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# *Aleuria aurantia* AGGLUTININ. A NEW ISOLATION PROCEDURE AND FURTHER STUDY OF ITS SPECIFICITY TOWARDS VARIOUS GLYCOPEPTIDES AND OLIGOSACCHARIDES*

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ABSTRACT

A new procedure for isolating a L-fucose-specific lectin from the mushroom *Aleuria aurantia* is described. The fine specificity of the purified lectin was determined by inhibition of agglutination of human red blood cells by various glycopeptides and oligosaccharides, and by studying the affinity of the immobilized lectin towards glycopeptides and oligosaccharides. Results of inhibition of hemagglutination showed that the lectin presents the highest affinity towards α-(1→6)-linked L-fucosyl groups. Immobilized *Aleuria aurantia* agglutinin interacts strongly with all *N*-glycosylpeptides or related glycans possessing an α-L-fucopyranosyl group linked to O-6 of the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue involved in the glycosylamine linkage. In addition, presence of α-(1→3)-linked L-fucosyl groups greatly enhances the affinity of the lectin for the α-(1→6)-L-fucosylated glycans. The immobilized *Aleuria* lectin is a powerful tool for the resolution of the microheterogeneity of L-fucosylated glycopeptides and glycans of the *N*-acetyllactosamine type.

## INTRODUCTION

It has been recently demonstrated that some lectins (*Lens culinaris, Vicia faba, Pisum sativum,* and *Lathyrus ochrus* agglutinins) having a specificity towards α-D-mannose or α-D-glucose possess, in fact, the ability to recognize fine differences in more complex glycans belonging to *N*-glycosylproteins, and that the presence of an α-L-fucosyl group linked to O-6 of the 2-acetamido-2-deoxy-β-D-gluco-

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pyranosyl residue involved in the glycosylamine linkage is a major determinant for binding[2–4]. This property can be used to fractionate glycopeptides of the *N*-acetyllactosamine type possessing this type of α-L-fucosyl group[5]. However, the aforementioned lectins are not L-fucose-specific lectins. As two well known L-fucose specific lectins (*i.e.*, *Ulex europeus* agglutinin I and *Lotus tetragonolobus* agglutinin) possess a very weak affinity towards glycans of the *N*-acetyllactosamine type, substituted either by an α-(1→6)- or an α-(1→3)-linked L-fucopyranosyl group, or both[2,6], an α-L-fucose-specific lectin having such a specificity could be a very powerful tool for the fractionation of L-fucosylated glycans.

In the present study, we describe a new procedure to isolate an α-L-fucose-specific lectin from the fruiting bodies of the mushroom *Aleuria aurantia*, reported earlier by Kochibe and Furukawa[7,8]. In addition, we have defined precisely the specificity of the lectin by inhibition of hemagglutination with various glycopeptides and oligosaccharides, and by studying the affinity of the immobilized lectin towards glycopeptides and oligosaccharides of the *N*-acetyllactosamine type.

Our results confirm those of Yamashita *et al.*[9] showing that the lectin presents a higher affinity for *N*-glycosylpeptides or related oligosaccharides possessing an α-L-fucopyranosyl group at O-6 of the 2-acetamido-2-deoxy-D-glucopyranosyl residue. In addition, we demonstrated further that the presence of external α-L-(1→3)-fucopyranosyl groups greatly enhances the affinity of the lectin for the oligosaccharides.

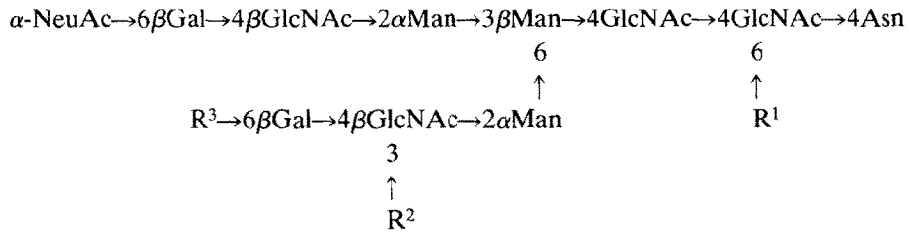
Thus, the immobilized *Aleuria aurantia* agglutinin (AAA) is a powerful tool for the resolution of the microheterogeneity of glycopeptides of the *N*-acetyllactosamine type and related oligosaccharides owing to the presence of β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-Glc*p*NAc, α-L-Fuc*p*-(1→6)-β-D-Glc*p*NAc-(1→4)-Asn, or both, residues.

