

## Core $\alpha$ 1,3-fucose is a key part of the epitope recognized by antibodies reacting against plant N-linked oligosaccharides and is present in a wide variety of plant extracts

Iain B.H. Wilson<sup>3</sup>, Jean E. Harthill<sup>1,3</sup>, Nicholas P. Mullin<sup>1,4</sup>, David A. Ashford<sup>1,5</sup> and Friedrich Altmann<sup>2</sup>

Institut für Chemie der Universität für Bodenkultur, Muthgasse 18, A-1190, Wien, Austria and <sup>1</sup>Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, United Kingdom

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<sup>2</sup>To whom correspondence should be addressed

<sup>3</sup>Present address: Department of Biochemistry, University of Dundee, Dundee, DD1 4HN, Scotland

<sup>4</sup>Present address: Centre for Protein Technology, Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JJ, Scotland

<sup>5</sup>Present address: Glycobiology: Research & Analytical, Department of Biology, University of York, P.O. Box 373, York, YO1 5YW, United Kingdom

Carbohydrates have been suggested to account for some IgE cross-reactions between various plant, insect, and mollusk extracts, while some IgG antibodies have been successfully raised against plant glycoproteins. A rat monoclonal antibody raised against elderberry abscission tissue (YZ1/2.23) and rabbit polyclonal antiserum against horseradish peroxidase were screened for reactivity in enzyme-linked immunosorbent assay against a range of plant glycoproteins and extracts as well as neoglycoproteins, bee venom phospholipase, and several animal glycoproteins. Of the oligosaccharides tested,  $\text{Man}_3\text{Xyl-FucGlcNAc}_2$  (MMXF<sup>3</sup>) derived from horseradish peroxidase was the most potent inhibitor of the reactivity of both YZ1/2.23 and anti-horseradish peroxidase to native horseradish peroxidase glycoprotein. The reactivity of YZ1/2.23 and anti-horseradish peroxidase against *Sophora japonica* lectin was most inhibited by a neoglycoconjugate of bromelain glycopeptide cross-linked to bovine serum albumin, while the defucosylated form of this conjugate was inactive as an inhibitor. A wide range of plant extracts was found to react against YZ1/2.23 and anti-horseradish peroxidase, with particularly high reactivities recorded for grass pollen and nut extracts. All these reactivities were inhibitable with the bromelain glycopeptide/bovine serum albumin conjugate. Bee venom phospholipase and whole bee venom reacted weakly with YZ1/2.23 but more strongly with anti-horseradish peroxidase in a manner inhibitable with the bromelain glycopeptide/bovine serum albumin conjugate, while hemocyanin from *Helix pomatia* reacted poorly with YZ1/2.23 but did react with anti-horseradish peroxidase. It is concluded that the  $\alpha$ 1,3-fucose residue linked to the chitobiose core of plant glycoproteins is the most important residue in the epitope recognized by the two antibodies studied, but that the polyclonal anti-horseradish peroxidase antiserum also contains antibody populations that recognize the xylose

linked to the core mannose of many plant and gastropod N-linked oligosaccharides.

**Key words:** N-glycans/cross-reactivity/anti-carbohydrate/pollens/food

### Introduction

Cross-reactions in immunoassays of plant, arthropod and mollusk extracts have been reported for a number of years, in particular a “carbohydrate cross-reacting determinant” has been hypothesized but not structurally analyzed (Aalberse *et al.*, 1981a). IgE and/or IgG reactivity against the carbohydrate moieties of *Lol p XI* (an antigen from the pollen of ryegrass, *Lolium perenne*) (van Ree *et al.*, 1995a), *Olea europaea* (de Cesare *et al.*, 1993), as well as specifically the major olive pollen allergen *Ole e I* (Batanero *et al.*, 1994, 1996), *Cry j I* (an allergen from the pollen of *Cryptomeria japonica*, Japanese cedar) (Hijikata *et al.*, 1994), buckwheat (Aalberse *et al.*, 1981b), *Parietaria judaica* (Mucci *et al.*, 1992), Bermuda grass BG60 allergen (Su *et al.*, 1996), tomato fruit and grass pollen (Petersen *et al.*, 1996), cereal flour (Garcia-Casado *et al.*, 1996), and a hydroxyproline-rich glycoprotein from *Parthenium hysterophorus* pollen (Gupta *et al.*, 1996) has been proposed on the basis of reduction of reactivity after chemical deglycosylation or retention of inhibition of immunoassays after protease treatment. That caution should be applied to interpreting results of chemical deglycosylation with trifluoromethanesulfonic acid is borne out by the finding that the circular dichroism spectrum of *Ole e I* reveals complete loss of the native conformation of the protein (Batanero *et al.*, 1994). Cross-reactions in assays to a number of plant extracts of IgE from patients allergic to caddis fly could be inhibited by periodate treatment (Koshte *et al.*, 1989), while a series of studies have shown that rabbit anti-bee venom phospholipase cross-reacts with plant glycoproteins (Prenner *et al.*, 1992) and that a portion of the IgE of some patients allergic to bee venom has the  $\alpha$ 1,3-fucose residue of the core of the N-linked oligosaccharides as an epitope (Weber *et al.*, 1987; Tretter *et al.*, 1993). Grasshopper and *Drosophila* neural tissue as well as *Drosophila* male reproductive tissue has been stained with anti-horseradish peroxidase (Snow *et al.*, 1987; Katz *et al.*, 1988; Kurosaka *et al.*, 1991; Wang *et al.*, 1994). An antiserum to carrot  $\beta$ -fructosidase was found to react against a wide range of plant and arthropod glycoproteins and the authors believed xylose was the common epitope (Faye and Chrispeels, 1988), although xylose has yet to be detected in insects. Indeed anti-fructosidase and anti-horseradish peroxidase could be fractionated into a bee venom phospholipase A<sub>2</sub> binding fraction and a remaining xylose-active fraction (Faye *et al.*, 1993). Carbohydrate-reactive antibodies have also been raised against *Wistaria floribunda* lectin (Kaladas *et al.*, 1983), *Erythrina cristagalli* lectin (Harthill, 1991), and elderberry bark abscission tissue (McManus *et al.*, 1988).

| Glycan abbreviation | Structure | Example sources                                                                            |
|---------------------|-----------|--------------------------------------------------------------------------------------------|
| M0XF <sup>3</sup>   |           | Bromelain                                                                                  |
| MMXF <sup>3</sup>   |           | Horseradish peroxidase<br><i>Sophora japonica</i> agglutinin<br>Zucchini ascorbate oxidase |
| MMF <sup>3</sup>    |           | BG60 allergen<br>Bee venom phospholipase                                                   |
| MMX                 |           | Zucchini ascorbate oxidase                                                                 |
| MMXF <sup>6</sup>   |           | <i>Helix pomatia</i> haemocyanin                                                           |
| MM                  |           |                                                                                            |
| 00XF <sup>3</sup>   |           |                                                                                            |
| M3                  |           |                                                                                            |
| M2(3)               |           |                                                                                            |
| M2(6)               |           |                                                                                            |

**Fig. 1.** Structures of oligosaccharides referred to in this study. Oligosaccharides referred to in the study are shown with the abbreviation, structure and (where appropriate) example natural sources.

Unfortunately, many of these observations have not been followed up with structural analysis and so the nature of many of these putative carbohydrate IgG and IgE epitopes has yet to be determined. Of relevance for the antisera mentioned above raised against known glycoproteins, the N-glycan structures from *Cry j* I (Ogawa *et al.*, 1996), bee venom phospholipase A<sub>2</sub> (*Api m* I)

(Kubelka *et al.*, 1993), horseradish peroxidase (McManus *et al.*, 1988; Harthill, 1991; Kurosaka *et al.*, 1991; Yang *et al.*, 1996), carrot  $\beta$ -fructosidase (Sturm, 1991), *Wistaria floribunda* lectin (Ramirez-Soto and Poretz, 1991), and *Erythrina cristagalli* lectin (Ashford *et al.*, 1987) have been determined (see Figure 1 for structures). All contain  $\alpha$ 1,3-fucose residues attached to the glycan core, with a portion of the bee venom structures featuring a  $\alpha$ 1,3-fucose residue and a  $\alpha$ 1,6-fucose residue attached to the same *N*-acetylglucosamine residue. However, the glycan structures of only three plant pollen allergens have been determined: Japanese cedar *Cry j* I (Ogawa *et al.*, 1996), Bermuda grass BG60 (Ohsuga *et al.*, 1996), and mugwort (*Artemisia vulgaris*) *Art v* II (Nilsson *et al.*, 1991). *Cry j* I contains elaborate glycans with core xylose and  $\alpha$ 1,3-linked fucose, BG60 allergen contains core  $\alpha$ 1,3-linked fucose (but no xylose) and the PNGase F released glycans of *Art v* II are all of the oligomannose type. That  $\alpha$ 1,3-fucose and  $\beta$ 1,2-xylose residues are more common in plants than just on the glycoproteins against which antisera have been prepared is evidenced by many studies such as those on legume lectins (Ashford *et al.*, 1991), pineapple stem bromelain (van Kuik *et al.*, 1986), bean phytohemagglutinin (Sturm *et al.*, 1992), bean phaseolin (Sturm *et al.*, 1987), mistletoe lectin (Debray *et al.*, 1992), miraculin (Takahashi *et al.*, 1990), red kidney bean purple acid phosphatase (Stahl *et al.*, 1994), soybean peroxidase (Gray *et al.*, 1996), zucchini ascorbate oxidase (Altmann, 1998), and *Sophora japonica* lectin (Fournet *et al.*, 1987).  $\beta$ 1,2-Xylosylated oligosaccharides lacking  $\alpha$ 1,3-linked fucose have been found in rice amylase oligosaccharides (Hayashi *et al.*, 1990), zucchini ascorbate oxidase oligosaccharides (D'Andrea *et al.*, 1988; Altmann, 1998), algal oligosaccharides (Balshüsemann and Jaenicke, 1990), and self-incompatibility ribonucleases of *Nicotiana glauca* (Oxley *et al.*, 1996), as well as on snail hemocyanin oligosaccharides (with core  $\alpha$ 1,6-fucose rather than  $\alpha$ 1,3-fucose; van Kuik *et al.*, 1985). Thus, on this indirect evidence, the  $\alpha$ 1,3-fucose residue is a candidate to account for cross-reaction of the same antibodies against plant and insect materials, but any cross-reaction between plant and gastropod is probably due to  $\beta$ 1,2-linked xylose.

