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Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins

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Many plant proteins, particularly those found in foods and pollen, are known to act as sensitizing agents in humans upon repeated exposure. Among the cereal flour proteins involved in asthmatic reactions, those members of the α-amylase inhibitor family which are glycosylated, polypeptides BMAI-1, BTAI-CMb*, and WTAI-CM16* are particularly reactive both *in vivo* and *in vitro*. We show here that these major glycoprotein allergens carry a single asparagine-linked complex glycan that contains both β1→2 xylose and α1→3 fucose. Evidence is presented that the xylosyl residue and, to a lesser extent, the fucosyl residue are key IgE-binding epitopes and largely responsible for the allergenicity of these and unrelated proteins from plants and insects. Our results suggest that the involvement of xylose- and fucose-containing complex glycans in allergenic responses may have been underestimated previously; these glycans provide a structural basis to help explain the cross-reactivities often observed between pollen, vegetable food, and insect allergens.

Key words: complex glycans/fucose/glycoprotein allergens/xylose

Introduction

A significant proportion of individuals repeatedly exposed to sensitizers of plant origin develop some sort of atopic allergy, an IgE-mediated clinical disorder that is accompanied by severe symptoms. Many plant proteins are known to act as sensitizing agents (allergens), particularly those present in pollens and foods. By using standard techniques to study the role of isolated proteins in IgE-mediated immunity, we have identified during the past few years a number of allergenic proteins of wheat, barley, and rye flour associated with baker's asthma, an occupational disease with elevated prevalence among workers of the cereal industry (Barber *et al.*, 1989; Gomez *et al.*, 1990; Mena *et al.*, 1992; Sanchez-Monge *et al.*, 1992; Armentia *et al.*, 1993; Garcia-Casado *et al.*, 1995, 1996). Major salt-soluble flour allergens belong to a single family of 12–15 kDa seed-specific inhibitors of insect and mammalian α-amylases (see Carbonero *et al.*, 1993, and references therein). Purified members of this family have been shown to bind specific IgE from allergic patients (Barber *et al.*, 1989; Gomez *et al.*, 1990; Sanchez-Monge *et al.*, 1992; Garcia-Casado *et al.*, 1995) and provoke positive responses in skin prick tests (Armentia *et al.*, 1993; Garcia-Casado *et al.*, 1996). Nonetheless, very different reactivities have been reported among these allergens. The highly allergenic wheat and barley glycoproteins WTAI-CM16* and BTAI-CMb*, for example, are considerably more reactive both *in vivo* and *in vitro* than their nonglycosylated homologues WTAI-CM16 and BTAI-CMb (Sanchez-Monge *et al.*, 1992). An additional strongly allergenic component from barley, BMAI-1, is also a glycosylated inhibitor of insect α-amylase (Mena *et al.*, 1992). Nonglycosylated forms have not yet been reported for BMAI-1. The lower or null allergenicity of most nonglycosylated members of this family in wheat and barley, compared to the strong reactivities observed for the glycosylated ones, led us to further investigate the role of their oligosaccharide-side chains as IgE-binding determinants in symptomatic patients. Besides BMAI-1, BTAI-CMb* and WTAI-CM16*, an increasing number of plant glycoproteins have been associated with allergic diseases over the past few years (Haavik *et al.*, 1985; Sward-Nordmo *et al.*, 1988; Nilsen *et al.*, 1991; Taniai *et al.*, 1993; Batanero *et al.*, 1994; Hijikata *et al.*, 1994). However, little is know about the structure of their attached glycans or the involvement of these glycans in the allergenic responses. To date, the complete structures of the glycans of only two plant allergens, *Art v* II from mugwort pollen (Nilsen *et al.*, 1991) and *Cry j* I from Japanese cedar pollen (Hino *et al.*, 1995) have been determined. They are typical plant asparagine-linked glycans (see Faye *et al.*, 1989), and the only glycan moiety present in *Ole e* I, the major allergen of olive tree pollen, has also been shown to be linked to an asparagine residue (Batanero *et al.*, 1994). To our knowledge, no O-linked glycans have been found in plant allergens.

