FEBS Letters 427 (1998) 134–138 FEBS 20178

Multi-antennary Galβ1→4GlcNAc and Galβ1→3GalNAc clusters as important ligands for a lectin isolated from the sponge *Geodia cydonium*

June H. Wu[b], Shuh-Chyung Song[a], Yuen-Yuen Chen[a], Ming-Chi Tsai[a], Elvin A. Kabat[c], Albert M. Wu[a,*]

[a] *Glyco-Immunochemistry Research Laboratory, Institute of Molecular and Cellular Biology, Chang-Gung Medical College, Kwei-san, Tao-yuan 333, Taiwan*
[b] *Department of Microbiology and Immunology, Chang-Gung Medical College, Kwei-san, Tao-yuan 333, Taiwan*
[c] *Department of Microbiology, Human Genetics and Development, and Cancer Center/Institute for Cancer Research, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA*

Received 23 March 1998

Abstract The affinity of a lectin from the sponge *Geodia cydonium* (GCL-I) for multi-antennary Galβ1→4GlcNAc and Galβ1→3GalNAc ligands was studied by both the biotin/avidin-based microtiter plate lectin binding assay and the inhibition of lectin-glycoform interaction. Among the glycoforms tested for binding, GCL-I reacted strongly with three multi-antennary Galβ1→4GlcNAc clusters containing glycoproteins (asialo human and bovine α_1-acid gps and asialo fetuin), T (Galβ1→3GalNAc) rich glycoprotein from porcine salivary gland, asialo bird nest gp, and human blood group A active cyst gp, while human and bovine α_1-acid gps, fetuin, and Tn containing gps were inactive. Among the haptens tested for inhibition, tri-antennary Galβ1→4GlcNAc (Tri-II) was about 1500, 72, and 72 times more active than GalNAc, Galβ1→4GlcNAc (II), and Galβ1→3GalNAc (T), respectively.

Based on the present and previous results, it is proposed that tri-antennary Galβ1→4GlcNAc and Galβ1→3GalNAc clusters, in addition to GalNAcα1→3GalNAc and GalNAcα1→3Gal, are also important ligands for binding; and sialic acid of glycoprotein does interfere with binding.

© 1998 Federation of European Biochemical Societies.

Key words: Lectin binding property; *Geodia cydonium* lectin I; Multi-antennary Galβ1→4GlcNAc; Galβ1→3GalNAc cluster

1. Introduction

The soluble agglutinins from the marine sponge *Geodia cydonium* (GCL) are involved in the sorting-out of cells during reaggregation of allogenic cells [1]. In the early nineties, GCL-I, which is one of the important soluble agglutinins from sponge *G. cydonium* [2,3], has been sequenced on the protein and DNA levels and shown to exhibit striking homology to the carbohydrate recognition domain characteristic for mammalian galectin-1 [2,3]. The lectin molecule binds two moles of lactose per mole of native lectin in a calcium independent manner [4]. Recently, it was shown that GCL binds with high affinity for the blood group A trisaccharide and even more strongly for the Forssman di- and pentasaccharides [5]. In order to clarify the relationship of the affinity of this lectin for various ligands reported by different investigators [2,4,5], the combining site of GCL-I was here further characterized by both lectin-enzyme binding assay and inhibition profile of lectin-glycoform interaction. The results provide evidence that multi-antennary Galβ1→4GlcNAc and Galβ1→3GalNAc clusters, in addition to GalNAcα1→ derivatives at nonreducing ends, also play a major role in binding GCL-I.

*Corresponding author. Fax: +886 (3) 328-6456 (Lab.); +886 (3) 328-3031 (Col.).