## EXPERIMENTAL

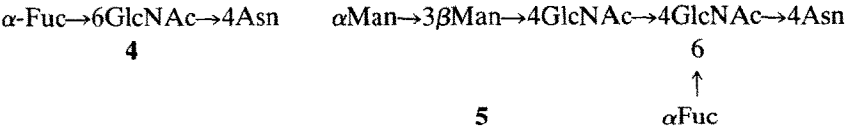
*Reagents.* — Fruiting bodies of *Aleuria aurantia* were collected locally and stored at −20° until used. *N*-(ε-Aminocaproyl)-β-L-fucopyranosylamine, prepared according to Blumberg *et al.*[10], was a gift from Prof. Nathan Sharon (The Weizmann Institute of Science, Rehovot, Israel). [1-$^{14}$C]Acetic anhydride (0.26 gBq/mM) was obtained from the C.E.A. (Saclay, France), and Aqualyte liquid scintillation cocktail from Baker (Deventer, The Netherlands).

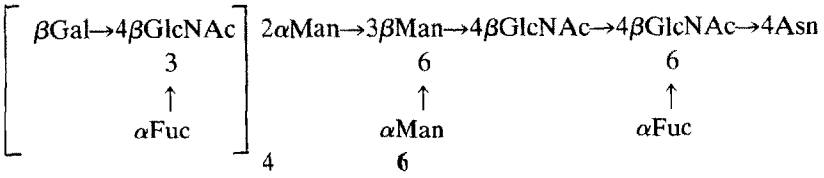
*Glycosylasparagines and oligosaccharides.* — Glycopeptides isolated from human serotransferrin (**1**), from human lactotransferrin (**2** and **3**), as well as the native glycoproteins were gifts from Prof. Geneviève Spik. Fucosylated glycopeptides (**4–6**), as well as a fucosylated oligosaccharide (**7**) were isolated by Dr. Gérard Strecker from the urine of patients with fucosidosis[11]. Glycopeptides **15–18** were isolated by Dr. Annick Pierce-Crétel from human milk sIgA. Human $\alpha_1$-acid glycoprotein, as well as glycopeptide **19**, were gifts of Prof. Bernard Fournet. In order to determine the affinity of immobilized AAA, all these glycopeptides were

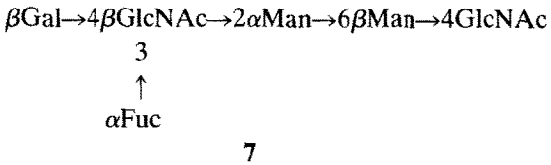
*N*-[$^{14}$C]acetylated according to Koide *et al.*[12]. *N*-Acetyllactosamine-type glycans from human lactotransferrin (**20** and **21**) were obtained by hydrazinolysis of the native glycoprotein, followed by *N*-reacetylation with [$^{14}$C]acetic anhydride of the liberated glycans. Oligosaccharides **8**–**14** were isolated by Dr. Gérard Strecker from human milk.

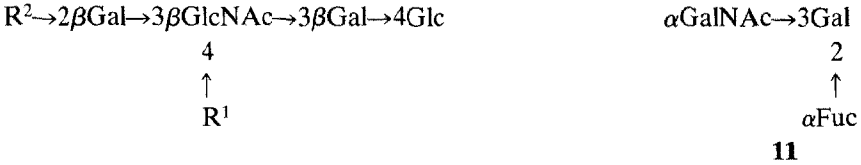



**1** $R^1 = R^2 = H$, $R^3 = \alpha$NeuAc
**2** $R^1 = \alpha$Fuc, $R^2 = H$, $R^3 = \alpha$NeuAc
**3** $R^1 = R^2 = \alpha$Fuc, $R^3 = H$