**Table I.** Titers of YZ1/2.23 and anti-horseradish peroxidase

| Antigen                                | YZ1/2.23 titer                           | Anti-HRP titer                           |
|----------------------------------------|------------------------------------------|------------------------------------------|
| Horseradish peroxidase                 | <sup>a</sup> 65,600, <sup>a</sup> 92,100 | <sup>a</sup> 13,700, <sup>a</sup> 12,900 |
| <i>Erythrina cristagalli</i> lectin    | 98,900                                   | 6000                                     |
| Bee venom phospholipase A <sub>2</sub> | 2990                                     | 1050                                     |
| Bee venom                              | 3710                                     | 970                                      |
| Keyhole limpet hemocyanin              | 10                                       | 60                                       |

Plates were coated with 50 mM sodium bicarbonate buffer pH 9.6 containing 20  $\mu$ g/ml glycoproteins or 50  $\mu$ g/ml bee venom. Titers for YZ1/2.23 and the anti-glycan fraction of polyclonal rabbit anti-horseradish peroxidase antiserum were determined by 5-fold serial dilution from 1:250 to 1:1.25  $\times$  10<sup>10</sup>. Bound antibody was measured using alkaline phosphatase conjugated anti-rat or anti-rabbit IgG antiserum as appropriate. The calculated dilution giving 50% binding was taken as the titer.

<sup>a</sup>Independent assays by different operators.

In this report, further studies have been performed on the specificity of YZ1/2.23 a monoclonal antibody raised in rat against elderberry abscission tissue. Previous studies have shown that binding of this antibody is inhibited by addition of the major xylose/fucose containing oligosaccharide of horseradish peroxidase (McManus *et al.*, 1988). In the present study two different

sets of experiments lead to the same conclusion on the specificity of YZ1/2.23 and on the carbohydrate binding of polyclonal anti-horseradish peroxidase antiserum. Additionally, a wide range of plant extracts, including pollens, have been screened for reactivity against these antibodies. The results show that the  $\alpha$ 1,3-fucose residue is an important epitope for antibody binding and that reactivity to YZ1/2.23 and anti-horseradish peroxidase is widespread, but not ubiquitous, in plant extracts.

**Table II.** Oligosaccharide inhibitors of YZ1/2.23 and anti-horseradish peroxidase

| Inhibitor             | IC <sub>50</sub> values of glycans for inhibition of binding of antibody to HRP ( $\mu$ M) |                    |
|-----------------------|--------------------------------------------------------------------------------------------|--------------------|
|                       | YZ1/2.23                                                                                   | Anti-HRP           |
| HRP glycoprotein      | 0.2 <sup>a</sup>                                                                           | 0.1 <sup>a</sup>   |
| HRP N-glycans         | 3.6                                                                                        | 5.1                |
| MMXF <sup>3</sup>     | 5.8                                                                                        | 1.4                |
| MMXF <sup>3</sup> -ol | 42                                                                                         | 10                 |
| MMX                   | 160                                                                                        | <190 <sup>b</sup>  |
| MMX-ol                | 74                                                                                         | <190 <sup>b</sup>  |
| 00XF <sup>3</sup>     | 29                                                                                         | 41                 |
| 00XF <sup>3</sup> -ol | 230                                                                                        | <230 <sup>b</sup>  |
| MMX-Gn                | 200                                                                                        | 91                 |
| MM                    | <1100 <sup>b</sup>                                                                         | <1100 <sup>b</sup> |
| M2(6)                 | <2900 <sup>b</sup>                                                                         | <2900 <sup>b</sup> |
| M2(3)                 | <2900 <sup>b</sup>                                                                         | <2900 <sup>b</sup> |
| M3                    | <2000 <sup>b</sup>                                                                         | <2000 <sup>b</sup> |

Horseradish peroxidase glycoprotein and potential oligosaccharide inhibitors (5-fold serial dilution, initial concentration 0.2–1.0 mg/ml) were preincubated with an equal volume of YZ1/2.23 (1:50,000 dilution) or the anti-glycan fraction of polyclonal rabbit anti-horseradish peroxidase antiserum (1:1000 dilution) for 1 h. Binding of the antibodies to horseradish peroxidase coated plates was then determined by ELISA. The concentration of glycan giving 50% inhibition (IC<sub>50</sub>) of binding was calculated from the titer. The abbreviations used for the structures are those given in Figure 1. MMXF<sup>3</sup>-ol, MMX-ol, and 00XF<sup>3</sup>-ol are the reduced forms of MMXF<sup>3</sup>, MMX, and 00XF<sup>3</sup> respectively. MMX-Gn is the form of MMX lacking the reducing terminal GlcNAc residue.

<sup>a</sup>Calculation assumes 8 mol glycan/mol protein.

<sup>b</sup>Not inhibitory at maximum concentration tested.

## Results

### Antibody titers with plant and arthropod glycoproteins

Titers for both YZ1/2.23 and the anti-glycan fraction of anti-horseradish peroxidase were measured using ELISA method I (Table I). These results show similar YZ1/2.23 titers for the two glycoproteins which contain both xylose and  $\alpha$ 1,3-linked fucose, horseradish peroxidase and *Erythrina cristagalli* lectin, while with bee venom phospholipase A<sub>2</sub> and whole bee venom the titer required for 50% binding is more than a magnitude higher. This lower affinity may be due to a variety of reasons separately or together: truly lower affinity of YZ1/2.23 for structures lacking xylose; differences in the amount of N-glycan actually bound to the plates; that only 15% in total of phospholipase A<sub>2</sub> glycans have core  $\alpha$ 1,3-linked fucose (Kubelka *et al.*, 1993). However, the affinity of YZ1/2.23 for phospholipase A<sub>2</sub> glycans is sufficient for the antibody to bind to a column of phospholipase