Abbreviations: Gal, D-galactopyranose; Glc, D-glucopyranose; LFuc, L-fucopyranose; GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; F, GalNAcα1→3GalNAc; Fα, GalNAcα1→3GalNAcα1→Ser/Thr; A_h, GalNAcα1→3(LFucα1→2)Gal; II, Galβ1→4GlcNAc; mII, multivalent II, including bi-, tri- and tetra-antennary; Tα, Galβ1→3GalNAcα1→Ser/Thr; cTα, cluster of Tα, indicating Tα containing glycopeptides; Tri-II, tri-antennary Galβ1→4GlcNAc:

```
                Galβ1→4GlcNAcβ1→2Man
                                 ↓ α1,6
Galβ1→4GlcNAcβ1→2Manα1→3Manβ1→4GlcNAcβ1→4GlcNAcβ1-N-Asn
                                 ↑ β1,4
                Galβ1→4(3)GlcNAc
```

; GCL-I, *Geodia cydonium* lectin I; ELLSA, enzyme linked lectinosorbent assay

2. Materials and methods

2.1. Lectin

The sponge *G. cydonium* was collected in the Mediterranean Sea near Banyuls sur Mer, France. The sponge tissue was minced and dried at 35° to 40°C. The dried material was homogenized in a blender and stored at −20°C until use. To prepare saline extracts, 50 g of sponge tissue were stirred with 1 l of 1 N NaCl for 16 h at 4°C. One normality (1 N) NaCl solutions were used because the lectin precipitated in 0.9% NaCl during concentration. The extract was centrifuged at 2000 rpm for 30 min and then for 5 h at 15 000 rpm in the cold. The dark brown supernatant was passed through a 45-μm pore size Millipore filter; 0.02% NaN_3 was added to this crude extract as a preservative.

G. cydonium lectin I was isolated by adsorption to a column of hog A+H coupled to Sepharose 4B and eluted by 0.2 M lactose [2].

2.2. Glycoproteins and polysaccharide

Fetuin, human and bovine α_1-acid glycoprotein, and mannan were purchased from Sigma Chemical Co. (St. Louis, MO). MSS and Mcdon cyst human blood group A active gps were prepared from human ovarian cyst fluid as described previously [6–9]. Porcine and ovine salivary glycoproteins were purified according to the method of Tettamanti and Pigman [10] and its modification [10–12]. Hamster submaxillary glycoprotein and its preparation are described by Downs et al. [13] and Wu et al. [14]. The crude salivary gland mucus glycoprotein (native BN gp) of the Chinese swiftlet (genus *Collocalia*) was extracted at 66°C with distilled H_2O for 20 min from commercial bird nest (BN) [15,16]. For desialylation, a glycoprotein sample in 0.01 N HCl was hydrolyzed at 80°C for 90 min and dialyzed against distilled H_2O [10,14,17]. Native ASG-Tn, an armadillo salivary glycoprotein containing only Tn (GalNAcα1→Ser/Thr) as carbohydrate side

0014-5793/98/$19.00 © 1998 Federation of European Biochemical Societies. All rights reserved.
PII S0014-5793(98)00411-6

chains, was isolated from the 0.01 M PBS pH 6.8 gland extract after removal of ASG-A, which is one of the sialic acid containing glycoproteins in armadillo glands [17–19].

2.3. Sugar inhibitors

Monosaccharides, their derivatives and most oligosaccharides were purchased from Sigma Chemical Company (St. Louis, MO).

Tri-antennary Galβ1→4GlcNAc glycopeptides were prepared from asialo fetuin by pronase digestion and repeatedly fractionated by Bio-Gel P-4 400 mesh, column chromatography. Peak II was used for this assay. The chemical structure of this peak was analyzed by NMR at the Complex Carbohydrate Research Center, The University of Georgia, Athens, GA, USA on a Bruker AMX500 instrument. The samples were exchanged twice with D_2O, and finally dissolved in 0.5 ml 99.96% D_2O. Acetone was added as an internal standard. The proton one-dimensional spectra were obtained at 25°C at 500.139 MHz. The signal due to residual HDO at δ 4.78 was suppressed with low power preirradiation at that frequency. Chemical shifts were referenced to the internal standard, acetone, at δ 2.225.

