503.08	2503.35	-0.27
	75.1,78.7,82.2,84.3	515.69	5	10	5	5	0	11	2.2	2583.04	2583.45	-0.41
	75.1,78.7,82.3,84.6	531.62	5	10	5	5	0	12	2.4	2663.00	2663.10	-0.10
	79.0,82.1,84.4,85.1	547.62	5	10	5	5	0	13	2.6	2742.95	2743.10	-0.15
	81.2,82.2,84.3,85.1	563.65	5	10	5	5	0	14	2.8	2822.91	2823.25	-0.34
	84.2,88.6	579.59	5	10	5	5	0	15	3.0	2902.87	2902.95	-0.08
Dodecasaccharides	73.7	506.07	6	12	6	5	1	12	2.0	3042.11	3042.42	-0.31
	87.4,90.1	499.06	6	12	6	6	0	12	2.0	3000.10	3000.36	-0.26
	87.5,90.3	512.38	6	12	6	6	0	13	2.2	3080.05	3080.28	-0.23
	87.4,90.1,93.8	525.69	6	12	6	6	0	14	2.3	3160.01	3160.14	-0.13
	86.2,87.9,90.2	539.03	6	12	6	6	0	15	2.5	3239.97	3240.18	-0.21
	88.1,90.3	552.39	6	12	6	6	0	16	2.7	3319.92	3320.34	-0.42
	90.1,92.6,94.7	565.69	6	12	6	6	0	17	2.8	3399.88	3400.14	-0.26
	92.7,94.7	578.90	6	12	6	6	0	18	3.0	3479.84	3479.40	0.44
Tetradecasaccharides	90.0,92.7	592.32	6	12	6	6	0	19	3.2	3559.79	3559.92	-0.13
	89.8,93.0	581.84	6	14	7	7	0	14	2.0	3497.11	3497.04	0.07
	89.8,93.0	595.25	6	14	7	7	0	15	2.1	3577.07	3577.50	-0.43
	89.8,93.8	608.51	6	14	7	7	0	16	2.3	3657.03	3657.06	-0.03
	89.9,93.8	621.88	6	14	7	7	0	17	2.4	3736.98	3737.28	-0.30
	94.8	635.20	6	14	7	7	0	18	2.6	3816.94	3817.20	-0.26

a) HexA: グルクロン酸及びイズロン酸, HexN: グルコサミン, HexNAc: *N*-アセチルグルコサミン, sulfate: 硫酸基. b) 繰り返し構造あたりの硫酸基の数. c) Monoisotopic values.

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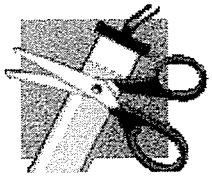
Glycotechnology

Japanese

Application of Bioinformatics to Glycoresearch: Glycoinformatics



The realm of bioinformatics has markedly advanced to analyze huge amounts of genome sequence data which are the blueprints of life. To understand systematically the functions of glycans as informational molecules not coded in DNA, it is necessary to construct databases by collecting basic data of glycans, genes for proteins which participate in synthesis or degradation of glycans, and molecules interactive with glycans in order to obtain useful information from the database. Constructing databases is proceeding internationally in the glycoscience field. Researchers can browse and retrieve data in the database and withdraw information concerning the classification of glycans, molecular evolution (see The Evolutionary History of Glycosyltransferase Genes), and relationship with hereditary diseases, etc., using the statistical approaches of bioinformatics so as to make the best of for a particular research project. Moreover, such an approach may lead to the prediction and discovery of novel substances, properties, or dynamic changes in the system, resulting in great advances in the glycoscience field. Some of the public databases focusing on glycans are introduced here with their characteristics and applications.

Glycan Structure Database

The Kyoto Encyclopedia of Genes and Genomes (KEGG)/GLYCAN utilized the graph-theoretic approach to draw glycan structures which enabled scoring of structural homologies between glycans and searching for similar glycan structures. The direct link with the KEGG Pathway database makes information on biosynthetic and metabolic pathways of glycans and participating enzymes accessible to users. Utilizing the bioinformatics approach, a repertoire of glycan structures of the organism has been predicted from the expression profiles of glycosyltransferases in the transcriptome and glycan-related pathways (1). Moreover, CarbBank (CCSD, now discontinued) which is the first worldwide carbohydrate database is available via several databases including KEGG. LIPIDBANK for Web provides information on related glycolipids, biological activities, genes, etc., by inputting a glycan structure.