The most reactive allergens identified by us in the α-amylase inhibitor family also have single N-glycosylation sites in their primary structures (see Carbonero *et al.*, 1993). The N-linked glycans found in plant glycoproteins are either of the high-mannose type, with a structure identical to that found in mammalian and yeast glycans, or of the complex type. Unlike those of mammalian cells, the complex glycans of plants lack sialic acid, are much smaller, and almost invariably contain a β1→2 xylose residue attached to the β-linked mannose residue of the core (Faye *et al.*, 1989). The complex N-linked glycans of invertebrates have similar epitopes as those found in plants (Faye and Chrispeels, 1988). It is tempting to speculate that some of these structural features of plant and invertebrate, but not of mammalian N-linked oligosaccharides might increase the allergenicity of the proteins that harbor them. So far, the involvement of these oligosaccharide-side chains in the allergenic response has not yet been well established. In addition to the low recoveries of most allergens, chemical methods aimed at removing or modifying their glycans may also alter other groups of the protein, thus leading, as we show in this paper, to losses of allergenicity not related to the glycans themselves. Until very recently, commercially available glycosidases were not able to remove complex glycans from plant glycoproteins.

Here we report that each of the highly-reactive allergens BMAI-1, BTAI-CMb*, and WTAI-CM16* has epitopes corresponding to β1→2 xylose- and α1→3 fucose-containing complex glycan attached to its single N-glycosylation site. We also show that only the endo-Lys peptide of BMAI-1 that harbors its single glycan is recognized by specific IgE from allergic patients, and this recognition is lost upon enzymatic deglycosylation. Furthermore, IgE antibodies from these patients are able to recognize other unrelated glycoproteins if they carry N-linked β1→2 xylose- or α1→3 fucose-containing complex glycans, with the xylosyl residue being the most reactive. These results are discussed in the context of the IgE-mediated cross-reactivities commonly found between plant and insect proteins.

Results

The asparagine-linked glycans of plant glycoproteins can be either of the high mannose type or of the complex type (Faye *et al.*, 1989). By using a glycan specific serum described previously (Laurière *et al.*, 1989) we determined that each of the three highly allergenic polypeptides of the α-amylase inhibitor family BMAI-1, BTAI-CMb*, and WTAI-CM16*, has a glycan of the complex type attached to its single N-glycosylation site (not shown). Immunoblotting of eight purified allergens with sera specific for β1→2 xylose and α1→3 fucose residues (Faye *et al.*, 1993) showed that these glycan moieties react with both serum fractions suggesting that they contain both a β1→2 xylose residue on the β-linked mannose of the core and an α1→3 fucose residue on the proximal GlcNAc (Figure 1). No other members of this protein family appear to contain such residues (Figure 1) or even glycan moieties at all (Mena *et al.*, 1992; Sanchez-Monge *et al.*, 1992; Garcia-Casado *et al.*, 1996).

