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Concanavalin A binding and endoglycosidase D resistance of β1,2-xylosylated and α1,3-fucosylated plant and insect oligosaccharides

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The binding to concanavalin A (Con A) by pyridylaminated oligosaccharides derived from bromelain (Manα1,6(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc), horseradish peroxidase (Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc), bee venom phospholipase A_2 (Manα1,6Manβ1,4GlcNAcβ1,4GlcNAc and Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc) and zucchini ascorbate oxidase (Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4 GlcNAcβ1,4GlcNAc) was compared to the binding by $Man_3GlcNAc_2$, $Man_5GlcNAc_2$ and the asialo-triantennary complex oligosaccharide from bovine fetuin. While the fetuin oligosaccharide did not bind, bromelain, zucchini, $Man_2GlcNAc_2$ and horseradish peroxidase were retarded (in that order). The α1,3-fucosylated phospholipase, $Man_3GlcNAc_2$ and $Man_5GlcNAc_2$ structures were eluted with 15 mM α-methylmannoside. It is concluded that core α1,3-fucosylation has little or no effect on ConA binding while xylosylation decreases affinity for ConA.

In a parallel study comparing the endoglycosidase D (Endo D) sensitivities of $Man_3GlcNAc_2$, IgG-derived GlcNAcβ1,2Manα1,6(GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc, the phospholipase Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc, and horseradish and zucchini pyridylaminated N-linked oligosaccharides, it was found that only the $Man_3GlcNAc_2$ structure was cleaved. The IgG structure was sensitive only when β-hexosaminidase was also present. Thus, in contrast to core α1,6-fucosylated structures, such as those present in mammals, the presence of core α1,3-fucose, as found in structures from plants and insects, and/or β1,2-xylose, as found in plants, causes resistance to Endo D.

***Keywords*: concanavalin A, endoglycosidase D, plant oligosaccharides, bee venom oligosaccharides**

***Abbreviations*: ConA, concanavalin A; Endo D, endo-β-*N*-acetylglucosaminidase D (from *Streptococcus pneumoniae*); HRP, horseradish peroxidase; PNGase A, peptide: N-glycosidase from almonds; pyridylaminated oligosaccharides: M0, Manα1,6Manβ1,4GlcNAcβ1,4GlcNAc; MM, Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc;M5, Manα1,6(Manα1,3)Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc; MMF^3, Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc;$M0XF^3$,Manα1,6(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc; $MMXF^3$,Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc; MMX, Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4GlcNAc; $GnGnF^6$, GlcNAcβ1,2Manα1,6(GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc**

Introduction

In general, there appears to be a dearth of published information on the specificities of lectins and glycosidases towards plant and insect oligosaccharides. Standard textbooks and reviews neglect this area, and the literature that otherwise exists is fragmentary and sometimes seemingly contradictory. For instance the horseradish peroxidase (HRP)-type structure (Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc) is sometimes claimed not to bind concanavalin A (ConA). In two different studies [1,2], glycopeptides from phytohaemagglutinin were fractionated into $ConA^+$ and $ConA^-$ and the subsequent nuclear magnetic resonance (NMR) spectra indicated the former were oligomannosidic glycopeptides, while the latter were glycopeptides of the HRP-type. Foxwell *et al.* [3], when studying different molecular weight isoforms of ricin A chain, suggested (based on monosaccharide composition) that the $ConA^+$ form of the protein contains an oligomannosidic and an HRP-type oligosaccharide, while

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the lower molecular weight ConA$^-$ form has only an HRP-type oligosaccharide. (It should be noted however that Kimura *et al.* concluded that hydrazinolysis of ricin yields xylosylated but not fucosylated oligosaccharides [4], even though hydrazinolysis of other castor bean glycoproteins does yield the HRP-type oligosaccharide that is both xylosylated and fucosylated as a major product [5]). From the results of Debray *et al.* on mistletoe lectin glycopeptides [6], where the actual ConA elution profile was shown, it can be calculated that the ConA$^-$ material was not retarded, but was washed off with one column volume of starting buffer; this ConA$^-$ material was found by NMR to be a glycopeptide of the HRP type.

In contrast, however, there exists a method where HRP is added to detect ConA binding to proteins after Western blotting [7]. HRP as a whole protein can be purified on Con A [8, 9] as can miraculin [10], which apparently only has HRP-type oligosaccharides [11]. The $Man_3GlcNAc_2$ structure which is known to bind ConA [12] was found by Harthill to account for less than 2% of the oligosaccharides of HRP [13], and so it is unlikely this 2% would account for binding of HRP as a protein to ConA even though HRP has eight glycosylation sites. In addition, an early study on HRP glycopeptides contradicts others in that 86% of HRP glycopeptides were purified by chromatography on ConA [14]. Cross-linking of ConA with *Erythrina* lectins has been described [15] and these lectins are known to contain HRP-type oligosaccharides [16].