Glycan-related enzymes and carbohydrate-binding proteins

The CAZy database (CAZy) describes the families of catalytic and carbohydrate-binding modules or functional domains of enzymes that degrade, modify, or create glycosidic bonds. The Glycogene Database (GGDB) provides comprehensive information on human glycogenes focusing on glycosyltransferases and sugar transporters. Based on these databases, the prediction of novel genes (*in silico* cloning) has been done and the common characteristics among carbohydrate-binding modules has been drawn out from bioinformatic analyses of glycosyltransferase families (2). The 3D lectin database consists of information on lectins from various origins. Every database is linked to versatile sites such as PDB (protein data bank) to offer a wide range of related information on proteins and genes.

Consortium for Functional Glycomics is a research initiative to understand the role of carbohydrate-protein interactions at the cell surface in cell-cell communication. It is divided into four categories, i.e., Central (glycan mass data, lectin-ligand interactions, mouse phenotype, glycan profiling of tissue, glycogene, etc.), CBP (carbohydrate-binding proteins), GT (glycosyltransferase) and Glycan (structure and biological activities), and also provides resources such as glycan array, glycogene chips, glycosyltransferases and mutant animals (3).

There are many open databases useful for glycoscientists besides the above, and their number and mutual links among them are increasing. Research will be greatly promoted by additions to the database and using the predictions made by computer calculation which should be performed by various researchers in experimental and bioinformatics fields cooperating with each other. The incorporation of glycoinformatics into systems glycobiology where the bioorganisms are viewed as biomolecular networks will open a paradigm of understanding complex glycan functions in the cell, organ, and individual organism.