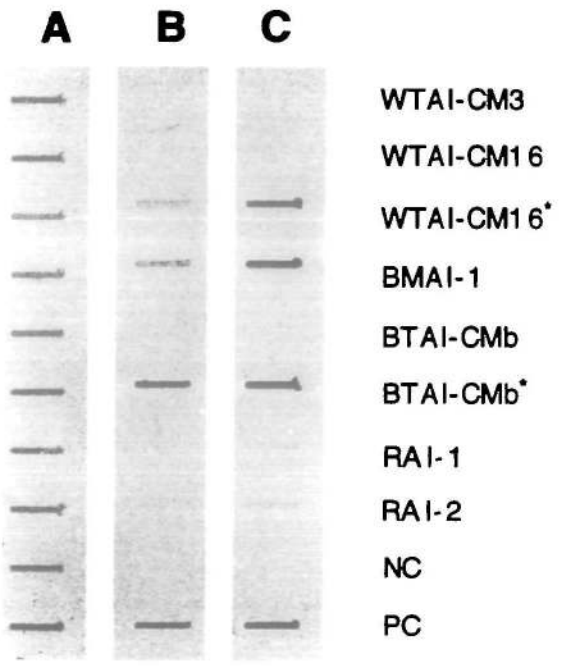

Fig. 1. Immunodetection of asparagine-linked complex glycans. Purified allergens of the cereal α-amylase inhibitor family were adsorbed onto nitrocellulose and then stained with Coomassie blue (**A**), or probed with antisera specific for glycans containing either β1→2 xylose (**B**) or α1→3 fucose (**C**). Leaf extracts from wild-type *A.thaliana* and the *cgl* mutant, whose glycoproteins lack complex glycans (von Schaewen *et al.*, 1993), were included as positive (PC) and negative (NC) controls, respectively.

Since the most allergenic members of the inhibitor family in wheat and barley are those that are glycosylated, we decided to investigate the involvement of their xylose- and fucose-containing complex glycans in the allergenic response. Thus, BMAI-1, the most allergenic protein of this family in barley (Armentia *et al.*, 1993), was cleaved with endoproteinase Lys-C and the resulting peptidic fragments fractionated by HPLC (Figure 2A). Peptides 1–4 were dot-blotted onto PVDF and assayed for the presence of glycan moieties (Figure 2B) and for their capacity to bind IgE from allergic patients (Figure 2C). The peptide carrying the single asparagine-linked glycan of BMAI-1 (number 4 in Figure 2) showed specific IgE-binding, whereas no reactivity was found for the remaining unglycosylated peptides (1 to 3). An approximately equimolar amount of uncleaved BMAI-1 was included as a positive control. A fifth small peptide, Glu^{68}-Lys^{77}, expected from the primary structure of BMAI-1 (Mena *et al.*, 1992), could not be detected in the HPLC chromatogram. This peptide does not contain sugar-binding residues and is unlikely to carry an IgE-binding epitope, given its short length.