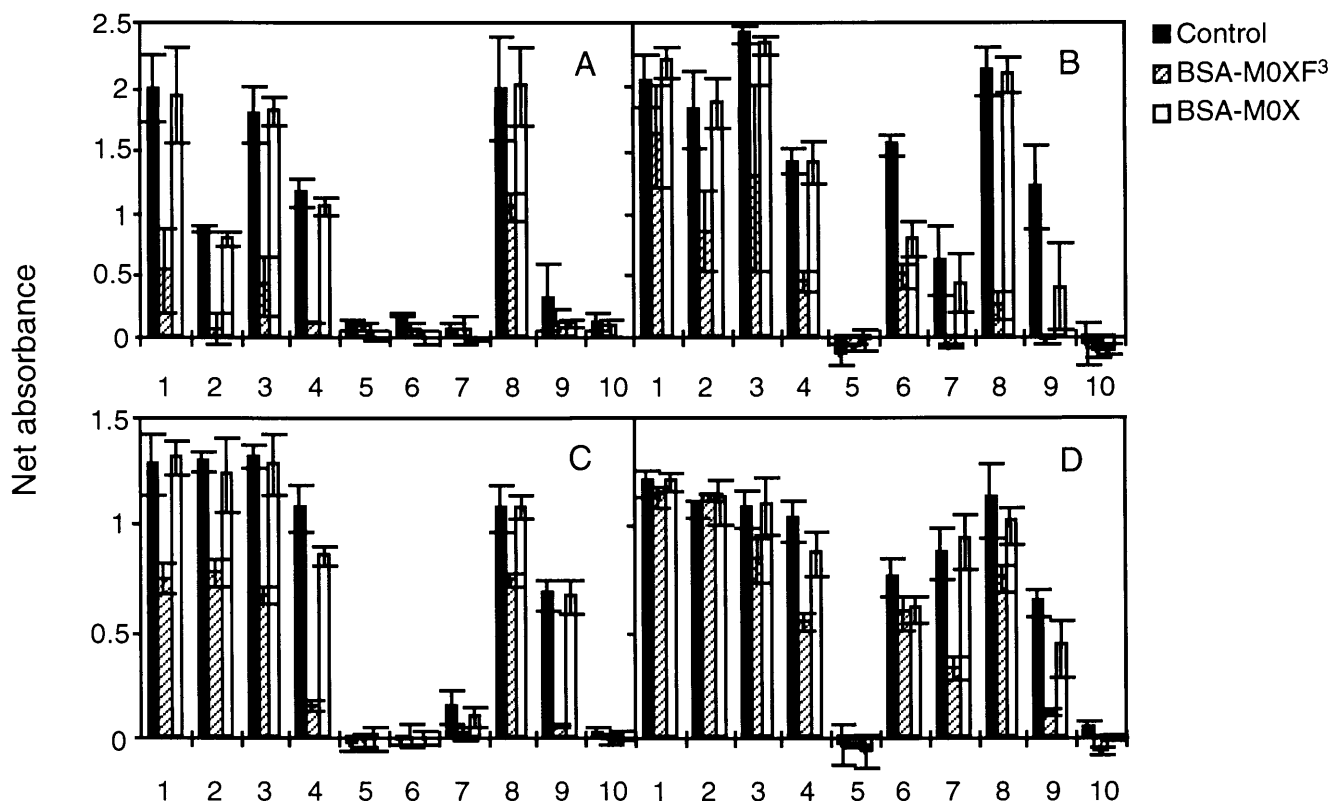


**Fig. 2.** ELISA of YZ1/2.23 and anti-horseradish peroxidase with glycoproteins. ELISA plate wells were coated with glycoproteins at a GlcNAc content concentration of either 0.0675  $\mu$ M (color reaction terminated at 10 min; **A** and **B**) or 1.35  $\mu$ M (color reaction terminated at 6 min; **C** and **D**). The primary antibodies were YZ1/2.23 monoclonal (**A** and **C**) and anti-horseradish peroxidase polyclonal (**B** and **D**) and were preincubated for 1 h prior to addition to the wells in the absence or presence of neoglycoproteins BSA-M0XF<sup>3</sup> or BSA-M0X (40 nM GlcNAc content). The net absorbance was recorded at 405/620 or 405/492 respectively. Glycoprotein coats: 1, *S.japonica* lectin; 2, carboxymethylated horseradish peroxidase; 3, carboxymethylated bromelain; 4, zucchini ascorbate oxidase; 5, bovine ribonuclease B; 6, *Helix pomatia* (snail) hemocyanin; 7, bee venom phospholipase A<sub>2</sub>; 8, BSA-M0XF<sup>3</sup>; 9, BSA-M0X; 10, BSA-MM.

A<sub>2</sub> glycopeptide conjugated Sepharose in a manner reversible by elution with 0.2 M glycine, pH 2.2 (data not shown). Broadly similar relative titers as found for YZ1/2.23 were found for the anti-glycan fraction of anti-horseradish peroxidase, except that the affinity of *Erythrina cristagalli* lectin for the anti-glycan antibodies was much less than that of native, noncarboxymethylated horseradish peroxidase. Another way to see the results is that the YZ1/2.23 titer for *Erythrina cristagalli* lectin is about 30 times greater than that for phospholipase A<sub>2</sub>, compared with a 6-fold difference in the titers for the anti-glycan fraction of anti-horseradish peroxidase.

#### Competition ELISA with horseradish peroxidase and related free N-glycans

In order to probe the specificity of the two antibodies further, free N-glycans were used to inhibit binding of the antibodies to horseradish peroxidase using ELISA method I (Table II). The results indicated that the presence of the  $\alpha$ 1,3-fucose residue and its orientation relative to the rest of the glycan were important for recognition. This was demonstrated by the strong inhibition titer of MMXF<sup>3</sup> and 00XF<sup>3</sup> relative to MMX, and the decrease in inhibition titer of MMXF<sup>3</sup>-ol and 00XF<sup>3</sup>-ol relative to their respective unreduced glycans (the structures of these N-glycans are given in Figure 1). The presence of the  $\alpha$ -mannose residues were also required for optimal binding as it was seen that 00XF<sup>3</sup>

was a weaker inhibitor than MMXF<sup>3</sup>. The inhibition due to the mannoses required the presence of the xylose residue as MMX was weakly inhibitory but MM did not inhibit at all nor did the M2 dimannoside isomers nor the trimannoside M3. Only the  $\alpha$ 1,6-linked mannose may be required since YZ1/2.23 also binds to bromelain (see Figure 2) which has an M0XF<sup>3</sup> glycan lacking the  $\alpha$ 1,3-linked mannose (van Kuik *et al.*, 1986). The anti-glycan fraction of anti-horseradish peroxidase showed a similar pattern of inhibition to YZ1/2.23, and these results are compatible with the conclusions of Kurosaka *et al.* (Kurosaka *et al.*, 1991) that the  $\alpha$ 1,6-linked mannose and  $\alpha$ 1,3-linked fucose of plant N-glycans are the predominant residues recognized by polyclonal anti-horseradish peroxidase.

#### Binding on antibodies to a range of standard glycoproteins

In some initial experiments using ELISA method II, it appeared that a coating concentration of 0.0675  $\mu$ M (in terms of GlcNAc) and dilutions of 1:50,000 YZ1/2.23 gave the right balance between detection and the economic use of inhibitors. In other initial experiments, plates were coated with *S.japonica* lectin (0.5  $\mu$ g/ml) and YZ1/2.23 was preincubated in the absence or presence of five fold dilutions of different glycoconjugates. It was found that concentrations (in terms of glycopeptide) of approximately 10 nM BSA-M0XF<sup>3</sup> gave around 50% inhibition of binding of YZ1/2.23 by *S.japonica* lectin, while bromelain

glycopeptide was nowhere as effective as an inhibitor (50% inhibition with approximately 500 nM). With plant extracts as inhibitors, 50% inhibition was seen for YZ1/2.23 generally in the range 1–10  $\mu$ M (in terms of GlcNAc) but reproducibility was lacking. Adding plant extracts to an interferon/anti-interferon ELISA showed the possibility of false inhibitions (data not shown); thus, another format of inhibition ELISA was used to test binding by plant extracts (see below).

Based on the results of the initial experiments with YZ1/2.23, in subsequent experiments, a single concentration of inhibitor was used with both antibody systems, but with a range of coating glycoproteins. Two different coating concentrations (in terms of GlcNAc content) were used, and BSA-M0XF<sup>3</sup> and BSA-M0X were used as inhibitors. The same concentrations of inhibitors and antibody dilutions were used for ELISA with both anti-horseradish peroxidase and the YZ1/2.23 monoclonal. The results are shown in Figure 2. Glycoproteins used fell into a number of categories. *S.japonica* lectin, carboxymethylated horseradish peroxidase, carboxymethylated bromelain, and BSA-M0XF<sup>3</sup> bound well to both YZ1/2.23 and anti-horseradish peroxidase with binding to them inhibited to a moderate extent by BSA-M0XF<sup>3</sup> (40 nM in terms of GlcNAc), but with no significant inhibition when the BSA-M0X was used. A trimannosyl glycopeptide conjugated to bovine serum albumin (BSA-MM) and ribonuclease B did not bind either antibody. Hemocyanin from the snail *Helix pomatia* (whose low molecular weight glycans include the MMXF<sup>6</sup> structure and so it has xylosylated glycans with  $\alpha$ 1,6-linked rather than  $\alpha$ 1,3-linked core fucose (van Kuik *et al.*, 1985)) did not bind YZ1/2.23 but did bind anti-horseradish peroxidase in a manner inhibitable by both BSA-M0XF<sup>3</sup> and BSA-M0X; this would be in keeping with YZ1/2.23 requiring core  $\alpha$ 1,3-linked fucose, but also in keeping with polyclonal anti-horseradish peroxidase containing antibodies binding xylose as well as antibodies binding  $\alpha$ 1,3-linked core fucose. Bee venom phospholipase A<sub>2</sub> would only bind to any extent to YZ1/2.23 (1: 50,000) at a coating concentration of 1.35  $\mu$ M, but would moderately bind anti-horseradish peroxidase (1: 50,000) at 0.0675  $\mu$ M. The result of phospholipase A<sub>2</sub> with YZ1/2.23 is compatible with its relatively low titer for the monoclonal (Table I). BSA-M0X showed low binding to YZ1/2.23 at the 0.0675  $\mu$ M coating concentration, but would bind moderately at 1.35  $\mu$ M. This could be due to some remaining fucose (certainly less than 5%, as judged by the monosaccharide composition) or that there is some low affinity of YZ1/2.23 for structures with xylose alone (consistent with the data in Table II showing that MMX as a free glycan has some ability to inhibit YZ1/2.23 binding to horseradish peroxidase). However, the explanation that there is low affinity of the monoclonal for xylose residues is not borne out by the result with hemocyanin, although the presence of  $\alpha$ 1,6-linked fucose could mean that the conformation of the xylose in comparison to the rest of the glycan is not necessarily the same and so like cases are not being compared. BSA-M0X bound quite well to anti-horseradish peroxidase and binding to it was inhibited by both native and defucosylated conjugates.

Due to poor inhibition with some glycoproteins when 1.35  $\mu$ M (in terms of GlcNAc) of glycoprotein was used to coat the plate and unfractionated anti-horseradish peroxidase used as the primary antibody, titrations were performed with the BSA-M0XF<sup>3</sup> (40, 400, or 4000 nM) preincubated with anti-horseradish peroxidase prior to binding to wells coated with either *S.japonica* lectin, carboxymethylated horseradish peroxidase and carboxymethylated bromelain. Similar experiments with

YZ1/2.23 were performed with either 4, 40, or 400 nM of BSA-M0XF<sup>3</sup>. The results of these titrations with both antibodies are shown in Figure 3. It appears that increasing coating concentration for experiments with anti-horseradish peroxidase results in very tight binding of antibodies. Binding of total anti-horseradish peroxidase to carboxymethylated bromelain, which carries the M0XF<sup>3</sup> structure, is seemingly more easily inhibited than that to carboxymethylated horseradish peroxidase or to *S.japonica* lectin which both carry mainly MMXF<sup>3</sup>. Binding of all these glycoproteins to YZ1/2.23 appears more easily inhibitable, but like is not being compared with like since YZ1/2.23 is a monoclonal.