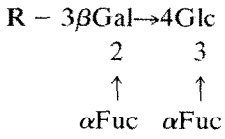


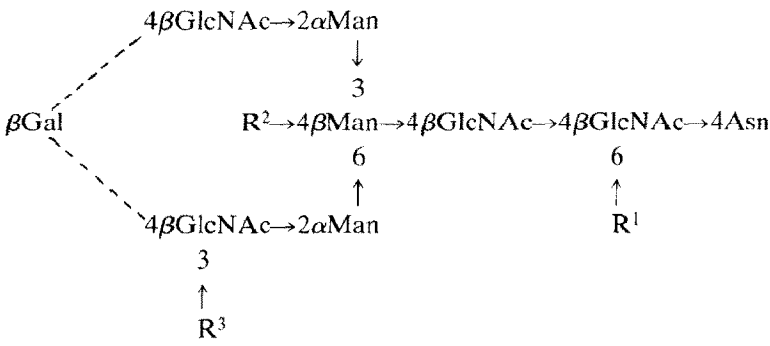



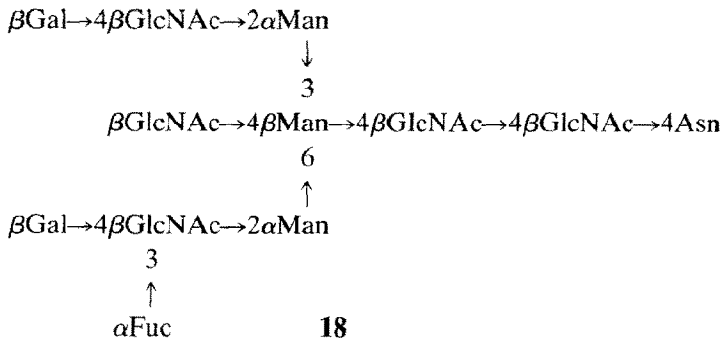

**8** $R^1 = R^2 = H$
**9** $R^1 = H$, $R^2 = \alpha$Fuc
**10** $R^1 = R^2 = \alpha$Fuc



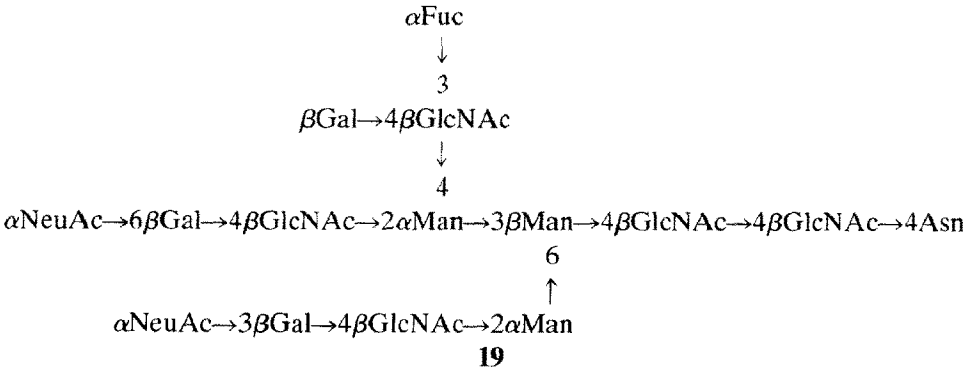

**12** R = H
**13** R = αGal
**14** R = αGalNAc



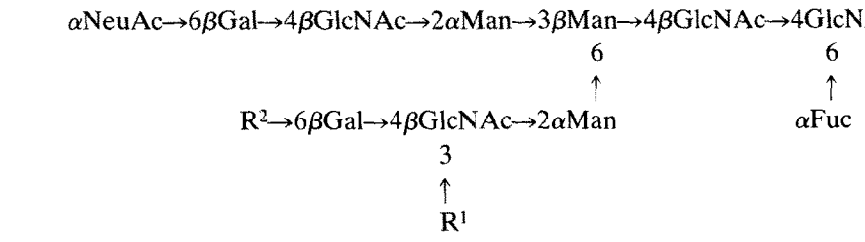

**15** $R^1$ = αFuc, $R^2$ = $R^3$ = H
**16** $R^1$ = $R^3$ = αFuc, $R^2$ = βGlcNAc
**17** $R^1$ = αFuc, $R^2$ = βGlcNAc, $R^3$ = H




**18**




**19**




**20** $R^1$ = H, $R^2$ = αNeuAc
**21** $R^1$ = αFuc, $R^2$ = H

Scheme 1. Glycan structures for Tables III and IV. The pyranose form, the D configuration for the Gal, Glc, GalNAc, and GlcNAc, residues, the L configuration for the Fuc and Asn residues, and the glycosyl linkages at C-2 for the NeuAc residue and at C-1 for the other residues are assumed.

*Preparation of the fucosylated resin.* — *N*-(ε-Aminocaproyl)-β-L-fucopyranosylamine was coupled to CNBr-activated Sepharose 4B by the method of March *et al.*[13] at a concentration of 5 mg/mL of settled gel. The resin bound 2 mg of AAA per mL of gel.