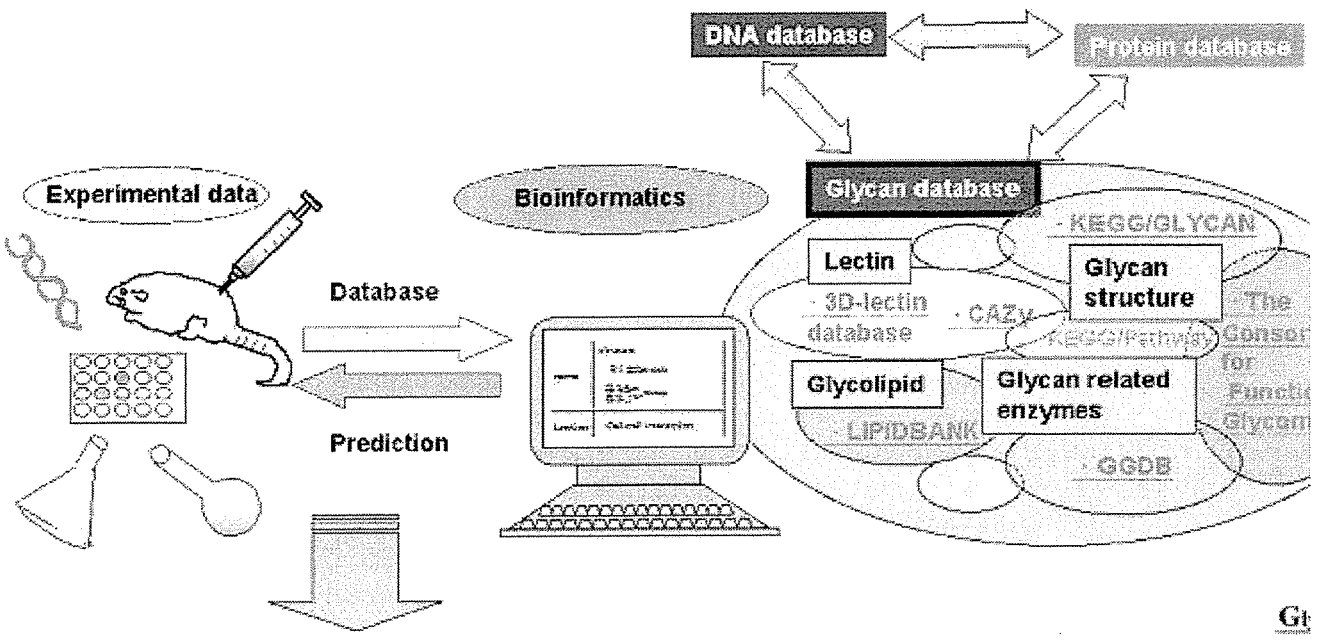


Figure The understanding of the complex interaction of all levels of biological glycan informati

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Novel Carbohydrate-binding Activity of Pancreatic Trypsins to *N*-Linked Glycans of Glycoproteins*

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How glycosylation affects the reactivity of proteins to trypsin is not well understood. Bovine and porcine pancreatic trypsins were discovered to bind to α-Man, Neu5Acα2,6Galβ1,4Glc, and α-galactose sequences by binding studies with biotinylated sugar-polymers. Quantitative kinetic studies supported that phenylmethylsulfonyl fluoride (PMSF)-treated trypsin binds to glycolipid analogues possessing α-Man or α-NeuAc but not to those possessing β-galactose or β-GlcNAc residue. Enzyme-linked immunosorbent assay (ELISA) showed that trypsin binds to six kinds of biotinylated glycoproteins possessing high mannose-type and complex-type *N*-glycans but not to bovine submaxillary mucin, which possesses only *O*-glycans. Further, the binding of trypsin to glycoproteins was differentially changed by treatments with sequential exoglycosidases, endoglycosidase H, or *N*-glycosidase F. Quantitative kinetic studies indicated that PMSF-treated trypsin binds with bovine thyroglobulin with the affinity constant of 10^{10} M^{-1}, which was the highest among the glycoproteins examined, and that α-galactosidase treatment decreased it to 10^5 M^{-1}. PMSF-treated trypsin bound to other glycoproteins, including ovomucoid, a trypsin inhibitor, with the affinity constants of 10^8-10^5 mol^{-1} and were markedly changed by glycosidase treatments in manners consistent with the sugar-binding specificities suggested by ELISA. Thus, the binding site for glycans was shown to be distinct from the catalytic site, allowing trypsin to function as an uncompetitive activator in the hydrolysis of a synthetic peptide substrate. Correspondingly the carbohydrate-binding activities of trypsin were unaffected by treatment with PMSF or soybean trypsin inhibitor. The results indicate the presence of an allosteric regulatory site on trypsin that sugar-specifically interacts with glycoproteins in addition to the proteolytic catalytic site.

Numerous biological phenomena are mediated by recognition of specific oligosaccharide signals. This recognition implies quality control in polypeptide folding, cellular interactions, and protein targeting (1–3). In contrast, some functions of protein glycosylation seem to be widely applicable to various types of glycosylation, for example, protecting against proteolysis, stabilizing active conformations, and affording solubility to proteins (3). These functions have been attributed to ambiguous steric effects of glycosylation in the absence of clear structural specificity, but the involvement of glycan recognition in achieving these functions has not yet been elucidated. Clarification of the molecular mechanism by which glycosylation plays a role in protecting or stabilizing the active conformation of proteins would enable the use of glycosylation in molecular engineering of recombinant products for therapeutic purposes.

Trypsin is a principal pancreatic serine protease that plays a key role in digestion in the duodenum by activating zymogens and degrading dietary proteins. Trypsin acts specifically on peptide bonds of the carboxyl side of positively charged lysine and arginine and catalyzes the activation of many pancreatic proenzymes, such as trypsinogen, chymotrypsinogen, proelastase, and carboxypeptidase, and protease-activated receptors to control digestive efficiency in the intestines (4, 5). When, however, trypsin is activated in the pancreas, the activated proteinases induce the destruction of pancreatic cells. The modulation of trypsin activity is therefore important for controlling digestive efficiency and preventing pancreatitis.