### Screening of plant extracts

Twenty-eight crude plant extracts were analyzed for binding to YZ1/2.23 and polyclonal anti-horseradish peroxidase. Additionally bee venom and housedust mite (*Dermatophagoides pteronyssinus*) extract were examined: bee venom was examined since it contains phospholipase A<sub>2</sub> as well as hyaluronidase which both carry some core  $\alpha$ 1,3-linked fucose (Kubelka *et al.*, 1993, 1995) while *Dermatophagoides* extract was included in the set of samples due to reported periodate-sensitive cross-reactions between the Dp6 monoclonal raised against *Dermatophagoides* extract and plant glycoproteins (van Ree *et al.*, 1995a). Plates were coated at a concentration of 0.0675  $\mu$ M in terms of GlcNAc; antibodies applied at 1:50,000 and BSA-M0XF<sup>3</sup> and BSA-M0X used as inhibitors at 40 nM in terms of GlcNAc. Many of the extracts bound both antibodies (see Figure 4), predicting a widespread occurrence of core  $\alpha$ 1,3-linked fucose, although some extracts appeared to have reduced binding to YZ1/2.23 as compared to binding to anti-horseradish peroxidase. Binding of either antibody to plant extracts was in general far more inhibited by BSA-M0XF<sup>3</sup> than by BSA-M0X. As regards YZ1/2.23, grass and ragweed pollen extracts and the nut extracts gave the highest readings, which would suggest the presence of  $\alpha$ 1,3-linked core fucosylated oligosaccharide in types of substance which are commonly allergenic. Bee venom appeared to bind anti-horseradish peroxidase well but only at a low level to YZ1/2.23 (as with bee venom phospholipase). At 0.675  $\mu$ M in terms of GlcNAc coating concentration (i.e., 10 times more GlcNAc than for the other samples), there was no sign of binding of either antibody by *Dermatophagoides* extract.

In other experiments to test cross-reaction between plant and insect, selected plant extracts were used in ELISA with anti-bee venom antiserum at 1:50,000 dilution, but the net absorbances were generally very low. The highest result was with ryegrass (*Lolium perenne*) pollen which gave a net absorbance approaching 50% of that for bee venom phospholipase A<sub>2</sub>. The binding of anti-bee venom to ryegrass pollen extract was 50% inhibited by BSA-M0XF<sup>3</sup> while the binding of anti-bee venom to bee venom phospholipase A<sub>2</sub> was not inhibited by either BSA-M0XF<sup>3</sup> or BSA-M0X under the conditions used (data not shown). This would suggest that anti- $\alpha$ 1,3-linked fucose activity is a minor portion of total anti-bee venom antiserum, but isolation of a glycopeptide-binding fraction of this antiserum, which cross-reacts with plant glycoproteins, has been described by others (Prenner *et al.*, 1992).

### Discussion

The monoclonal antibody YZ1/2.23 has previously been shown to react with the plant N-glycan Man<sub>3</sub>XylFucGlcNAc<sub>2</sub> (MMXF<sup>3</sup>; see Figure 1) (McManus *et al.*, 1988). In the present article, we

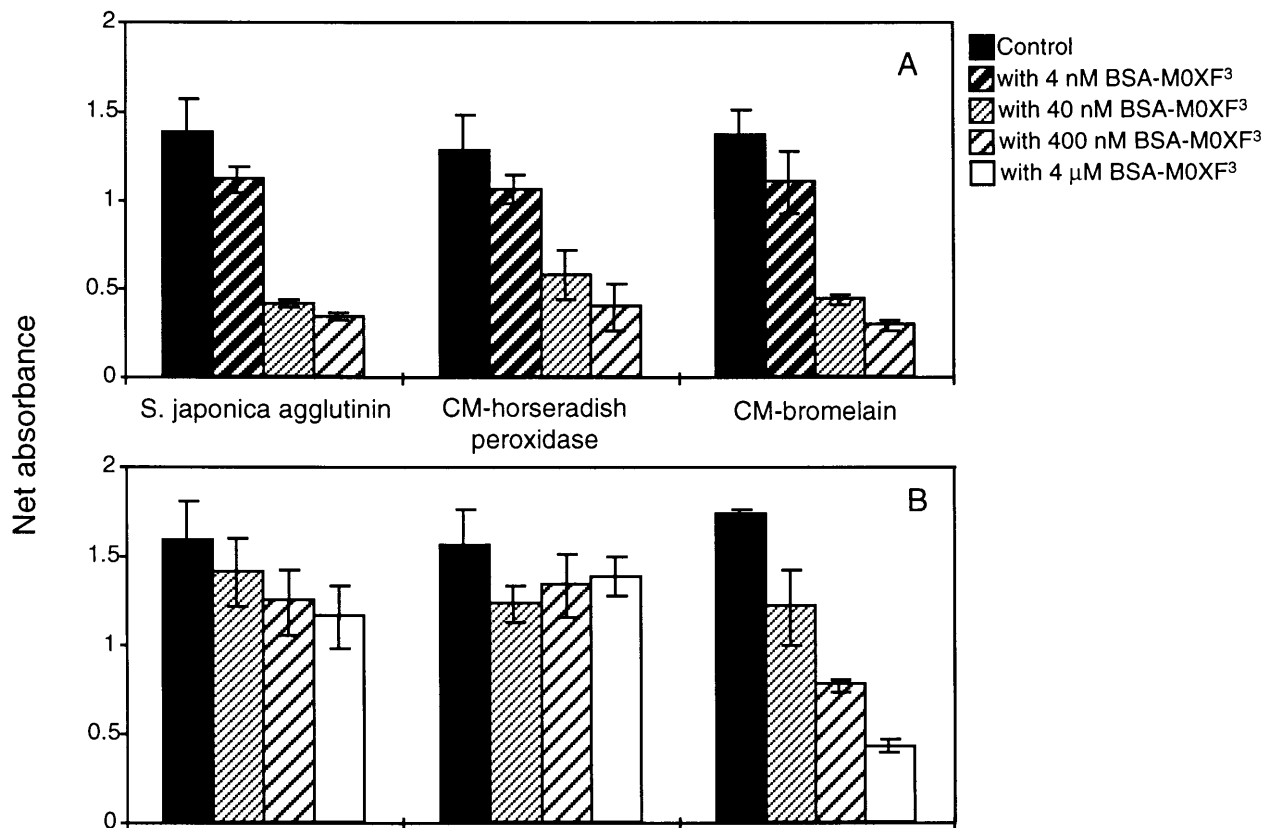


**Fig. 3.** Titration of inhibition of glycoprotein binding to YZ1/2.23 and anti-horseradish peroxidase. ELISA plate wells were coated with *S. japonica* agglutinin, carboxymethylated horseradish peroxidase, or carboxymethylated bromelain at a GlcNAc content concentration of 1.35  $\mu$ M. The primary antibodies were YZ1/2.23 monoclonal (A) and anti-horseradish peroxidase polyclonal (B) and were preincubated for one h prior to addition to the wells in the absence or presence of the neoglycoprotein BSA-M0XF<sup>3</sup> (4, 40, or 400 nM GlcNAc content in the case of YZ1/2.23 and 40, 400 or 4000 nM GlcNAc in the case of anti-horseradish peroxidase). The color reaction was terminated after 6 min and net absorbance was recorded at 405/620 or 405/492, respectively.

present data that defines more specifically the epitope of the antibody. The evidence we have obtained indicates that the most important requirement for binding is the presence of a core  $\alpha$ 1,3-linked fucose residue, with an additional involvement of, at least, the terminal  $\alpha$ 1,6-linked mannose residue for optimal binding. The presence of  $\beta$ 1,2-linked xylose alone is not sufficient for strong binding, but it does appear to confer some low degree of binding (see Table II). As previously noted, the titer of YZ1/2.23 with bee venom phospholipase is about 30-fold less than the titer with *Erythrina cristagalli* lectin, while for the titer of anti-horseradish peroxidase the difference is only 6-fold less. This could indicate that YZ1/2.23 may also require the presence of the  $\beta$ -xylose residue for optimal binding, while anti-horseradish peroxidase may include populations of antibodies that recognize  $\alpha$ 1,3-linked fucose regardless of the presence of xylose or substitution of the mannose residues. Ideally pure core  $\alpha$ 1,3-fucosylated glycan should be tested to determine the precise role of the xylose in the structure necessary for optimal binding; however, neither a convenient source which has this pure structure in a sufficient amount, nor a xylosidase of the required purity or specificity in order to generate this structure from MMXF<sup>3</sup>, are currently available.