2.4. Lectinochemical assays

The assay was performed according to the procedures described by Duk et al. [20]. The volume of each reagent applied to the plate was 50 μl/well, and all incubations, except for coating, were performed at 20°C. The reagents, if not indicated otherwise, were diluted with TBS containing 0.05% Tween 20 (TBS-T). The TBS buffer or 0.15 M NaCl containing 0.05% Tween 20 was used for washing the plate between incubations.

For inhibition studies, the serially diluted inhibitor samples were mixed with an equal volume of lectin solution containing a fixed amount of GCL-I. The control lectin sample was diluted two-fold with TBS-T. After 30 min at 20°C, the samples were tested by the binding assay, as described by Duk et al. [20,21]. The inhibitory activity was estimated from the inhibition curve and is expressed as the amount of inhibitor (nmole/well) giving 50% inhibition of the control lectin binding.

3. Results and discussion

Sponges are a rich source of carbohydrate binding proteins. Crude extracts of the sponge *G. cydonium* contain at least two different lectins [2,3,5]. Two of them bound to affinity chromatography resins composed of hog A+H, a glycoprotein with blood group A and H activities, coupled covalently to Sepharose 4B. Lectin I could be eluted in a highly purified form with 0.2 M lactose; lectin II was removed from the column by 3 M $MgCl_2$, but the eluate may be contaminated with lectin I. *G. cydonium* lectin I has a molecular mass of about 60 000 daltons of four identical subunits [2,3]. The lectin is mitogenic for human lymphocytes in which the lymphocytes are triggered after 24 h incubation with the lectin in the absence of fetal calf serum.

In an earlier study, it was demonstrated that GCL-I precipitates well with Galβ1→3/4GlcNAc (I/II) and Galβ1→3GalNAcα1→ (Tα) containing glycoproteins [2,22–24]. Recently, studies on the cross reactivities of terminal structural carbohydrates revealed that the affinity of GCL-I for sugar ligands is in the decreasing order of GalNAcα1→3GalNAcβ > GalNAcα1→3(LFucα1→2)Galβ ≫ Galβ1→3GlcNAcβ > Galβ1→4Glc [5]. Thus, the different binding abilities of this lectin toward ligands described in previous reports [2,5,22–24] deserve to be clarified. In this communication, the combining site of GCL-I was further characterized by both lectin-enzyme binding assay and inhibition of lectin-glycoform interaction.

The binding profile of GCL-I with various glycans, studied by a microtiter plate lectin-enzyme binding assay [20,21], is shown in Fig. 1, and its avidity toward glycoproteins is summarized in Table 1. When 2 ng of biotinylated lectin was added to concentrations of glycans ranging from 0.081 ng to 1 μg, GCL-I reacted strongly with three multi-antennary Galβ1→4GlcNAc clusters containing gps (asialo human α_1-acid gp in Fig. 1a; asialo bovine α_1-acid gp in Fig. 1b; and asialo fetuin in Fig. 1c); Tα (Galβ1→3GalNAcα1→Ser/Thr) containing gp from porcine salivary gland (Fig. 1d); Fα (GalNAcα1→3GalNAcα1→Ser/Thr) containing gp (asialo bird nest gp in Fig. 1e); and two human blood group A active glycoproteins (Fig. 1f). All reactions reached A_{405} more than 2.0 within 1 h, indicating that GCL recognizes strongly all of the following ligands: multivalent Galβ1→4GlcNAc (mII); cluster forms of Galβ1→3GalNAc (cTα); GalNAcα1→3GalNAc (F); and GalNAcα1→3(LFucα1→2)Gal (A_h). Among the other glycans tested for binding, all Tn containing gps, human and bovine α_1-acid gps, fetuin, and mannan were inactive. These results suggest that: (1) GCL-I recognizes effi-

Table 1
Comparison of avidity of GCL-I lectin for human blood group A active glycoproteins as well as sialo, asialo glycoproteins and polysaccharide[a]