Porcine pancreatic α-amylase (PPA)[2] is activated by interaction with glycoproteins. Previously we reported that PPA exhibits carbohydrate-binding activity toward *N*-glycans of glycoproteins (6). To further elucidate the biological functions of the carbohydrate-binding activity found in PPA, we investigated whether other pancreatic digestive enzymes possess similar carbohydrate-binding activity. In this study, we found that trypsin exhibits remarkable carbohydrate-binding activities to the sequences present in the *N*-glycans of glycoproteins with a specificity distinct from PPA. This finding provides new insights into the interaction between the proteases and glycoproteins related to protease resistance and the biological functions of carbohydrate-specific interactions in the digestive organs.

EXPERIMENTAL PROCEDURES

Materials—Porcine pancreatic trypsin (PPT), *N*-α-benzoyl-L-arginine ethyl ester (BAEE), *N*-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPA), soybean trypsin inhibitor, bovine serum albumin (BSA), 3,3′-diaminobenzidine tetrahydrochloride, methyl-α-D-mannoside, and mannitol were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Bovine pancreatic trypsin (BPT), bovine submaxillary gland mucin (BSM), human holotransferrin, fetuin from fetal calf serum, hen ovomucoid, human orosomucoid, bovine thyroglobulin, streptavidin-biotinylated horseradish peroxidase complex (ABC complex), and 4-nitrophenyl phosphate magnesium salt were purchased from Sigma. Sugar-biotinylated polyacrylamide probes (sugar-BP probes) were purchased from Lectinity Holdings, Inc. Moscow,

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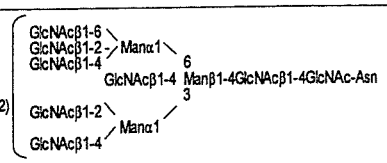
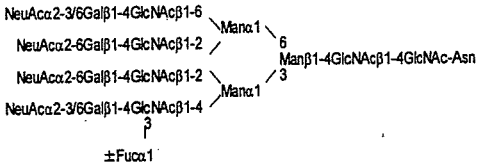
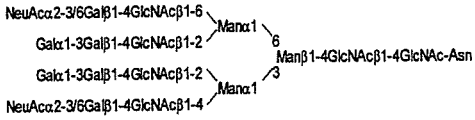
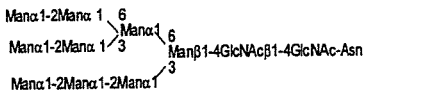
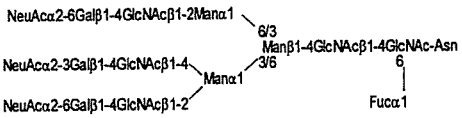
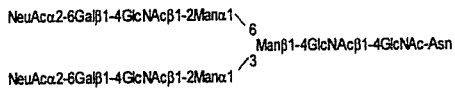
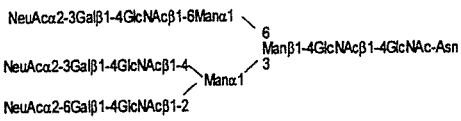
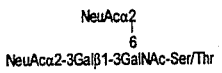
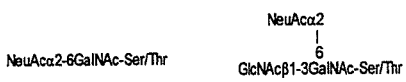
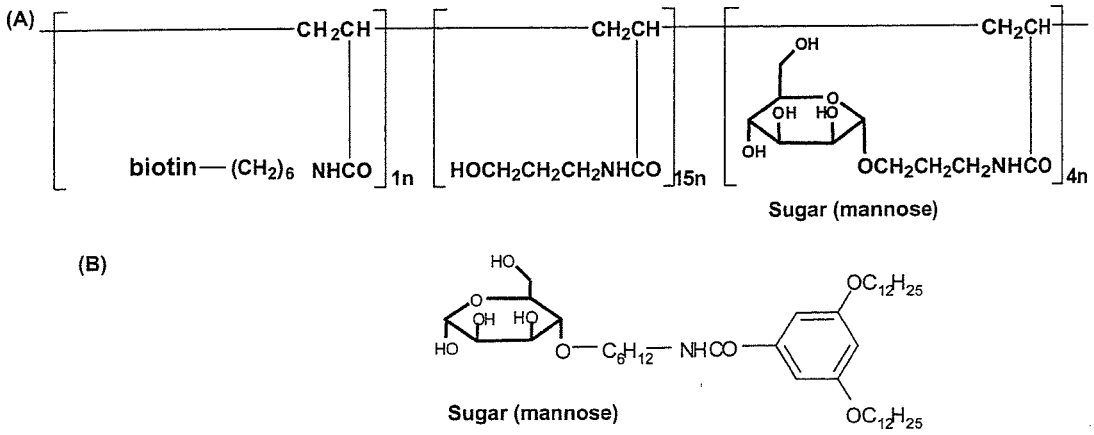
[2] The abbreviations used are: PPA, porcine pancreatic α-amylase; BPT, bovine pancreatic trypsin; PPT, porcine pancreatic trypsin; BAEE, *N*-α-benzoyl-L-arginine ethyl ester; BAPA, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride; BSA, bovine serum albumin; BSM, bovine submaxillary mucin; ABC complex; streptavidin-biotinylated horseradish peroxidase complex; sugar-BP probe, sugar-biotinylated polyacrylamide probe; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline; K_A, affinity constant; k_a, association rate constant; k_d, dissociation rate constant; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; RU, resonance units; Me, methyl.