Our results on binding of unfractionated anti-horseradish peroxidase antiserum to known glycoproteins (Figure 2), included for comparative purposes, are consistent with previous reports to indicate the presence of reactivities directed against the

xylose residue, as well as against the core  $\alpha$ 1,3-fucose residue, in antisera to plant N-glycoproteins. Indeed, the affinity separation of polyclonal antisera to plant N-glycoproteins into different anti-carbohydrate antibody populations that react preferentially with either fucosylated or xylosylated glycans has been reported (Faye *et al.*, 1993). Overall, therefore, it is concluded that binding to anti-horseradish peroxidase antiserum can indicate the presence of either xylose or  $\alpha$ 1,3-linked fucose, and thus binding to this polyclonal cannot alone demonstrate the presence of horseradish peroxidase type glycans with *both* xylose and core  $\alpha$ 1,3-linked fucose in nonplant extracts. The conformational analyses that have been published on xylose/fucose-type glycans would suggest that the xylose and  $\alpha$ 1,3-fucose are on opposite faces of the core (Bouwstra *et al.*, 1990; Lommerse *et al.*, 1995), and so it could be hypothesized that xylose-reactive antibodies could recognize one face and fucose-reactive antibodies the other face. Indeed, Kurosaka *et al.* (Kurosaka *et al.* 1991), based on their results, presented such a model of anti-horseradish peroxidase binding to the predominant epitope formed by the  $\alpha$ 1,6-linked mannose and  $\alpha$ 1,3-linked fucose. In the case of gastropod glycoproteins (such as *Helix pomatia* hemocyanin; see Figure 2), it is expected that binding to anti-horseradish peroxidase is mediated by the xylose residue and not by cross-reaction with core  $\alpha$ 1,6-linked fucose; certainly recent results on a monoclonal raised against *Dictyostelium discoideum* glycoproteins that recognizes  $\alpha$ 1,6-fucosylated, but not core

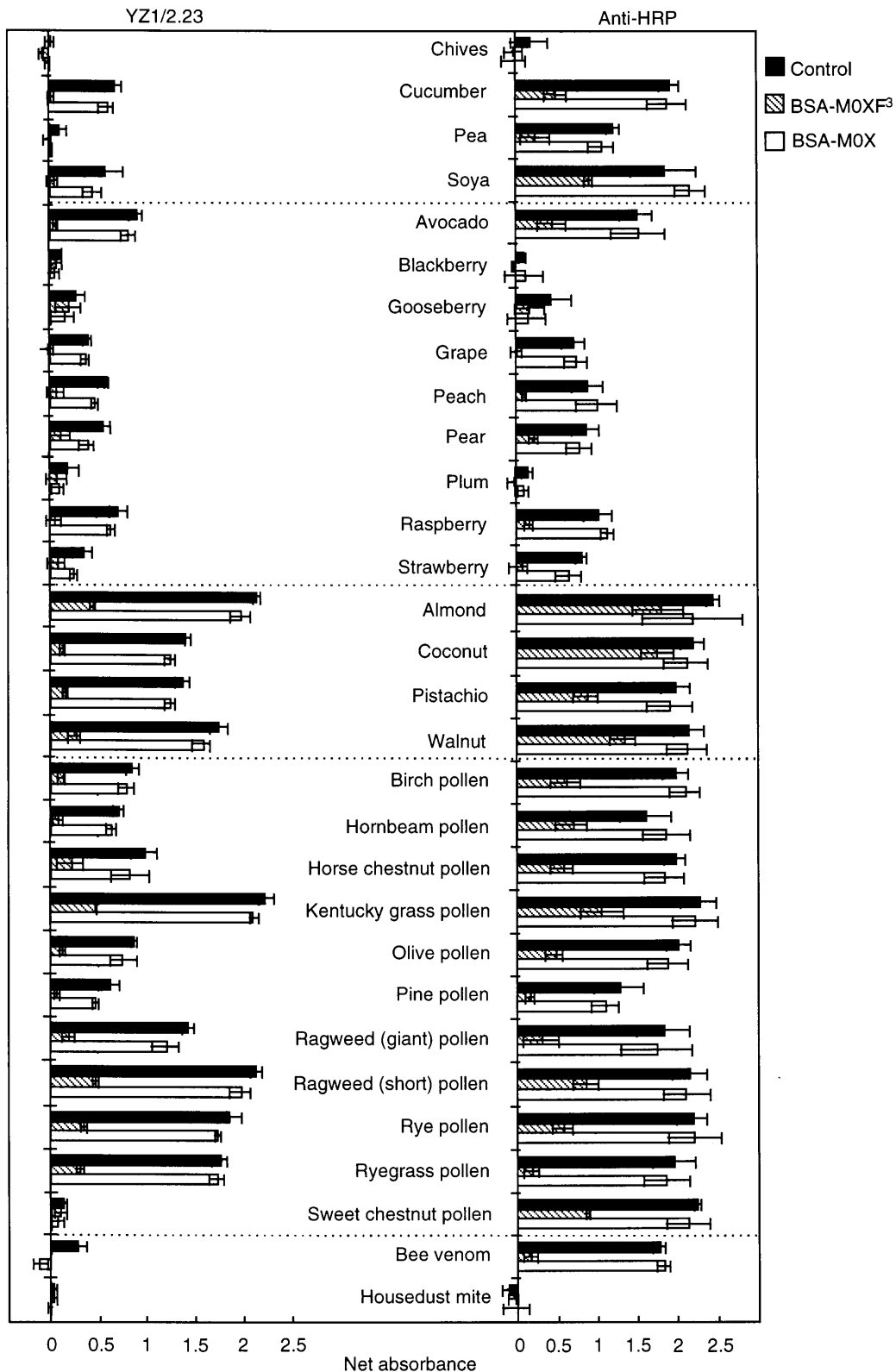


**Fig. 4.** ELISA of YZ1/2.23 and anti-HRP with plant extracts. ELISA plate wells were coated with plant extracts at a concentration of  $0.0675 \mu\text{M}$  GlcNAc content. Additionally, *Dermatophagoides* extract ( $0.675 \mu\text{M}$  GlcNAc) and whole bee venom ( $0.0675 \mu\text{M}$ ) were tested. The primary antibodies were YZ1/2.23 monoclonal and anti-horseradish peroxidase polyclonal and were preincubated for 1 h prior to addition to the wells in the absence or presence of neoglycoproteins BSA-M0XF<sup>3</sup> or BSA-M0X ( $40 \text{ nM}$  GlcNAc content). The net absorbance was recorded at 405/620 or 405/492, respectively.

$\alpha$ 1,3-fucosylated, glycoproteins would suggest that core  $\alpha$ 1,3-linked fucose and  $\alpha$ 1,6-linked fucose are not cross-reacting epitopes (Srikrishna *et al.*, 1997).

A range of plant extracts have also been examined with particular emphasis on known extracts from known allergens such as pollens and food extracts. The results suggest that potential carbohydrate allergens are present in a wide range of these extracts (but not all) and structural analyses of pollen, as well as some fruit and nut, extracts show the presence of xylosylated and core  $\alpha$ 1,3-fucosylated N-glycans (I.B.H. Wilson and F. Altmann, unpublished observations). It is noticeable that some extracts, particularly tree pollens, react as well as grass pollens with the anti-horseradish peroxidase polyclonal but only about half as well as grass pollens with the YZ1/2.23 monoclonal. This may be due to a greater proportion of nonreducing terminal N-acetylglucosamine residues, as found by the structural analyses of tree pollen glycans as compared to grass pollen glycans (I.B.H. Wilson and F. Altmann, unpublished observations), which potentially may block part of the epitope recognized by the monoclonal.

In some recent studies, control glycoproteins with N-glycan structures containing xylose and/or  $\alpha$ 1,3-linked fucose have been tested against patients' sera and found to bind IgE (van Ree *et al.*, 1995a; Batanero *et al.*, 1996; Petersen *et al.*, 1996). It has also been reported that the carbohydrate of ryegrass (*Lolium perenne*) allergen *Lol p XI* is a cross-reactive epitope (van Ree *et al.* 1995a), that serum from a patient allergic to grass pollens and tomatoes has IgE cross-reacting with BSA-M0XF<sup>3</sup> (Petersen *et al.*, 1996), that Bermuda grass BG60 allergen has periodate-sensitive IgE epitopes (Su *et al.*, 1996), that a glycopeptide from olive (*Olea europaea*) *Ole e I* is an IgE epitope (Batanero *et al.*, 1994) and that cross-reactions of IgE from fruit-allergic patients have been found to be partly due to carbohydrate as well as profilin (van Ree *et al.*, 1995b). These findings with *Lol p XI* and *Ole e I* are explicable by the present results, and those of the companion structural studies mentioned above, if the presence of xylose and/or  $\alpha$ 1,3-linked fucose in the overall extracts is reflected in the glycosylation of the individual allergens. In the case of purified Bermuda grass BG60 allergen, the presence of  $\alpha$ 1,3-linked fucose has been proven (Ohsuga *et al.*, 1996). The absence of any cross-reactivity of either YZ1/2.23 or anti-horseradish peroxidase with *Dermatophagoides* extract makes it difficult to explain the finding of van Ree *et al.* (van Ree *et al.*, 1995a) that an anti-*Dermatophagoides* monoclonal, Dp6, cross-reacts with plant extracts. They concluded that Dp6 must be against the carbohydrate because of periodate-sensitive cross-reaction with a wide range of pollen and food extracts. However, together with preliminary structural analysis of *Dermatophagoides* N-glycans which as yet have not indicated the presence of MMXF<sup>3</sup> or related structures (data not shown), the present findings are not compatible with the binding of Dp6 being due to the presence of N-glycans containing xylose and/or  $\alpha$ 1,3-fucose. The lack of knowledge of *Dermatophagoides* glycosylation, though, means that cross-reactions of Dp6 to other N-glycans, O-glycans, or polysaccharides or other periodate-sensitive components cannot be absolutely ruled out.