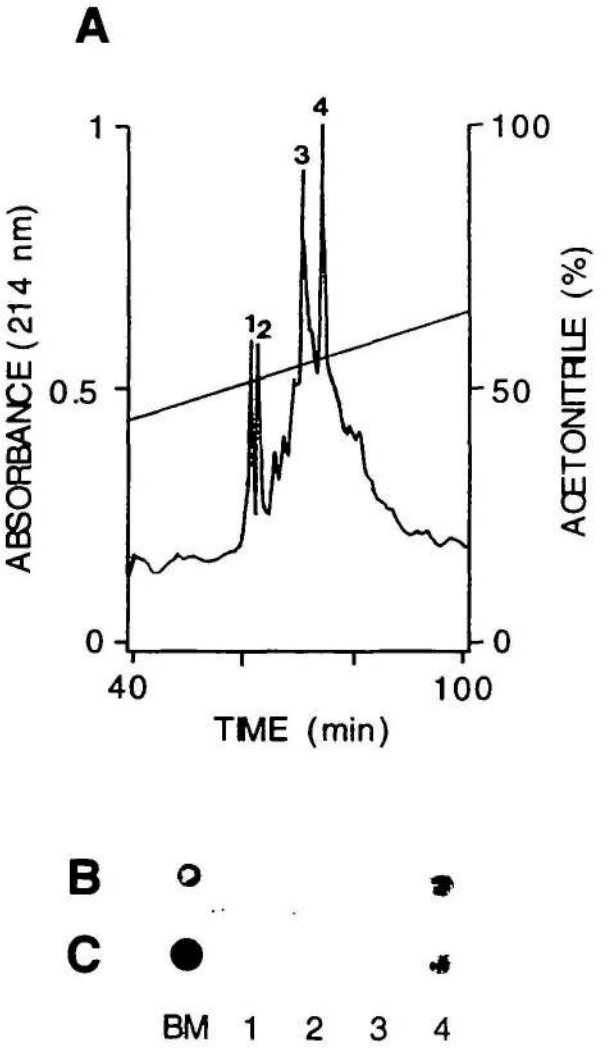

Fig. 2. (**A**) HPLC fractionation of the peptides obtained after cleavage of allergen BMAI-1 with endoproteinase Lys-C. Peptides 1–4 were dot blotted and assayed for the presence of glycans using a commercial (Boehringer) glycan detection kit (**B**) and for the binding of IgE from allergic patients (**C**). An equimolar amount of uncleaved BMAI-1 (BM) was also included.

To further delimit the involvement of their glycan moieties in the allergenic response, BMAI-1 and WTAI-CM16* were subjected to chemical deglycosylation with TFMS, fractionated by SDS–PAGE, and then immunoblotted with sera of allergic patients. The TFMS treatment completely removed the glycan moieties of both allergens (Figure 3A,B; compare lanes 1 and 2, or lanes 3 and 4) and this resulted in a total loss of their IgE-binding capacity (Figure 3C). However, this treatment also abolished the reactivity of the unglycosylated barley dimeric inhibitor BDAI-1, which was included as a negative control (Figure 3C; proteins were dot-blotted in this case because BDAI-1 reacts poorly after SDS–PAGE). Our results indicate that chemical deglycosylation with TFMS, which is widely used for evaluating the role of glycan moieties as IgE-binding epitopes, may alter the peptide backbone as well and lead to losses of reactivity not related to the glycan removal.

Enzymatic deglycosylation, on the other hand, has not been feasible until very recently because commercially available glycosidases were not able to remove the complex glycans of plant glycoproteins. A new glycosidase (PNGase A, Boehringer) became recently available that is able to remove N-linked $\alpha 1 \rightarrow 3$ fucose-containing glycans, although the extent of this digestion largely depends on the nature of the glycoprotein. After incubation with PNGase A for 24 h, about half of the BMAI-1 molecules had lost their single glycan (lower band in lane 2, Figure 4A) and were not recognised by a glycan detection kit (Figure 4B) or by a serum that reacts specifically with the complex glycans of plant glycoproteins (not shown). The unglycosylated molecules had also lost their capacity to ligate IgE from allergic patients (Figure 4C), thus indicating that the complex glycan bound to BMAI-1 is largely responsible for its reactivity. The upper band in lane 2 (Figure 4A), which corresponds to undigested BMAI-1, remained fully reactive after the PNGase treatment. The efficiency of the digestion could not be improved by heating the allergen prior to its incubation with the enzyme or by using different amounts of SDS and/or mercaptoethanol. The glycan of WTAI-CM16* could not be removed even after incubation with PNGase A for 48 h (lane 4 in Figure 4A).