The specificity of endoglycosidases towards the plant and insect oligosaccharides is also an area which is poorly documented. Endoglycoside H has been used in studies on plant glycosylation, such as following biosynthesis [17], and it appears that the HRP-type structure is resistant. With respect to endoglycosidase D (Endo D) and plant oligosaccharides, it appears that only resistance of the ricin A chain to Endo D has been reported [3].

In the present study, the interaction of ConA and Endo D with selected plant and insect oligosaccharides has been examined in comparison to some standard oligosaccharides.

Materials and methods

Oligosaccharides

Pyridylaminated oligosaccharides were obtained after almond peptide:N-glycosidase (PNGase A) digestion of glycopeptides, with subsequent reductive derivatization with 2-aminopyridine [18] (and references therein). In all cases, the oligosaccharides had been analysed (and, as necessary, purified) by size fractionation and reversed-phase chromatographies together with exoglyosidase digestions. The plant and insect oligosaccharides used were from the following sources (with references to the original relevant structural determination data): M0 and MMF^3 were from bee venom phospholipase A_2 [18]; M0XF^3 from bromelain [19]; MMXF^3 was from horseradish peroxidase [13, 20]; MMX was from zucchini ascorbate oxidase fraction 1 [21]. Human IgG was acidically desialylated [8], trypsinized and enzymatically degalactosylated prior to PNGase A digestion and pyridylamination to generate GnGnF^6. Bovine fetuin glycopeptides, carrying triantennary oligosaccharides [22], were acidically desialylated prior to PNGase A digestion and pyridylamination. M5 was obtained from *Aspergillus oryzae* α-amylase [23] and MM was obtained after desialylation and galactosidase treatment of bovine fibrin glycopeptides with subsequent hexosaminidase treatment of the pyridylaminated oligosaccharides.

Chromatography on ConA

A 0.5 ml column of ConA-Sepharose (Sigma Chemical Co.) was equilibrated at 4 °C with fifty column volumes of 20 mM Tris HCl, pH 7.4, 0.5 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ Pyridylaminated oligosaccharides were diluted to 150 µl with equilibration buffer and applied in one of three mixtures (feutin, MXF^3, MMX, MMF^3; fetuin, M, M5, MMXF^3; fetuin, MMXF^3, MM). After standing for 30 min the column was washed with five column volumes of equilibration buffer, followed by five column volumes of buffer containing 15 mM α-methylmannoside and five column volumes of buffer containing 500 mM α-methylmannoside. One-column volume fractions were dried before reversed-phase HPLC.

Digestion with Endo D

Approximately 0.1 nmol of pyridylaminated oligosaccharides (MM, GnGnF^6, MMX, MMF^3 or MMXF^3) were incubated with 1 mU (or 2 mU) Endo D (Boehringer Manheim) overnight (or for 2 days) at 37 °C in a toluene atmosphere in 0.1 M citrate–phosphate buffer, pH 6.0 in a total volume of 50 µl. The incubations were suitable for immediate use with reversed-phase HPLC.

Reversed-phase HPLC

Pyridylaminated oligosaccharides were fractionated on a 5 µ Hypersil ODS column (4 × 250 mm, ÖFZ Seibersdorf) with a gradient starting from 10% (v/v) methanol in 0.1 M ammonium acetate, pH 4.0 (flow rate, 1.5 ml min^{-1}) and increasing with 1% (v/v) methanol per min using a Bio-Rad 700 chromatography workstation. Fluorescence of the eluate was detected with a Shimadzu RF-551 fluorescence monitor (excitation 320 nm, emission 400 nm).

Results and discussion

The results of ConA chromatography of pyridylaminated oligosaccharides with subsequent detection with reversed-phase HPLC are shown in Table 1. The triantennary complex oligosaccharide from bovine fetuin was washed off with

Table 1. Fractionation of pyridylaminated oligosaccharides on ConA followed by reversed-phase HPLC. Pyridylaminated oligosaccharides were chromatographed as three mixtures as described in Materials and methods. Fraction 7 was the first column volume after addition of buffer with 15 mM *α*-methylmannoside and fraction 12 the first after addition of 500 mM *α*-methylmannoside. The peaks were integrated and the percentage of an oligosaccharide present in each fraction calculated. The fetuin results are the means from the fractionation of all three mixtures.