N-Glycans are, of course, not the only potential "pan-allergens" and doubt has been cast by van Ree and Aalberse on the clinical significance of carbohydrate cross-reactive determinants (van Ree and Aalberse, 1993), but that conclusion was based on one study of seven patients (Aalberse *et al.*, 1981b). In contrast, there is much data on protein "pan-allergens" such as profilins (which are components of the eukaryotic cytoskeleton

homologous to the birch *Bet v 2* allergen) in plants (Valenta *et al.*, 1992; van Ree and Aalberse, 1993; van Ree *et al.*, 1995b) and tropomyosins in crustacea and mollusca (Leung *et al.*, 1996). One factor to consider in assessing the importance of N-glycans as "pan-allergens" is that since plant glycans are immunogenic, antibodies raised against native plant glycoprotein allergens will contain a component directed against the glycan, although anti-carbohydrate antibodies may not account for all the antibody species present. Therefore even though a polyclonal antiserum raised against a glycoprotein (or a mixture of proteins including glycoproteins) may recognize recombinant protein expressed in *E. coli*, there may well be an anti-glycan component as well. Only careful examination of the antibody titer or inhibition studies could rule out the importance of the glycan as an epitope. What maybe true of antibodies raised in rabbits could also be true of IgE pools from patients allergic to plant materials and if patients have IgE against the glycan, then cross-reactions not dependent on known conserved proteins are possible. Indeed, the xylosylated and/or fucosylated N-linked carbohydrate structures in plants are probably more conserved across plant species than any protein sequence, as shown by the studies referred to above (van Kuik *et al.*, 1986; Ashford *et al.*, 1987, 1991; Fournet *et al.*, 1987; Sturm *et al.*, 1987, 1992; D'Andrea *et al.*, 1988; Balshusemann and Jaenicke, 1990; Hayashi *et al.*, 1990; Takahashi *et al.*, 1990; Ramirez-Soto and Poretz, 1991; Sturm, 1991; Debray *et al.*, 1992; Stahl *et al.*, 1994; Gray *et al.*, 1996; Ogawa *et al.*, 1996; Ohsuga *et al.*, 1996; Oxley *et al.*, 1996; Yang *et al.*, 1996; Altmann, 1998). Also, xylosylated N-glycans are present in snails (van Kuik *et al.*, 1985), while  $\alpha$ 1,3-linked fucosylated N-glycans are present in insects (Kubelka *et al.*, 1993, 1995).

It appears that cross-reactions between IgE and plant and invertebrate materials could be due to a wide range of protein and carbohydrate materials. With more structural analysis or the use of antibodies with proven anti-carbohydrate reactivity, it should prove possible to give a proper structural basis for understanding many of the data suggesting anti-carbohydrate IgE cross-reactions. Once the structures responsible for the observed carbohydrate-mediated cross-reactions are identified then it will be possible to use defined glycoconjugates to verify that IgE from patients actually does bind certain types of glycan, causing IgE-dependent allergy-related events and to facilitate better screening of patients' sera. This would provide definitive proof that the carbohydrate-based cross-reactions so frequently observed are clinically relevant.

## Materials and methods

### Materials

YZ1/2.23 was the kind gift of Professor Daphne Osborne (Open University, Oxford); rabbit anti horseradish peroxidase, alkaline phosphatase conjugated anti-rat IgG and horseradish peroxidase conjugated anti-rabbit IgG were from Sigma. The glycan-specific fraction of anti-horseradish peroxidase was obtained by passing the antiserum through a column of agarose-linked *Erythrina cristagalli* lectin. Bound antibodies were eluted with 0.1 M glycine HCl buffer pH 2.8. Pooled fractions were immediately adjusted to pH 7.4 with 1 M KH<sub>2</sub>PO<sub>4</sub> and concentrated by ultrafiltration to the original volume applied to the column. The following plant extracts were the kind gift of Professor Christof Ebner (Allgemeines Krankenhaus der Stadt Wien) and had been prepared by the method of Hirschwehr *et al.* (Hirschwehr *et al.*, 1992): soya, pea, cucumber, chive, hornbeam pollen, horse chestnut pollen, sweet chestnut pollen, ryegrass pollen, blackberry,

pear, peach, plum, avocado, gooseberry, grape, strawberry, raspberry, almond, coconut, walnut, and pistachio. Pine (*Pinus leucodermis*) pollen collected from a tree in a local park and birch, Kentucky blue grass, olive, ragweed, and rye pollens purchased from Sigma were extracted overnight with carbonate buffer by the method of Ipsen and Løwenstein (Ipsen and Løwenstein, 1983), prior to a brief centrifugation to remove particulate material. *Helix pomatia* hemocyanin was from Serva, Heidelberg, Germany, and zucchini ascorbate oxidase from Boehringer Mannheim. Other glycoproteins were from Sigma. *Dermatophagoides pteronyssinus* extract was from Greer Laboratories, USA. The mannose dimers M2(6) and M2(3) and branched mannose trimer M3 were purchased from Dextra Laboratories Ltd., Reading, UK. Oligosaccharides, glycopeptides, and neoglycoproteins were prepared as detailed below.

#### Preparation of oligosaccharides

Total horseradish peroxidase N-glycans, MMXF<sup>3</sup>, and MMX-Gn were isolated as described previously (Ashford *et al.*, 1987; McManus *et al.*, 1988; Harthill, 1991); MM was isolated from hen egg-white ovomucoid by methods described in (Ashford *et al.*, 1987); MMXF<sup>3</sup>-ol, was prepared by reduction of MMXF<sup>3</sup> with 0.1 M NaBD<sub>4</sub> for 4 h at 30°C as described previously (Ashford *et al.*, 1987). MMX and MMX-ol were prepared by chemical defucosylation of MMXF<sup>3</sup> and MMXF<sup>3</sup>-ol, respectively, with 0.1 M HCl for 20 min at 100°C; 00XF<sup>3</sup> and 00XF<sup>3</sup>-ol were prepared by enzymatic digestion of MMXF<sup>3</sup> and MMXF<sup>3</sup>-ol, respectively, with jack bean  $\alpha$ -mannosidase (10 U/ml, 300 h, 37°C). All the above glycan preparations were checked for purity and integrity by matrix-assisted laser-desorption time-of-flight mass spectrometry using a Lasermat instrument (Finnegan MAT Ltd., UK) with 2,5-dihydroxybenzoic acid as the matrix. The composition and concentration of these glycans was determined by monosaccharide analysis after acid hydrolysis with 2 M trifluoroacetic acid for 4 h at 100°C using HPAEC on a Dionex BioLC system (Dionex, UK).

#### Preparation of bromelain and fibrin glycopeptides

Bromelain and fibrin were digested with pepsin in 5% formic acid. The digests were subject to gel filtration steps and finally to anion exchange. Glycopeptides were analyzed by amino acid and amino sugar analysis (Altmann, 1992) and by analysis of sugar composition on HPLC after labeling with 1-phenyl-3-methyl-5-pyrazolone (PMP) (Fu and O'Neill, 1995). Bromelain glycopeptide was defucosylated by treatment with 100% (v/v) trifluoroacetic acid at room temperature for 2 days and then subjected to gel filtration. The MM glycopeptide was generated by enzymatic removal of galactose and *N*-acetylglucosamine from fibrin glycopeptide. Removal of sugar residues was monitored by the PMP method.

#### Preparation of neoglycoproteins

Cross-linking of fucosylated and defucosylated bromelain glycopeptide to bovine serum albumin (to prepare BSA-M0XF<sup>3</sup> and BSA-M0X, respectively) or cross-linking of the MM glycopeptide to bovine serum albumin (to prepare BSA-MM) was performed as follows: 3 mg of glycopeptide was dissolved in 100  $\mu$ l cold 0.1 M KH<sub>2</sub>PO<sub>4</sub>, pH 7.2, to which 70  $\mu$ l 7 M guanidine hydrochloride and 500  $\mu$ l 2,4-dinitro-1,5-difluorobenzene (60 mg/ml in methanol) were added. After 15 min at room temp, the reaction mixture was placed on ice and extracted three times

with diethylether. The lower phase was retained and dried under a stream of nitrogen and the residue was taken up in 100  $\mu$ l of 100 mg/ml bovine serum albumin in 0.4 M sodium borate, pH 10, and left in the dark overnight at room temperature prior to gel filtration on P30 (50 mM ammonium acetate, pH 5). The resulting neoglycoproteins were subject to amino sugar analysis as described above and found to contain an estimated 3–4 mol glycopeptide per mole of protein.

#### Enzyme-linked immunosorbent assay (method I)

The method described by McManus *et al.* (McManus *et al.*, 1988) was used for the data in Table I and II.