*Purification of the* Aleuria aurantia *agglutinin.* — An 80% saturation ammonium sulfate fraction was prepared from frozen fruiting bodies (100 g) according to Kochibe and Furakawa[7] and dialyzed against 10mM phosphate buffer, pH 7.2, containing 0.15M NaCl (PBS) for 48 h at 4°. The dialyzate was applied onto a Sepharose-4B-*N*-(ε-aminocaproyl-β-fucopyranosylamine) column (2 × 7 cm) at 4°, at a flow rate of 9 mL/h, and 4-mL fractions were collected. The column was washed with PBS until no absorbing material could be detected in the effluent. The bound lectin was then released by washing the column with 50mM L-fucose in PBS. The fucose eluate was dialyzed against distilled water for 48 h at 4° and lyophilized.

*Poly(acrylamide)-gel electrophoresis.* — The purity and the molecular weight of the purified lectin were examined by sodium dodecylsulfate slab-gel electrophoresis according to Laemmli[14] in a 5–25% poly(acrylamide) gradient gel.

*Chemical analyses.* — The amino acid composition of AAA was determined as follows. Purified AAA was hydrolyzed *in vacuo* at 100° with 6M HCl for 24 h. One drop of 1% phenol was added to prevent excessive degradation of tyrosine and the amino acid analysis was performed with a Beckman 119 CL amino acid analyser.

The quantitative determination of protein was performed according to Lowry *et al.*[15].

*Determination of contaminating α-L-fucosidases.* — The presence of contaminating α-L-fucosidases in the purified lectin preparation was determined by incubation of an aliquot of the lectin solution (1 mg/mL) with 5mM *p*-nitrophenyl fucoside in 0.01M citrate–phosphate buffer (400 μl.). After 30 min at 37°, the reaction was stopped by the addition of M $Na_2CO_3$ (1 mL) and liberated *p*-nitro-

phenol was measured spectrophotometrically at 400 nm. The incubation was also performed with α-D-Gal*p*NAc-(1→3)-α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-[α-L-Fuc-(1→3)]-D-Glc and the L-fucose released detected by t.l.c.[16].

*Hemagglutination test.* — Agglutination of a 3% suspension of human red blood cells in PBS and inhibition of hemagglutination by oligosaccharides, glycopeptides, or glycoproteins was carried out in Linbro mitrotiter U-plates titertek (Linbro Scientific Co, Hamden, U.S.A.) by a two-fold serial dilution technique[17]. The results are expressed as the minimum concentration (mM) required to completely inhibit 4 hemagglutinating doses.

*Immobilization of AAA on Sepharose 4B.* — AAA was coupled to Sepharose 4B activated according to the procedure of March *et al.*[13], at a concentration of 3 mg of lectin per mL of gel.

*Affinity chromatography of glycopeptides on AAA–Sepharose 4B column.* — *N*-[$^{14}$C]Acetylated glycopeptides ($2–30 \cdot 10^3$ d.p.m.; 0.1–10 nmol in 0.5 mL of PBS) were applied to the column of AAA–Sepharose (0.5 × 20 cm), equilibrated at room temperature in a 10mM phosphate buffer, pH 7.2, containing 0.15M NaCl (PBS). Elution was carried out, first with the aforementioned buffer at a flow rate of 9 mL/h, and then with PBS containing 50mM L-fucose; 1.5-mL fractions were collected and the radioactivity was counted in aliquots by liquid scintillation in a Beckman LS-9000 scintillation counter. Recovery of radioactivity from the column was always >95%.

## RESULTS AND DISCUSSION

*Isolation of* Aleuria aurantia *agglutinin.* — About 80 mg of the purified lectin was obtained from 100 g of mushrooms by affinity chromatography on the Sepharose 4B–*N*-(ε-aminocaproyl-β-L-fucopyranosylamine) column (Table I). The fucosylated resin may be used for many successive purifications without any decrease in efficiency. The purified lectin was free from any contaminating α-L-