		Total carbohydrate contents (%, w/w)	Reference
Ovomucoid (hen)		25~30%	(30)
Orosomucoid (human)		36%	(31)
Bovine thyroglobulin		10%	(32,33)
Porcine thyroglobulin		10%	(34-36)
			
Transferrin (human)		5%	(37)
Fetuin (bovine)		22%	(38)
			(39)
BSM		57%	(40, 41)

SCHEME 1. **Major oligosaccharide structures of glycoproteins used in this study.**

Russia, except β-D-galactose-3-sulfate, which was purchased from Seikagaku Corp., Tokyo, Japan. Porcine thyroglobulin and *Galanthus nivalis* lectin were purchased from Cosmo Bio Co., Ltd, Tokyo, Japan. Neuraminidase from *Vibrio cholerae*, *N*-glycosidase F from *Flavobacterium meningosepticum*, and *O*-glycosidase were purchased from Roche Diagnostics Corp., Inc. (Indianapolis, IN). *Psathyrella velutina* lectin was prepared in our laboratory (7). β-Galactosidase and β-*N*-acetylhexosaminidase from jack bean and α-galactosidase from *Mortierella vinacea* were purchased from Seikagaku Corp. EZ-Link sulfo-*N*-hydroxysuccinimide-biotin and *Sambucus nigra* bark lectin were purchased from Funakoshi Co. Ltd., Tokyo, Japan. Phenylmethylsulfonyl fluoride (PMSF) and methyl-α-D-galactoside were purchased from Nacalai Tesque, Inc., Kyoto, Japan. *N*-Heptyl-β-D-thioglucoside was purchased from Dojindo Laboratories, Kumamoto, Japan. Peanut lectin and *Ricinus communis* agglutinin I were purchased from Seikagaku Corp. Lactose was purchased from Kanto Kagaku, Tokyo, Japan. SDS-PAGE molecular weight standards were purchased from Bio-Rad.