#### Enzyme-linked immunosorbent assay (method II)

For all ELISA data except those in Table I and II, plates were coated with extract or glycoprotein at a concentration of 0.0675  $\mu$ M or 1.35  $\mu$ M in terms of total GlcNAc (this is equivalent to 0.5  $\mu$ g/ml or 10  $\mu$ g/ml *Sophora japonica* lectin) in 50 mM sodium carbonate, pH 9.6 for 90 min at 37°C. Horseradish peroxidase and bromelain were inactivated prior to use by carboxymethylation. Only the inner 60 wells were used since “edge” effects were otherwise observed. After blocking with 3% w/v dried milk powder solution in TTBS (0.1 M Tris, pH 7.2, 0.1 M sodium chloride, 2.5 mM magnesium chloride, 0.05% (w/v) Tween-20) for 90 min at 37°C and subsequent washing, wells were incubated for 90 min at 37°C with YZ1/2.23 or unfractionated anti-horseradish peroxidase at a dilution of 1:50,000 in TTBS, either in the presence or absence of inhibitor which had been preincubated at room temperature with the antibody for 60 min. After washing three times with TTBS, the wells were incubated with a 1:2000 dilution of either alkaline phosphatase conjugated anti-rat (in the case of YZ1/2.23) or horseradish peroxidase conjugated anti-rabbit (in the case of anti-horseradish peroxidase). Then 200  $\mu$ l per well of either 1 mg/ml *p*-nitrophenylphosphate in 0.1 M diethanolamine, pH 9.8, or 0.55 mg/ml 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 50 mM sodium citrate, pH 5 with 0.075% (v/v) hydrogen peroxide. Color reactions were terminated (after 6 min or 10 min at room temperature as stated in the figure captions) with 50  $\mu$ l per well of either 5 M sodium hydroxide (alkaline phosphatase reaction) or 10 mM sodium azide (horseradish peroxidase reaction) with absorbances measured on an SLT Spectra reader at 405/620 or 405/492, respectively.

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#### Abbreviations

BSA-M0X, conjugate of bovine serum albumin with defucosylated bromelain glycopeptides; BSA-M0XF<sup>3</sup>, conjugate of bovine serum albumin with native bromelain glycopeptides.

## References

- Aalberse, R.C., Koshte, V. and Clemens, J.G.J. (1981a) Cross-reactions between vegetable foods, pollen and bee venom due to IgE antibodies to a ubiquitous carbohydrate determinant. *Int. Arch. Allergy Appl. Immunol.*, **66** (Suppl. 1), 259–260.
- Aalberse, R.C., Koshte, V. and Clemens, J.G.J. (1981b) Immunoglobulin E antibodies that crossreact with vegetable foods, pollen, and hymenoptera venom. *J. Allergy Clin. Immunol.*, **68**, 356–364.
- Altmann, F. (1992) Determination of amino sugars and amino acids in glycoconjugates using precolumn derivatisation with *o*-phthalaldehyde. *Anal. Biochem.*, **204**, 215–219.
- Altmann, F. (1998) Structures of the N-linked carbohydrate of ascorbic acid oxidase from zucchini. *Glycoconj. J.*, **15**, 79–82.
- Ashford, D., Dwek, R.A., Welply, J.K., Amatayakul, S., Homans, S.W., Lis, H., Taylor, G.N., Sharon, N. and Rademacher, T.W. (1987) The  $\beta$ 1 $\rightarrow$ 2-D-xylose and  $\alpha$ 1 $\rightarrow$ 3-L-fucose substituted N-linked oligosaccharides from *Erythrina cristagalli* lectin. *Eur. J. Biochem.*, **166**, 311–320.
- Ashford, D.A., Dwek, R.A., Rademacher, T.W., Lis, H. and Sharon, N. (1991) The glycosylation of glycoprotein lectins: Intra- and inter-genus variation in N-linked oligosaccharide expression. *Carbohydr. Res.*, **213**, 215–227.
- Balshuisemann, D. and Jaenicke, L. (1990) The oligosaccharides of the glycoprotein pheromone of *Volvox carteri* f. *naigariensis* Iyengar (Chlorophyceae). *Eur. J. Biochem.*, **192**, 231–237.
- Batanero, E., Villalba, M., Monsalve, R.I. and Rodríguez, R. (1996) Cross-reactivity between the major allergen from olive pollen and unrelated glycoproteins: evidence of an epitope in the glycan moiety of the allergen. *J. Allergy Clin. Immunol.*, **97**, 1264–1271.
- Batanero, E., Villalba, M. and Rodríguez, R. (1994) Glycosylation site of the major allergen from olive tree pollen: Allergenic implications of the carbohydrate moiety. *Mol. Immunol.*, **31**, 31–37.
- Bouwstra, J.B., Spoelstra, E.C., de Waard, P., Leeftang, B.R., Kamerling, J.P. and Vliegthart, J.F.G. (1990) Conformational studies on the N-linked carbohydrate chain of bromelain. *Eur. J. Biochem.*, **190**, 113–122.
- D'Andrea, G., Bouwstra, J.B., Kamerling, J.P. and Vliegthart, J.F.G. (1988) Primary structure of the xylose-containing N-linked carbohydrate moiety from ascorbic acid oxidase of *Cucurbita pepo medullosa*. *Glycoconj. J.*, **5**, 151–157.
- de Cesare, F., Pini, C., di Felice, G., Caiaffa, M.F., Macchia, L., Tursi, A., Tinghino, R., Palumbo, S., Sallustro, F. and Federico, R. (1993) Purification and fine characterisation of a major allergen from *Olea europaea* pollen extract. *Allergy*, **48**, 248–254.
- Debray, H., Wierszeski, J.M., Strecker, G. and Franz, H. (1992) Structural analysis of the carbohydrate chains isolated from mistletoe (*Viscum album*) lectin I. *Carbohydr. Res.*, **236**, 135–143.
- Faye, L. and Chrispeels, M.J. (1988) Common antigenic determinants in the glycoproteins of plants, molluscs and insects. *Glycoconj. J.*, **5**, 245–256.
- Faye, L., Gomord, V., Fitchette-Lainé, A.C., Chrispeels, M.J. (1993) Affinity purification of antibodies specific for Asn-linked glycans containing  $\alpha$ 1,3 fucose or  $\beta$ 1,2-xylose. *Anal. Biochem.*, **209**, 104–108.
- Fournet, B., Leroy, Y., Wierszeski, J.M., Montreuil, J., Poretz, R.D. and Goldberg, R. (1987) Primary structure of an N-glycosidic carbohydrate unit derived from *Sophora japonica* lectin. *Eur. J. Biochem.*, **166**, 321–324.
- Fu, D. and O'Neill, R.A. (1995) Monosaccharide composition analysis of oligosaccharides and glycoproteins by high-performance liquid chromatography. *Anal. Biochem.*, **227**, 377–384.
- García-Casado, G., Sánchez-Monge, R., Chrispeels, M.J., Armentia, A., Salcedo, G. and Gomez, L. (1996) Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins. *Glycobiology*, **6**, 471–477.
- Gray, J.S.S., Yang, B.Y., Hull, S.R., Venzke, D.P. and Montgomery, R. (1996) The glycans of soybean peroxidase. *Glycobiology*, **6**, 23–32.
- Gupta, N., Nartin, B.M., Metcalfe, D.D. and Rao, P.V.S. (1996) Identification of a novel hydroxyproline-rich glycoprotein as the major allergen in *Parthenium* pollen. *J. Allergy Clin. Immunol.*, **98**, 903–912.
- Harthill, J.E. (1991) *N-Glycosylation of horseradish peroxidase*. D.Phil. thesis, University of Oxford.
- Hayashi, M., Tsuru, A., Mitsui, T., Takahashi, N., Hanzawa, H., Arata, Y. and Akazawa, T. (1990) Structure and biosynthesis of the xylose-containing carbohydrate moiety of rice  $\alpha$ -amylase. *Eur. J. Biochem.*, **191**, 287–295.
- Hijikata, A., Matsumoto, I., Kojima, K. and Ogawa, H. (1994) Antigenicity of the oligosaccharide moiety of the Japanese cedar (*Cryptomeria japonica*) pollen allergen, Cry j 1. *Int. Arch. Allergy Immunol.*, **105**, 198–202.
- Hirschwehr, R., Valenta, R., Ebner, C., Ferreira, F., Sperr, W.R., Valent, P., Rohac, M., Rumpold, H., Scheiner, O. and Kraft, D. (1992) Identification of common allergenic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J. Allergy Clin. Immunol.*, **90**, 927–936.
- Ipsen, H., and Løwenstein, H. (1983) Isolation and immunochemical characterisation of the major allergen of birch pollen (*Betula verrucosa*). *J. Allergy Clin. Immunol.*, **72**, 150–159.
- Kaladas, P.M., Goldberg, R. and Poretz, R.D. (1983) Rabbit anti-carbohydrate antibody elicited by the lymphocyte mitogenic glycoprotein from *Wistaria floribunda* seeds. *Mol. Immunol.*, **20**, 727–735.
- Katz, F., Moats, W. and Jan, Y.N. (1988) A carbohydrate epitope expressed uniquely on the cell surface of *Drosophila* neurons is altered in the mutant nac (neurally altered carbohydrate). *EMBO J.*, **11**, 3471–3477.
- Koshte, V.L., Kagen, S.L. and Aalberse, R.C. (1989) Cross-reactivity of IgE antibodies to caddis fly with arthropoda and mollusca. *J. Allergy Clin. Immunol.*, **84**, 174–183.
- Kubelka, V., Altmann, F., Staudacher, E., Tretter, V., März, L., Hård, K., Kamerling, J.P. and Vliegthart, J.F.G. (1993) Primary structures of the N-linked carbohydrate chains from honeybee venom phospholipase A<sub>2</sub>. *Eur. J. Biochem.*, **213**, 1193–1204.
- Kubelka, V., Altmann, F. and März, L. (1995) The asparagine-linked carbohydrate of honeybee venom hyaluronidase. *Glycoconj. J.*, **12**, 77–83.
- Kurosaka, A., Yano, A., Itoh, N., Kuroda, Y., Nakagawa, T. and Kawasaki, T. (1991) The structure of a neural specific carbohydrate epitope of horseradish peroxidase recognised by anti-horseradish peroxidase antiserum. *J. Biol. Chem.*, **266**, 4168–4172.
- Leung, P.S.C., Chow, W.K., Duffey, S., Kwan, H.S., Gershwin, M.E. and Chu, K.H. (1996) IgE reactivity against a cross-reactive allergen in crustacea and mollusca: evidence for tropomyosin as the common allergen. *J. Allergy Clin. Immunol.*, **98**, 954–961.
- Lommerse, J.P.M., Kroon-Batenburg, L.M.J., Kamerling, J.P. and Vliegthart, J.F.G. (1995) Conformational analysis of the xylose-containing N-glycan of pineapple stem bromelain as part of the intact glycoprotein. *Biochemistry*, **34**, 8196–8206.
- McManus, M.T., McKeating, J., Secher, D.S., Osborne, D.J., Ashford, D., Dwek, R.A. and Rademacher, T.W. (1988) Identification of a monoclonal antibody to abscission tissue that recognises xylose/fucose-containing N-linked oligosaccharides from higher plants. *Planta*, **175**, 506–512.
- Mucci, N., Liberatore, P., Federico, R., Forlani, F., di Felice, G., Afferni, C., Tinghino, R., de Cesare, F. and Pini, C. (1992) Role of carbohydrate moieties in cross-reactivity between different components of *Parietaria judaica* pollen extract. *Allergy*, **47**, 424–430.
- Nilsson, B.M., Sletten, K., Paulsen, B.S., O'Neill, M. and van Halbeek, H. (1991) Structural analysis of the glycoprotein allergen Art v II from the pollen of mugwort (*Artemisia vulgaris* L.). *J. Biol. Chem.*, **266**, 2660–2668.
- Ogawa, H., Hijikata, A., Amano, M., Kojima, K., Fukushima, H., Ishizuka, I., Kurihara, Y. and Matsumoto, I. (1996) Structures and contribution to the antigenicity of oligosaccharides of Japanese cedar (*Cryptomeria japonica*) pollen allergen Cry j 1. *Glycoconj. J.*, **13**, 555–566.
- Ohsuga, H., Su, S.N., Takahashi, N., Yang, S.Y., Hakagawa, H., Shimada, I., Arata, Y. and Lee, Y.C. (1996) The carbohydrate moiety of the bermuda grass antigen BG60: new oligosaccharides of plant origin. *J. Biol. Chem.*, **271**, 26653–26658.
- Oxley, D., Munro, S.L.A., Craik, D.J. and Bacic, A. (1996) Structure of N-glycans on the S<sub>3</sub>- and S<sub>6</sub>-allele stylar self-incompatibility ribonucleases of *Nicotiana glauca*. *Glycobiology*, **6**, 611–618.
- Petersen, A., Vieths, S., Aulepp, H., Schlaak, M. and Becker, W.M. (1996) Ubiquitous structures responsible for IgE cross-reactivity between tomato fruit and grass pollen allergens. *J. Allergy Clin. Immunol.*, **98**, 805–815.
- Prenner, C., Mach, L., Glössl, J. and März, L. (1992) The antigenicity of the carbohydrate moiety of an insect glycoprotein, honey-bee (*Apis mellifera*) venom phospholipase A<sub>2</sub>: the role of  $\alpha$ 1,3-fucosylation of the asparagine-bound N-acetylglucosamine. *Biochem. J.*, **284**, 377–380.
- Ramírez-Soto, D. and Poretz, R.D. (1991) The (1–3)-linked  $\alpha$ -L-fucosyl group of the N-glycans of the *Wistaria floribunda* lectins is recognised by a rabbit antiserum. *Carbohydr. Res.*, **213**, 27–36.
- Snow, P.M., Patel, N.H., Harrelson, A.L. and Goodman, C.S. (1987) Neural-specific carbohydrate moiety shared by many surface glycoproteins in *Drosophila* and grasshopper embryos. *J. Neurosci.*, **7**, 4137–4144.
- Srikrishna, G., Varki, V.M., Newell, P.C., Varki, A. and Freeze, H.H. (1997) An IgG monoclonal antibody against *Dictyostelium discoideum* glycoproteins specifically recognises Fuc $\alpha$ 1,6GlcNAc $\beta$  in the core of N-linked glycans. Localised expression of core-fucosylated glycoconjugates in human tissues. *J. Biol. Chem.*, **272**, 25743–25752.
- Stahl, B., Klabunde, T., Witzel, H., Krebs, B., Steup, M., Karas, M. and Hillenkamp, F. (1994) The oligosaccharides of the Fe(III)-Zn(II) purple acid phosphatase of the red kidney bean: determination of the structure by a combination of matrix-assisted laser desorption/ionisation mass spectrometry and selective enzymic degradation. *Eur. J. Biochem.*, **220**, 321–330.