TABLE I

PURIFICATION OF LECTIN FROM *Aleuria aurantia* FRUITING BODIES[a]

| *Fraction* | *Total protein (mg)* | *Titer* | *Specific hemagglutinating activity (titer/mg)* | *Protein recovery (%)* |
|---|---|---|---|---|
| Crude extract | 4 600 | 8 | 1 | 100 |
| 80% Ammonium sulfate precipitate | 1 200 | 256 | 35 | 26 |
| Sepharose 4B–*N*-(ε-aminocaproyl-fucopyranosyl amine)affinity chromatography | 80 | 128 | 135 | 1.7 |

[a]Weight of starting material, 100 g.

fucosidase. It gave two polypeptide bands with an apparent mol. wt. of 31 000 ±1000 when analyzed by poly(acrylamide) slab-gel electrophoresis in the presence of SDS and 5% β-mercaptoethanol (Fig. 1). These results differ slightly from those obtained by Kochibe and Furukawa[7] who found a single polypeptide band when using poly(acrylamide) gel electrophoresis in the presence of SDS and β-mercaptoethanol according to Weber and Osborn[18]. On the basis of this result and of gel filtration analysis, the authors concluded that the native lectin probably exists as a dimer of two identical subunits. The highest resolution of the Laemmli's system showed that the native lectin contains two slightly different subunits differing in their charge, as it has been previously found for soybean agglutinin[19].

The amino acid composition of the lectin (Table II) indicated a high content of hydroxylic and acidic amino acids, and the absence of sulfur-containing amino acids. Analysis of monosaccharides by gas liquid chromatography according to

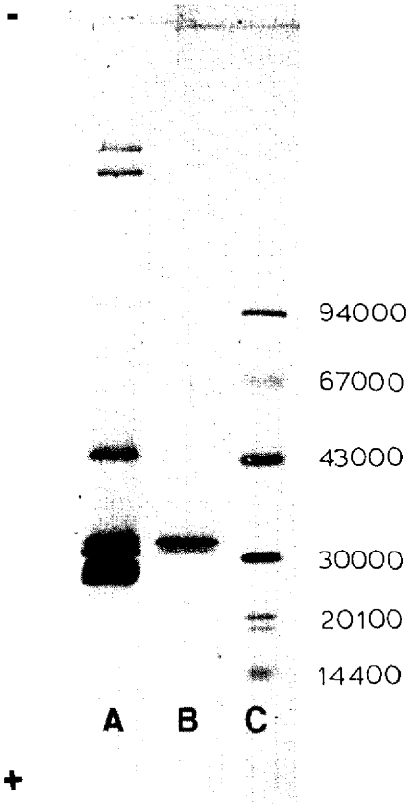



Fig. 1. Sodium dodecylsulfate slab-gel electrophoresis of the 80% ammonium sulfate fraction prepared from *Aleuria aurantia* (A) and of the purified lectin (B) in a 5–25% poly(acrylamide) gradient gel according to Laemmli[14]. On line C were applied mol. wt. markers for SDS gel calibration (Pharmacia, France): phosphorylase b (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100), and α-lactalbumin (14 400).

TABLE II

AMINO ACID COMPOSITIONS OF *Aleuria aurantia* AGGLUTININS[a]

| *Amino acids* | *Number of residues estimated* | |
|---|---|---|
| | *This study* | *Ref. 7* |
| Alanine | 62.7 | 52.6 |
| Valine | 29.6 | 33.3 |
| Leucine | 29.6 | 24.8 |
| Isoleucine | 34.9 | 42.5 |
| Proline | 33.1 | 33.8 |
| Phenylalanine | 18.6 | 18.1 |
| Glycine | 83.4 | 89.6 |
| Serine | 79.6 | 75.7 |
| Threonine | 36.7 | 37.5 |
| Tyrosine | 24.2 | 26.1 |
| Aspartic acid | 51.8 | 49.7 |
| Glutamic acid | 60.9 | 57.6 |
| Lysine | 26 | 28.5 |
| Arginine | 24.2 | 23.1 |
| Histidine | 7.1 | 3.7 |
| Methionine | 0 | 0 |
| Half-cystine | 0 | 0 |
| Total | 602.4 | 596.6 |

[a]Expressed as number of residues/mol, assuming a mol. wt. of 62 000 for the lectin.

Zanetta *et al.*[20] demonstrated that the *Aleuria aurantia* agglutinin is not glycosylated. These two series of results entirely confirm those of Kochibe and Furukawa[7].