Preparation of Glycoprotein Probes—All biotinylated glycoprotein probes, their deglycosylated derivatives, and biotinyl lectins were prepared in our laboratory. Biotinylation was performed using EZ-link™ sulfo-*N*-hydroxysuccinimide-biotin according to the instruction manual. Briefly, 2 mg of each glycoprotein was dissolved in 1 ml of 50 mM sodium bicarbonate buffer (pH 8.5), and 74 μl of sulfo-NHS-biotin (1 mg/ml) was added. After incubation for 30 min at room temperature, the reactant was dialyzed against water to remove excess biotin. Asialoglycoproteins, asialoagalactoglycoproteins, and asialoagalactoahexosaminoglycoproteins were prepared from biotinylated glycoproteins by sequential glycosidase treatments with neuraminidase (0.1 units/mg glycoprotein) in 20 mM acetate-buffered saline (pH 5.5), β-galactosidase (0.14 units/mg glycoprotein) in 50 mM sodium-citrate buffer (pH 3.5) overnight, and then β-*N*-acetylhexosaminidase (1.43 units/mg of glycoprotein) in 50 mM sodium citrate buffer (pH 5.0) at 37 °C overnight. The glycan structures and carbohydrate concentrations of the glycoproteins used in this study are summarized in Scheme 1. Besides the sequential treatments described above, biotinylated bovine thyroglobulin was treated with α-galactosidase (0.14 unit/mg of glycoprotein) in 20 mM acetate-buffered saline (pH 5.5) for agalactosylation of the major oligosaccharide, Galα1–3Galβ1–4GlcNAc. Fetuin was treated with *N*-glycosidase F (600 units/mg of glycoprotein) in 10 mM Tris-buffered saline (TBS) at pH 7, or de-*O*-glycosylated with a mixture of neuraminidase



SCHEME 2. **Structures of sugar-BP probes and glycolipid analogues used in this study.**

(0.1 unit/mg of glycoprotein) and *O*-glycosidase (2 milliunits/mg of glycoprotein) in 10 mM acetate buffer of pH 6 at 37 °C overnight.

Biotinylated porcine thyroglobulin (20 μg) was denatured in glycoprotein denaturing buffer (5% SDS, 10% β-mercaptoethanol) at 100 °C for 10 min, a 10% volume of 0.5 M sodium citrate buffer (pH 5.5) was added, and then it was incubated with 1500 units of endoglycosidase H at 37 °C overnight. Deglycosylation of all biotinylated glycoprotein probes was checked by ELISA for a change in reactivity with *Ricinus communis* agglutinin I for agalactosylation, *Psathyrella velutina* lectin for desialylation and ahexosaminylation, *Galanthus nivalis* lectin for endoglycosidase H treatment, and peanut lectin for de-*O*-glycosylation to recognize each carbohydrate structure, and by mobility on SDS-PAGE to demonstrate the decrease in molecular weight (data not shown).

SDS-PAGE—To check the purity of trypsin, SDS-PAGE was performed according to the method of Laemmli (8) using a 14% gel in the presence of 2-mercaptoethanol. The bovine and porcine trypsins (10, 20, or 40 μg of protein per lane) were loaded onto the gel together with a set of markers and run at 20 mA for 1.5 h. After electrophoresis, protein bands were visualized by Coomassie Brilliant Blue R-250.

Binding Studies with Sugar-BP Probes or Biotinylated Glycoprotein Probes—PPT and BPT were preincubated in the presence or absence of 0.5 mM PMSF, 0.5 mM soybean trypsin inhibitor, or 5 mM EDTA in 10 mM TBS (pH 7.5) for 1 h and then immobilized at concentrations of 0.01–0.5 μg/100 μl in wells of a microtiter plate (Immulon 1, Dynatech Laboratories) at 4 °C overnight. All other procedures were performed at room temperature using 10 mM TBS (pH 7.5) as the dilution buffer. After immobilization, the wells were blocked with 3% BSA for 2 h. Aliquots (100 μl) of various sugar-BP probes (shown in Scheme 1*A*) or biotinylated glycoprotein probes at concentrations of 10 μg/ml were added to each well, followed by incubation for 1 h. After incubation, the wells were washed three times, and 100 μl of ABC complex (1 μg/ml) was added, and the mixture was incubated for 1 h. After washing three times, color was developed by adding 200 μl of *o*-phenylenediamine/H_2O_2, and then 50 μl of 2.5 M H_2SO_4 was added to stop the reaction. Absorbance was measured with a microplate reader (Bio-Rad MPR-80) at 490 nm.