Fraction No.	*Fet*	*M0*	*M0XF³*	*MMX*	*MMXF³*	*MMF³*	*MM*	*M5*
1	12	—	—	—	—	—	—	—
2	80	8	47	20	11	—	—	—
3	7	39	45	37	16	—	—	—
4	—	37	8	35	20	—	—	—
5	—	16	—	8	20	—	—	—
6	—	—	—	—	19	—	—	—
7	—	—	—	—	14	21	10	16
8	—	—	—	—	—	17	16	19
9	—	—	—	—	—	22	16	15
10	—	—	—	—	—	14	17	16
11	—	—	—	—	—	9	11	12
12	—	—	—	—	—	16	14	7
13	—	—	—	—	—	—	10	15
14	—	—	—	—	—	—	5	—
15	—	—	—	—	—	—	—	—

one column volume as expected [12], while M0, M0XF^3 and MMX oligosaccharides were washed off by up to four column volumes equilibration buffer. The MMXF^3 oligosaccharide from HRP was more retarded: 85% was washed off with five column volumes of equilibrium buffer. The MMF^3, MM and M5 oligosaccharides bound to the column and were eluted slowly with 15 mM α-methylmannoside.

It is concluded that: (1) xylosylation has a negative effect on binding to ConA as shown by the comparison of MM and MMX and by the comparison of MMF^3 with MMXF^3; (2) the presence of α1,3-linked fucose may promote slightly greater retardation of the MMXF^3 structure as compared to the MMX structure, but does not confer full binding to the MMXF^3 structure; (3) the presence of α1,3-linked fucose does not affect the binding of non-xylosylated oligosaccharides as judged by the finding that MMF^3 binds approximately as well as MM; (4) comparison of the binding of M0 and M0XF^3 with MM and MMXF^3 respectively concurs with the findings of Baenziger and Fiete [12] that the absence of the α1,3-linked mannose reduces any interaction of the oligosaccharide with ConA. For instance, Baenziger and Fiete [12] found that a structure with Galβ1,4GlcNAc on the α1,6-arm has a K_d of 1.9×10^{-6} when the α1,3-mannose is absent and a K_d of 9.8×10^{-6} when it is present. Other studies, too, similarly conclude that at least two α-mannosyl residues are required for binding to ConA [24, 25]. Western blotting followed by ConA binding an HRP detection also suggests a difference between the binding of M0XF^3 and MMXF^3 since bromelain does not bind ConA, whereas HRP does (data not shown).

The present study does not concur with the study of Debray *et al.* where a MMXF^3 glycopeptide is washed off in one column volume [6], but neither does it agree, at first sight, with results indicating complete binding of the intact HRP glycoprotein to ConA. Perhaps, the polyvalency of the intact HRP glycoprotein or the exact chromatographic conditions may lead to intact HRP binding ConA, while studies with the pyridylaminated and reduced oligosaccharide or with individual glycopeptides show mere retardation or non-binding. However, the relative weakness of binding of intact HRP to ConA, as compared to that of ovalbumin, has been shown, since chromatography at 28 °C in the presence of SDS gives different results from chromatography at 4 °C or in the absence of SDS at 28 °C [9]. Overall, it is advised that some caution be exercised before making conclusions about oligosaccharide structure when considering ConA binding of plant glycoproteins. Consistent with the present results, though, the fraction of intact phospholipase A_2 glycoprotein which contains MMF^3 has been found to bind ConA [18].

The results of incubation with Endo D, as shown in Table 2, are consistent with data presented in the literature. The lower molecular weight form of the A subunit of ricin, which has a monosaccharide composition consistent with the presence of an HRP-type structure, is resistant to Endo

Table 2 Incubation of pyridylaminated oligosaccharides with Endo D. Pyridylaminated oligosaccharides were incubated as in the text. In the case of $GnGnF^6$ + hex, 60 mU jack bean hexosaminidase was added to the incubation and the portion not digested to Fuc*a*1,6GlcNAc had the same retention time as MMF^6.

Oligosaccharide	*Digestion overnight by 1 mU Endo D (%)*	*Digestion for 2 days by 2 mU Endo D (%)*
MM	47	100
$MMXF^3$	0	0
MMF^3	0	0
MMX	0	0
$GnGnF^6$	0	0
$GnGnF^6$ + hex	27	79

D [3] and both α1,3-fucosylated oligosaccharides examined in the present study are also resistant. With MM a new peak appeared with the retention time expected for pyridylaminated GlcNAc, but with $MMXF^3$, MMX and MMF^3 incubation resulted in no change in retention time. $MMXF^3$ and MMF^3 were found not to co-elute after the incubation (chromatograms not shown), which discounts the possibility that they had been converted by Endo D to the possible product (pyridylaminated Fucα1,3GlcNAc). For the IgG-derived $GnGnF^6$, *in situ* β-hexosaminidase treatment was required to result in digestion of $GnGnF^6$ to yield the pyridylaminated disaccharide Fucα1, 6GlcNAc. The formation of the intermediate MMF^6 was shown by the appearance of a peak of retention time between that of Fucα1,6GlcNAc and that of the intact $GnGnF^6$ (chromatograms not shown). This result with α1,6-fucosylated oligosaccharides is entirely consistent with the results of Mizuochi *et al*. [26]. In conclusion, it appears that xylosylation or α1,3-fucosylation of pyridylaminated oligosaccharides prevents digestion by Endo D.

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