Curve in Fig. 1	Glycoprotein (gp) or polysaccharide (ps)	Applied lectin determinants[b]	A_{405}	Avidity[c]
a	human α_1-acid gp	sialyl mII	0.2	±
a	asialo human α_1-acid gp	mII	2.1	++++
b	bovine α_1-acid gp	sialyl mII	0.3	±
b	asialo bovine α_1-acid gp	mII	2.4	++++
c	fetuin	sialyl mII, T	0.2	±
c	asialo fetuin	mII, T	2.4	++++
d	porcine salivary gp (PSM)	sialyl A, A_h, Tα, Tn	2.0	+++
d	asialo PSM	A, A_h, Tα, Tn	3.0	++++
d	asialo HSM	Tn	0.2	±
e	bird nest gp (BN)	sialyl II, E, Tα, Fα	1.6	+++
e	asialo BN	II, E, Tα, Fα	2.4	++++
e	mannan		0.03	−
f	MSS	$A_{h,A}$	2.7	++++
f	cyst Mcdon 15% EtOH	$A_{h,A}$	2.7	++++
f	asialo OSM	Tn	0.1	−
f	native ASG-Tn	Tn	0.2	±

[a]Two ng of biotinylated lectin was added to various concentrations of glycoprotein ranging from 0.081 ng to 1 μg.
[b]The following symbols indicate the human blood group activity and/or lectin determinants [22–24]: F, GalNAcα1→3GalNAc; A, GalNAcα1→3Gal; A_h, GalNAcα1→3[LFucα1→2]Gal; E, Galα1→4Gal; T, Galβ1→3GalNAc; Tn, GalNAcα1→Ser/Thr; I/II, Galβ1→3/4GlcNAc; mII, multivalent II.
[c]The results were interpreted according to the spectrophotometric absorbance value at 405 nm (A_{405}) after 1 h incubation as follows: ++++, A_{405}: > 2.0; +++, A_{405}: 2.0–1.5; ++, A_{405}: 1.5–0.75; +, A_{405}: 0.75–0.3; ±, A_{405}: 0.3–0.1; and −, A_{405}: < 0.1.