Quantification of Interactions between Trypsins and Glycolipid Analogues by Surface Plasmon Resonance—For binding studies between trypsins and glycolipid analogues or various glycoproteins, a BIAcore 2000 SPR apparatus (BIAcore AB, Uppsala, Sweden) was used. Structure of glycolipid analogues used in this study was illustrated in Scheme 2*B*, and their synthesis will be described elsewhere. Glycolipid analogues were immobilized on a HPA sensor chip (BIAcore AB) by preparing liposomes containing each glycolipid analogue/phosphatidylcholine at a molar ratio of 40/60 as described previously (9, 10). PMSF-treated PPT

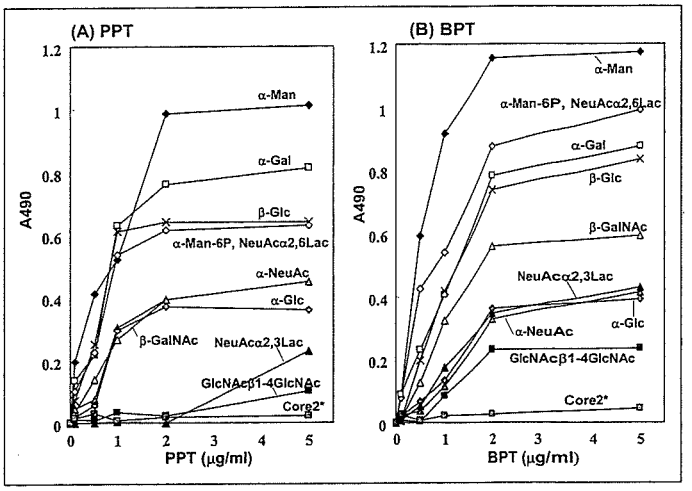


FIGURE 1. **Reactivities of PPT (*A*) and BPT (*B*) toward sugar-BP probes by ELISA.** PPT and BPT (100 μl) were coated onto the wells of a microtiter plate and reacted with various sugar-BP probes as described in the text. The bound sugar-BP probes were detected with ABC complex and *o*-phenylenediamine/H_2O_2 by ELISA. Symbols used are: ◆, α-Man-BP; ◇, α-Man-6-phosphate-BP and Neu5Ac α2–6Galβ1–4Glc-BP; □, α-Gal-BP; ×, β-Glc-BP; △, β-GalNAc-BP; ▲, Neu5Ac α2–3Galβ1–4Glc-BP; Δ, α-Neu5Ac-BP; ⊕, α-Glc-BP; ■, GlcNAcβ1–4GlcNAc-BP; ▨, GlcNAcβ1–6(Galβ1–3)GalNAc (core2)-BP, α-GalNAc-, β-Gal-, LacNAc-, Lac-, β-GlcNAc-, and β-Gal-3-sulfate-BP. The label "*core 2**" stands for the seven sugar-BP probes that bound very little with trypsin.

was injected onto the sensor chip at various concentrations in 10 mM TBS buffer (pH 7.5) at a flow rate of 20 μl/min at 25 °C using a BIAcore biosensor. The reference cell was prepared by immobilizing phosphatidylcholine and used to correct for bulk effect. The chip was regenerated each time by injection of 20 μl of 0.1 M phosphoric acid.

Quantification of Interactions between Trypsins and Various Glycoproteins by SPR—After equilibration of a CM5 sensor chip (BIAcore AB) with HEPES-buffered saline, the surface of the sensor chip was activated with an amine coupling kit. BPT or PPT (each 1.8 μM) in 10 mM sodium acetate buffer (pH 6) containing 0.1 mM PMSF and 0.2 M methyl α-D-mannoside was injected onto the activated surface, and then the remaining *N*-hydroxysuccinimide esters were blocked with 1.0 M ethanolamine hydrochloride (pH 8). Each step was performed for 14 min at a constant flow rate of 10 μl/min at 25 °C. The reference flow cell was prepared with BSA as a ligand.

To determine the pH dependence of the binding, fetuin, or porcine thyroglobulin were dissolved at 30 μg/ml in buffers of various pH, 10 mM acetate (pH 4.5, 5.5, and 6.5), 10 mM TBS (pH 7.0, 7.5, and 8.0), or 10 mM bicarbonate buffer (pH 9 and 10), and injected onto the trypsin-immobilized sensor chip. To measure binding curves, various glycoproteins in 10