Since complex glycans containing $\beta 1 \rightarrow 2$ xylose and/or $\alpha 1 \rightarrow 3$ fucose are commonly found in plant and some invertebrate glycoproteins (Faye *et al.*, 1989), we decided to investigate the cross-reactivity of different glycoproteins from various origins with sera from baker's asthma patients. Thus, protein extracts from plant, insect, mite and mammalian origin were fractionated by SDS–PAGE and electroblotted onto PVDF membranes in triplicate. One membrane was then stained with Coomassie blue, a second membrane was assayed for hypersensitive sera IgE binding, and the third membrane was immunoblotted with a serum that reacts with asparagine-linked complex glycans containing $\beta 1 \rightarrow 2$ xylose and/or $\alpha 1 \rightarrow 3$ fucose. The relevance of such glycan moieties as IgE-binding epitopes is shown in Figure 5. The majority of reactive (that is, IgE-binding) proteins from non-cereal seeds and from *T.molitor* larvae (Figure 5A, right panel) appear to carry complex glycans containing $\beta 1 \rightarrow 2$ xylose- or $\alpha 1 \rightarrow 3$ fucose epitopes (Figure 5A, central panel). Vertebrate or mite proteins, that lack such residues, did not react with the hypersensitive sera. Figure 5A also shows that the most reactive protein from the *T.molitor* extract (lane 4), which probably corresponds to a major allergen involved in baker's asthma (Schroeckenstein *et al.*, 1990), contains at least one glycan moiety of the complex type. To investigate to what extent the above cross-reactivities might be due to amino acid sequence similarities and/or to shared conformational epitopes, the reactivity of leaf proteins from *A.thaliana* and the *cgl* mutant was evaluated (Figure 5B). No proteins from the mutant, which lacks complex glycans (von Schaewen *et al.*, 1993), reacted with IgE from allergic patients (lane 2 in Figure 5B, right panel), whereas a number of IgE-binding proteins could be detected in the wild type (lane 1). Again, the pattern of IgE-binding proteins (Figure 5B, right panel) is similar to that of proteins containing complex glycans (Figure 5B, central panel). These results clearly support the notion that the complex glycans of plant glycoproteins are relevant IgE-binding determinants by themselves. To find out the involvement of $\beta 1 \rightarrow 2$ xylose and $\alpha 1 \rightarrow 3$ fucose in the allergenic response we analysed the IgE-binding of five unrelated glycoproteins whose glycan moieties have been structurally determined. As shown in Figure 6, those glycoproteins carrying a $\beta 1 \rightarrow 2$-linked xylose were able to bind significant amounts of IgE from baker's asthma patients, although this binding was always lower (per microgram of protein) than that of the positive control, WTAI-CM16*. Interestingly, some IgE-binding was also detected for phospholipase A_2 from bee venom, which lacks xylose but contains, as many plant glycoproteins, an $\alpha 1 \rightarrow 3$ fucose residue attached to the proximal GlcNAc residue. Bovine ribonuclease, that contains only high mannose glycans (mostly $Man_5GlcNAc_2$), and the unglycosylated protein WTAI-CM16 did not bind detectable amounts of IgE.

Discussion

Allergenicity of the complex glycan attached to BMAI-1, BMAI-CMb, and WTAI-CM16**

Many proteins contain N- and O-linked oligosaccharide chains (glycans) as part of their covalent structure. Such glycans share common structural motifs in all eukaryotes and represent there-

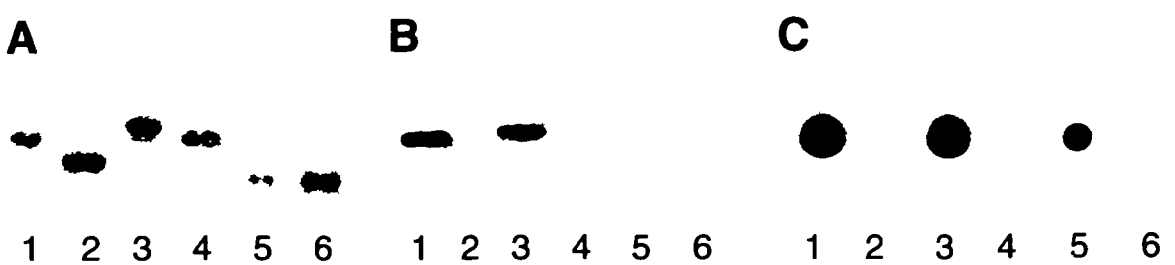

Fig. 3. Effect of chemical deglycosylation on the reactivity of purified allergens. Glycoprotein allergens BMAI-1 (1,2) and WTAI-CM16* (3,4), as well as the unglycosylated allergen BDAI-1 (5,6), either untreated (odd lanes) or TMFS-treated (even lanes) were fractionated by SDS–PAGE, electrotransferred to PVDF and stained with Coomassie blue (**A**) or assayed for the presence of glycans (**B**). The reactivity of the same samples was tested with hypersensitive sera and ^{125}I-labeled anti-human IgE antibodies (**C**).