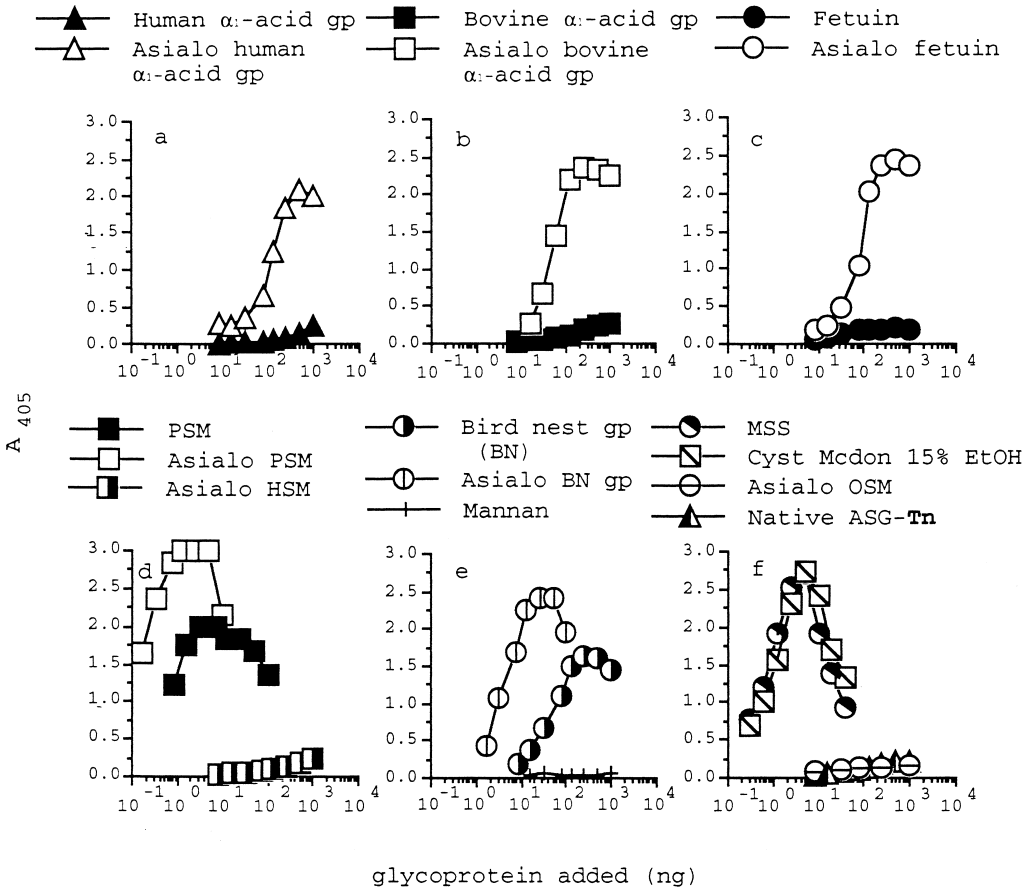

Fig. 1. Binding of GCL-I to microtiter plates coated with serially diluted glycoproteins and polysaccharide. The lectin was used at a constant amount of 2 ng per well. Total volume 50 μl. Read at 1 h.

ciently GalNAcα1→ linkages at nonreducing ends (A_h and Fα) but not Tn containing gps tested; (2) clusters of Galβ1→3GalNAc (cTα) and multivalent Galβ1→4GlcNAc (mII) are also potent ligands; (3) sialic acid does interfere with binding (masking effect on the binding).

In order to prove that the lectin-glycan interaction occurs through the above structural units (lectin determinants) [22–24], eleven sugar inhibitors, including six lectin determinants were used to inhibit the lectin-asialo bird nest gp interaction. As shown in Fig. 2 and Table 2, tri-antennary Galβ1→4GlcNAc (Tri-II) was the best inhibitory structural feature (curve 1 in Fig. 2). It was 1500 and 72 times more active than GalNAc (curve 8 in Fig. 2) and Galβ1→4GlcNAc (curve 3 in Fig. 2), respectively, while Galβ1→4GlcNAc and Galβ1→3GalNAc (curves 3 and 4), which were equally potent, were about 20 times stronger than Gal or GalNAc.

It has recently been reported that Galβ1→3GlcNAc (I) and Galβ1→4GlcNAc (II) are low affinity inhibitors as compared with F or A_h [5]. However, it was demonstrated that GCL-I precipitated strongly with Smith degraded or mild acid treated

Table 2
Amount of various saccharides giving 50% inhibition of binding of GCL-I (2 ng/50 μl) by asialo bird nest gp (10 ng/50 μl)[a]

Order of activity	Inhibitor	Quantity giving 50% inhibition (nanomoles)	Reciprocal of relative potency[b]
1	Tri-II	1.5	1.5×10^3
2	Galβ1→4Glc (L)	53	41.5
3	Galβ1→4GlcNAc (II)	105	20.9
4	Galβ1→3GalNAc (T)	110	20.0
5	Galβ1→3GlcNAc (I)	300	7.3
6	melibiose	1300	1.7
7	Gal	2200	1.0
8	GalNAc	2200	1.0
	Galα1→4Gal (E)	>243.3 (2.1% inhibition)	
	GlcNAc	>376.7 (−10.0% inhibition)	
	Glc	>2333.3 (8.0% inhibition)	

[a]The inhibitory activity was estimated from the inhibition curve in Fig. 2 and is expressed as the amount of inhibitor giving 50% inhibition. Total volume 50 μl.
[b]Reciprocal of relative potency of sugars when GalNAc was taken as 1.0 [25].