*Hemagglutination-inhibition tests.* — The results of the inhibition, by various oligosaccharides, of hemagglutination by *Aleuria aurantia* agglutinin (Table III) can be summarized as follows: The lectin presents the highest affinity towards α-(1→6)-linked L-fucosyl groups, the presence of which is a prerequisite for the interaction of the lectin with the oligosaccharide. The lectin can recognize also α-(1→2)-linked L-fucosyl groups, but only when the fucosyl group is linked to a sugar residue in a terminal reducing position, as in trisaccharide **11**. The lectin also reacted with α-(1→3)-linked L-fucosyl groups, but only when the fucosyl group is linked to a sugar residue, like D-glucose, in a terminal reducing position, as in oligosaccharides **12, 13** and **14**. The affinity of the lectin increased with the number of L-fucosyl groups linked to the oligosaccharide, indicating the existence of an "avidity effect"[21]. Thus, glycopeptide **6**, having four α-(1→3)-linked L-fucosyl groups and the α-L-Fuc*p*-(1→6)-β-D-Glc*p*NAc-(1→4)-Asn sequence was 8-fold more inhibitory than glycopeptide **3** which possesses only one external α-(1→3)-linked L-fucosyl group. From these results, it appears that AAA presents some analogies with such lectins as LCA, VFA, or PSA, for which the α-(1→6)-linked L-fucosyl group is a major determinant for binding[2,3]. However, in contrast to AAA, the

TABLE III

MINIMUM CONCENTRATION OF VARIOUS OLIGOSACCHARIDE STRUCTURES NECESSARY TO COMPLETELY INHIBIT RED BLOOD CELL AGGLUTINATION INDUCED BY *Aleuria aurantia* AGGLUTININ

| *Glycan structures* | *Minimum concentration* (mM)[a] |
|---|---|
| *Monosaccharides* | |
| α-L-Fucose | 0.31 |
| Methyl α-D-mannopyranoside | 90 |
| *Glycoproteins* | |
| Human serotransferrin | 0.033 |
| Human lactotransferrin | 0.001 |
| Human $\alpha_1$-acid glycoprotein | 0.0165 |
| *Glycoasparagines* | |
| **1** | 1.66 |
| **2** | 0.103 |
| **3** | 0.0257 |
| **4** | 0.0515 |
| **5** | 0.207 |
| **6** | 0.0031 |
| *Oligosaccharides* | |
| **7** | >1.66 |
| **8** | >1.66 |
| **9** | >1.66 |
| **10** | >1.66 |
| **11** | 0.103 |
| **12** | 0.0515 |
| **13** | 0.83 |
| **14** | 0.207 |

[a]Results are expressed as the minimum concentration of sugar (mM) necessary to completely inhibit four hemagglutinating doses.

aforementioned three lectins also need the presence of the mannotriose core with both α-(1→3)- and α-(1→6)-linked D-mannosyl residues either free or substituted at O-2 and O-6[3,6,22]. An L-fucosyl group α-(1→3)-linked to an external 2-acetamido-2-deoxy-D-glucopyranosyl residue did not play any role in the binding of LCA, PSV, or VFA, but it considerably increased the affinity of AAA.

*Affinity chromatography of* N*-glycosylpeptides and related oligosaccharides on immobilized AAA.* — Glycopeptides and oligosaccharides that do not interact with the immobilized lectin were eluted within the void volume and constitute the "nonretained fractions" (FNR of Table IV). Compounds weakly interacting with the lectin were eluted from the column by the starting buffer as "retarded fractions" (FR) according to their affinity for the lectin (Fractions FR-1 and FR-2 of Table IV, respectively). Under the experimental conditions used, FR1 was eluted between fractions 7 to 12 and FR-2 between fractions 10 to 22. The sharply-eluted fraction (FE of Table IV) obtained with 50mM L-fucose in starting buffer indicated

TABLE IV

BEHAVIOUR ON IMMOBILIZED *Aleuria aurantia* AGGLUTININ-SEPHAROSE OF *N*-GLYCOSYLPEPTIDES AND RELATED OLIGOSACCHARIDES

| *Glycan structures* | *FNR*[a] | *FR-1*[b] | *FR-2*[c] | *FE*[d] |
|---|---|---|---|---|
| **1** | + | | | |
| **18** | + | | | |
| **19** | | + | | |
| **2** | | | + | |
| **17** | | | + | |
| **20** | | | + | |
| **3** | | | | + |
| **4** | | | | + |
| **5** | | | | + |
| **15** | | | | + |
| **16** | | | | + |
| **21** | | | | + |
| **6** | | | | + |