- Sturm, A. (1991) Heterogeneity of the complex N-linked oligosaccharides at specific glycosylation sites of two secreted carrot glycoproteins. *Eur. J. Biochem.*, **199**, 169–179.
- Sturm, A., van Kuik, J.A., Vliegenthart, J.F.G. and Chrispeels, M.J. (1987) Structure, position, and biosynthesis of the high mannose and the complex oligosaccharides of the bean storage protein phaseolin. *J. Biol. Chem.*, **262**, 13392–13403.
- Sturm, A., Bergwerff, A.A. and Vliegenthart, J.F.G. (1992) <sup>1</sup>H-NMR structural determination of the N-linked carbohydrate chains on glycopeptides obtained from the bean lectin phytohemagglutinin. *Eur. J. Biochem.*, **204**, 313–316.
- Su, S.N., Shu, P., Lau, G.X., Yang, S.Y., Huang, S.W. and Lee, Y.C. (1996) Immunologic and physicochemical studies of Bermuda grass pollen antigen BG60. *J. Allergy Clin. Immunol.*, **98**, 486–494.
- Takahashi, N., Hitotsuya, H., Hanzawa, H., Arata, Y. and Kurihara, Y. (1990) Structural study of asparagine-linked oligosaccharide moiety of taste-modifying protein, miraculin. *J. Biol. Chem.*, **265**, 7793–7798.
- Tretter, V., Altmann, F., Kubelka, V., März, L. and Becker, W.M. (1993) Fucose  $\alpha$ 1,3-linked to the core region of glycoprotein N-glycans creates an important epitope for IgE from honeybee venom allergic individuals. *Int. Arch. Allergy Immunol.*, **102**, 259–266.
- Valenta, R., Duchene, M., Ebner, C., Valent, P., Sillaber, C., Deviller, P., Ferreira, F., Tejkl, M., Edlmann, H., Kraft, D. and Scheiner, O. (1992) Profilins constitute a novel family of functional plant pan-allergens. *J. Exp. Med.*, **175**, 377–385.
- van Kuik, J.A., van Halbeek, H., Kamerling, J.P. and Vliegenthart, J.F.G. (1985) Primary structure of the low-molecular-weight carbohydrate chains of *Helix pomatia*  $\alpha$ -hemocyanin: xylose as a constituent of N-linked oligosaccharides in an animal glycoprotein. *J. Biol. Chem.*, **260**, 13984–13988.
- van Kuik, J., Hoffmann, R.A., Mutsaers, J.H.G.M., van Halbeek, H., Kamerling, J.P. and Vliegenthart, J.F.G. (1986) A 500-MHz <sup>1</sup>H-NMR study on the N-linked carbohydrate chain of bromelain: <sup>1</sup>H-NMR structural-reporter-groups of fucose  $\alpha$ (1–3)-linked to asparagine-bound N-acetylglucosamine. *Glycoconj. J.*, **3**, 27–34.
- van Ree, R., Hoffman, D.R., van Dijk, W., Brodard, V., Mahieu, K., Koeleman, C.A.M., Grande, M., van Leeuwen, W.A. and Aalberse, R.C. (1995a) *Lol p* XI, a new major grass pollen allergen, is a member of a family of soybean trypsin inhibitor-related proteins. *J. Allergy Clin. Immunol.*, **95**, 970–978.
- van Ree, R., Fernández-Rivas, M., Cuevas, M., van Wijngaarden, M. and Aalberse, R.C. (1995b) Pollen-related allergy to peach and apple: an important role for profilin. *J. Allergy Clin. Immunol.*, **95**, 726–734.
- van Ree, R. and Aalberse, R.C. (1993) Pollen-vegetable food cross-reactivity: serological and clinical relevance of crossreactive IgE. *J. Clin. Immunoassay*, **16**, 124–130.
- Wang, X., Sun, B., Yasuyama, K. and Salvaterra, P.M. (1994) Biochemical analysis of proteins recognised by anti-HRP antibodies in *Drosophila melanogaster*: identification and characterisation of neuron specific and male specific glycoproteins. *Insect. Biochem. Mol. Biol.*, **24**, 233–242.
- Weber, A., Schröder, H., Thalberg, K. and März, L. (1987) Specific interaction of IgE antibodies with a carbohydrate epitope of honey bee venom phospholipase A<sub>2</sub>. *Allergy*, **42**, 464–470.
- Yang, B.Y., Gray, J.S.S. and Montgomery, R. (1996) The glycans of horseradish peroxidase. *Carbohydr. Res.*, **287**, 203–216.