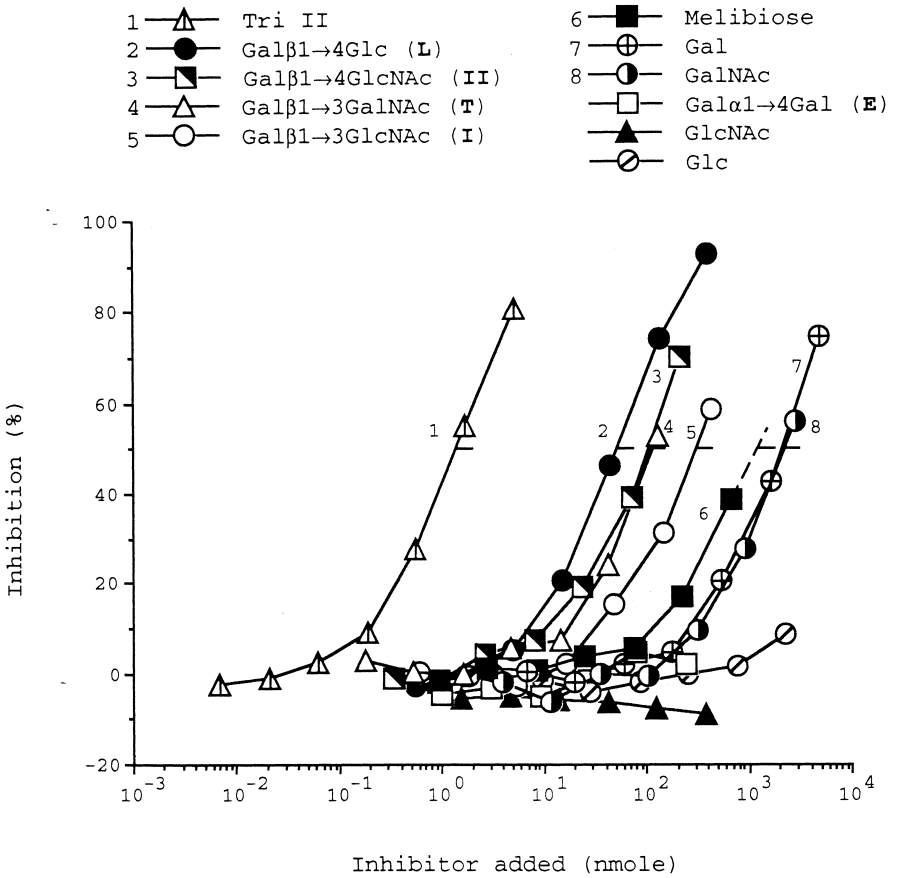

Fig. 2. Inhibition of GCL-I binding to asialo bird nest gp-coated microtiter plates by various saccharides. The amount of glycoprotein in the coating solution was 10 ng per well. The lectin was preincubated with an equal volume of serially diluted inhibitor solution. The final lectin content was 2 ng per well. Total volume, 50 μl.

cyst glycoproteins which contain no A and A_h, but a large number of branched or clustered forms of Galβ1→3GlcNAc (I), Galβ1→4GlcNAc (II) and Galβ1→3GalNAcα1→Ser/Thr (Tα) [4,22] indicating that the affinity of GCL-I for specific glycoforms can be enhanced by ligands occurring in a clustered or multivalent form. From our previous studies [4,22] and the present data, it is concluded that tri-antennary Galβ1→4GlcNAc and Galβ1→3GalNAc clusters, in addition to GalNAcα1→3GalNAc and GalNAcα1→3Gal, can be important ligands for binding.

Acknowledgements: This work was supported by grants from the Chang-Gung Medical Research Project (CMRP No. 676), Kwei-san, Tao-yuan, Taiwan and the National Science Council (NSC87-2314-B182-053), Taipei, Taiwan. This work was also supported by Grants 5RO1 A127508-03 and 2RO1 AI/GM19042-07 from the National Institutes of Health and DMB890-1840 from the National Science Foundation, USA and in part by the National Institutes of Health (NIH)-funded Resource center for Biomedical Complex Carbohydrates (NIH grant no. 2-P41-RR05351-06) to the Complex Carbohydrate Research Center, Athens, GA.