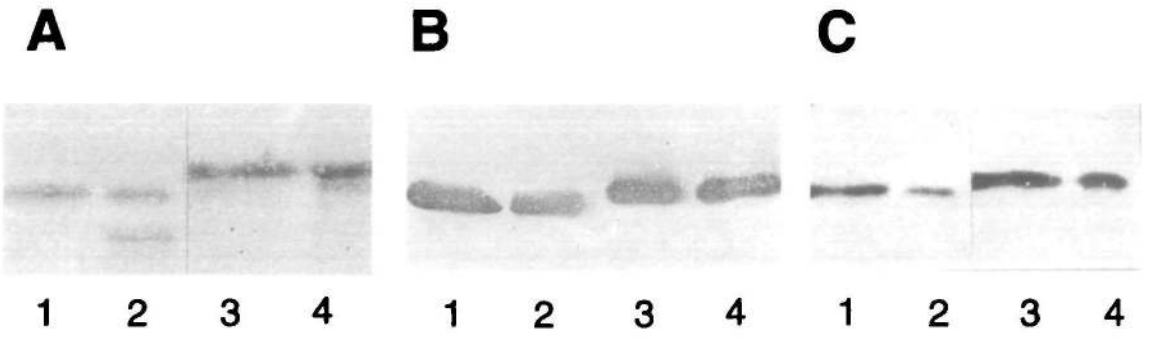

Fig. 4. Effect of incubation with PNGase A on the reactivity of glycoprotein allergens Untreated BMAI-1 (1) and WTAI-CM16* (3), as well as PNGase-treated BMAI-1 (2) and WTAI-CM16* (4) were fractionated by SDS–PAGE and adsorbed onto PVDF. Membranes were stained with Coomassie blue (**A**), assayed for the presence of glycans (**B**), or treated with hypersensitive sera and then with ^{125}I-labeled anti-human IgE antibodies (**C**).

fore a source of immunological cross-reactivity between different species. Thus, the N-linked glycans of plant glycoproteins have repeatedly been reported to be immunogenic in mammals (Faye and Chrispeels, 1988; Faye *et al.*, 1989; Kurosaka *et al.*, 1991; Ramirez-Soto and Poretz, 1991). In contrast, their allergenicity has received little attention so far, despite the increasing number of plant glycoproteins that have been associated with allergic diseases over the last decade (Haavik *et al.*, 1985; Sward-Nordmo *et al.*, 1988; Nilsen *et al.*, 1991; Sanchez-Monge *et al.*, 1992; Taniai *et al.*, 1993; Batanero *et al.*, 1994; Hijikata *et al.*, 1994).

In this report we show that wheat and barley flour allergens BMAI-1, BTAI-CMb* and WTAI-CM16* carry an asparagine-linked glycan of the complex type that contains, as most plant complex glycans, both a β1→2-linked xylosyl and an α1→3-linked fucosyl epitope (Figure 1). The possible involvement of this moiety in the hypersensitive response was suggested by the fact that the single endoLys-C glycopeptide of BMAI-1 largely accounts for the IgE-binding of the uncleaved allergen (Figure 2). Although the presence of IgE antibodies reacting with glycan moieties has been previously reported in individuals allergic to pollen glycoproteins, only a minor role has been assigned to such moieties in the hypersensitive response (Nilsen *et al.*, 1991; Mucci *et al.*, 1992; Taniai *et al.*, 1993; Hijikata *et al.*, 1994). Our results suggest, in contrast, that the complex glycan of BMAI-1 is the most prominent IgE-binding determinant of this allergen, as evidenced by the loss of detectable reactivity after its enzymatic removal with PNGase A (Figure 4). This seeming discrepancy with previous reports may arise, at least in part, from the fact that other researchers have used instead PNGase F (EC 3.2.2.18) (Nilsen *et al.*, 1991; Batanero *et al.*, 1994), which is not able to remove the complex fucose-containing glycans of plant glycoproteins (Tretter *et al.*,1991). For example, no significant effect on the binding of human IgE was found after treatment of the mugwort pollen allergen *Art v* II with this enzyme (Nilsen *et al.*, 1991), but only high-mannose glycans were removed by this treatment. In fact, incubation of BMAI-1 with PNGase F did not result in glycan release under conditions in which human transferrin was completely deglycosylated (not shown). Chemical deglycosylation with TFMS, on the other hand, has been used for the same purpose (Polo *et al.*, 1990; Mucci *et al.*,