[a]Nonretained fraction. [b]Slightly retarded. [c]Strongly retarded. [d]Fraction eluted with 50mM L-fucose.

a strong interaction between the lectin and the bound saccharides. Table IV describes the behavior of 13 glycopeptides and oligosaccharides on the immobilized *Aleuria aurantia* agglutinin. It could be summarized as follows: immobilized AAA interacts strongly with all the glycopeptides possessing an α-L-fucopyranosyl group at O-6 of the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue involved in the *N*-glycosylamine linkage. However, the interaction is modulated by the complexity of the glycan structure. For example, glycopeptides **4, 5,** and **6** were strongly bound to the lectin column and were eluted with PBS containing 50mM L-fucose, whereas glycopeptide **2** was strongly retarded but not retained. On this point, our results are not in agreement with those obtained by Yamashita *et al.*[9] who found that binding was not affected by the structures of the outer glycan chains. On the contrary, our results including those obtained by hapten inhibition of hemagglutination indicated that the lectin binding-site can accommodate only two to three saccharide units.

It is noteworthy that the glycans **20** and **21**, released by hydrazinolysis–*N*-reacetylation from human lactotransferrin, were still recognized by the immobilized lectin with the same affinity as the native glycopeptides **2** and **3**. This result confirms those obtained by Yamashita *et al.*[9], who also used an AAA–Sepharose column containing 3 mg of lectin per mL of gel, and shows that immobilized *Aleuria* agglutinin is very useful for the fractionation of glycans containing α-(1→6)-linked L-fucosyl groups that are released from *N*-glycosylproteins by hydrazinolysis. From this point of view, AAA differs from LCA, PSA, and VFA, for which such glycans liberated by hydrazinolysis and still possessing the α-(1→6)-linked L-fucosyl determinant were no longer retarded on VFA and PSA[22,23], or were retained on immobilized LCA[23,24]. Substitution of the β-D-mannosyl residue by a "bisecting"

2-acetamido-2-deoxy-β-D-glucopyranosyl group (glycopeptide **17**) decreased the affinity of the *Aleuria* lectin as compared to the unsubstituted one (**15**). In this respect, the *Aleuria* lectin is similar to $RCA_1$[6], to *Erythrina* lectins[25] and, to some extent, to Con A[6,26]. However, this result does not agree with that obtained by Yamashita *et al.*[9] who claimed that the binding was not affected by the presence of a "bisecting" 2-acetamido-2-deoxy-β-D-glucopyranosyl group.

The presence of at least one external α-L-Fuc*p*-(1→3)-D-Glc*p*NAc group (X-antigenic determinant) together with the α-L-Fuc*p*-(1→6)-D-GlcNAc sequence on the same glycopeptide (**3**) or glycan **21** greatly enhanced the interaction between the oligosaccharides and the *Aleuria* lectin. In this respect, immobilized *Aleuria* lectin represents a very useful tool for the fractionation of the two glycans found in human lactotransferrin: the monofucosylated glycopeptide **2** or glycan **20** were strongly retarded on the immobilized lectin, but the difucosylated glycopeptide **3** or glycan **21** were firmly bound and had to be eluted from the column with 50mM L-fucose. Compounds having structures **16** and **6**, with respectively one and four α-L-Fuc*p*-(1→3)-D-GlcNAc groups together with the α-L-Fuc*p*-(1→6)-β-D-Glc*p*NAc-(1→4)-Asn sequence, interacted also strongly with the immobilized lectin. This ability of the α-L-Fuc*p*-(1→3)-D-GlcNAc groups to enhance the interaction between the α-(1→6)-fucosylated glycopeptides or glycans and the immobilized *Aleuria* lectin was not emphasized by Yamashita *et al.*[9]. It is also noteworthy that the triantennary glycopeptide **19**, which possesses only one α-L-Fuc*p*-(1→3)-D-GlcNAc group, interacted weakly with the immobilized lectin, but that the bisected biantennary glycopeptide **18**, which possesses also this group, did not interact at all with the lectin.

In conclusion, as pointed out by Yamashita *et al.*[9] and Harada *et al.*[27], the present study also shows that, after careful calibration with well defined oligosaccharides and glycopeptides, the immobilized *Aleuria aurantia* agglutinin could provide a valuable tool for the resolution of the microheterogeneity due to the presence of different L-fucosyl substituents of glycopeptides of the *N*-acetyllactosamine type and related oligosaccharides.

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