References

[1] Müller, W.E.G. (1982) Int. Rev. Cytol. 77, 129–181.
[2] Bretting, H., Phillips, S.G., Klumpart, H.J. and Kabat, E.A. (1981) Immmunology 127, 1652–1658.
[3] Pfeifer, K., Haasemann, M., Gamulin, V., Bretting, H., Fahrenholz, F. and Müller, W.E.G. (1993) Glycobiology 3, 179–184.
[4] Müller, W.E.G., Conrad, J., Schröder, C., Zahn, R.K., Kurelec, B., Dreesbach, K. and Uhlenbruck, G. (1983) Eur. J. Biochem. 133, 263–267.
[5] Hanisch, F.C., Baldus, S.G. and Kiimmel, T.A. (1996) Glycobiology 6, 321–336.
[6] Kabat, E.A. (1956) Blood Group Substances. Their Chemistry and Immunochemistry, Academic Press, New York, pp. 135–139.
[7] Wu, A.M., Kabat, E.A., Pereira, M.E.A., Gruezo, F.G. and Liao, J. (1982) Arch. Biochem. Biophys. 215, 390–404.
[8] Wu, A.M., Kabat, E.A., Nilsson, B., Zopf, D.A., Gruezo, F.G. and Liao, J. (1984) J. Biol. Chem. 259, 7178–7186.
[9] Wu, A.M. (1988) Adv. Exp. Med. Biol. 228, 351–394.
[10] Tettamanti, G. and Pigman, W. (1968) Arch. Biochem. Biophys. 124, 41–50.
[11] Herp, A., Wu, A.M. and Moschera, J. (1979) Mol. Cell. Biochem. 23, 27–44.
[12] Herp, A., Borelli, C. and Wu, A.M. (1988) Adv. Exp. Med. Biol. 228, 395–435.
[13] Downs, F., Harris, M. and Herp, A. (1976) Arch. Oral Biol. 21, 307–311.
[14] Wu, A.M., Shen, F.S., Herp, A. and Wu, J.H. (1994) Mol. Immunol. 31, 485–490.
[15] Wieruszeski, J.M., Michalski, J.C., Montreuil, J., Strecker, G., Peter-Katalinic, J., Egge, H., van Halbeek, H., Mutsaers, J.H.G.M. and Vliegenthart, J.F.G. (1987) J. Biol. Chem. 262, 6650–6657.
[16] Wu, A.M., Wu, J.H., Song, S.C. and Kabat, E.A. (1996) FEBS Lett. 398, 183–186.
[17] Wu, A.M. and Pigman, W. (1977) Biochem. J. 161, 37–47.
[18] Wu, A.M., Wu, J.H. and Shen, F.S. (1994) Biochem. Biophys. Res. Commun. 198, 251–256.
[19] Wu, A.M., Shen, F.S., Herp, A., Song, S.C. and Wu, J.H. (1995) FEBS Lett. 360, 211–215.

[20] Duk, M., Lisowska, E., Wu, J.H. and Wu, A.M. (1994) Anal. Biochem. 221, 266–272.
[21] Lisowska, E., Duk, M. and Wu, A.M. (1996) in: T. Meier and F. Fahrenholz (Eds.), Laboratory Guide to Biotin-Labeling in Biomolecule Analysis, Birkhäuser Verlag, Basel, Boston, Berlin, pp. 115–129.
[22] Wu, A.M. and Sugii, S. (1988) Adv. Exp. Med. Biol. 228, 205–263.
[23] Wu, A.M. and Sugii, S. (1991) Carbohydr. Res. 213, 127–143.
[24] Wu, A.M., Song, S.C., Sugii, S. and Herp, A. (1997) Ind. J. Biochem. Biophys. 34, 61–71.
[25] Wu, A.M., Kabat, E.A., Gruezo, F.G. and Allen, H.J. (1980) Arch. Biochem. Biophys. 204, 622–639.