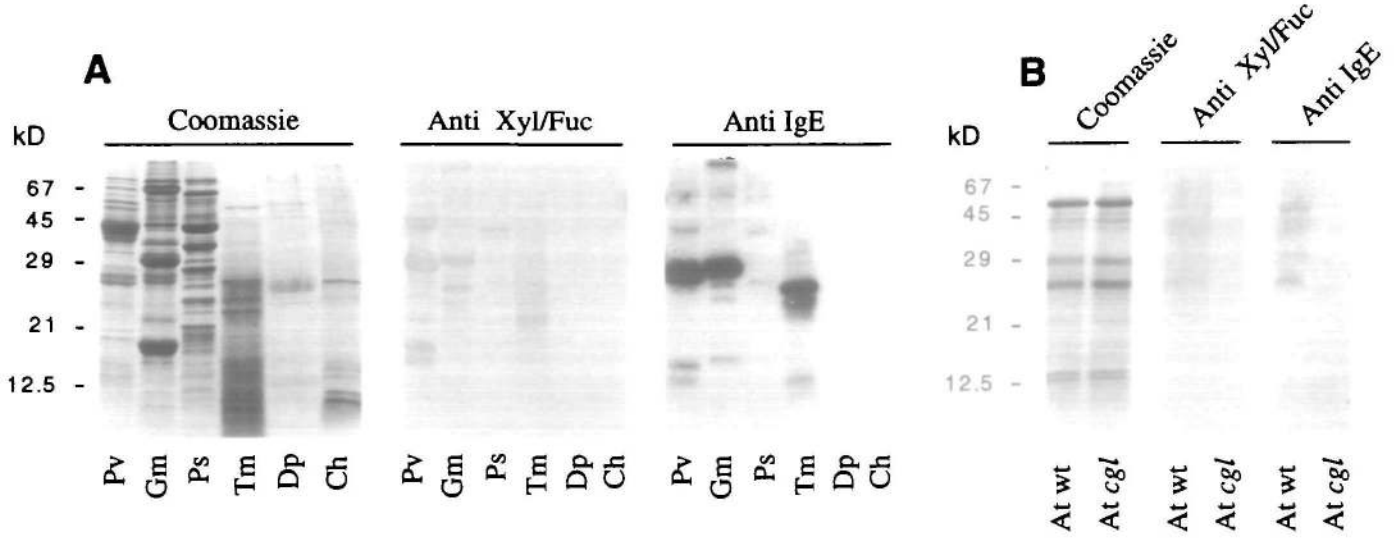

Fig. 5. (**A**) Glycosylation pattern and IgE immunoblotting of proteins (extracted as indicated in Materials and Methods) from plant, insect, mite and mammalian origin. After SDS–PAGE and electrotransfer to PVDF membranes, proteins were stained with Coomassie blue (left panel), immunoblotted with a serum specific for asparagine-linked glycans containing β1→2 xylose and/or α1→3 fucose (central panel), or assayed for hypersensitive sera IgE binding with ^{125}I-labeled anti-human IgE antibodies (right panel). Samples: (Pv) *Phaseolus vulgaris* seeds, (Gm) *Glycine max* seeds, (Ps) *Pisum sativum* seeds, (Tm) *Tenebrio molitor* larvae, (Dp) *Dermatophagoides pteronissinus*, and (Ch) chicken heart. (B) Differential reactivity of leaf proteins from *A.thaliana* wild type (At wt) or the *cgl* mutant (At *cgl*). Extracts were processed as in (A).

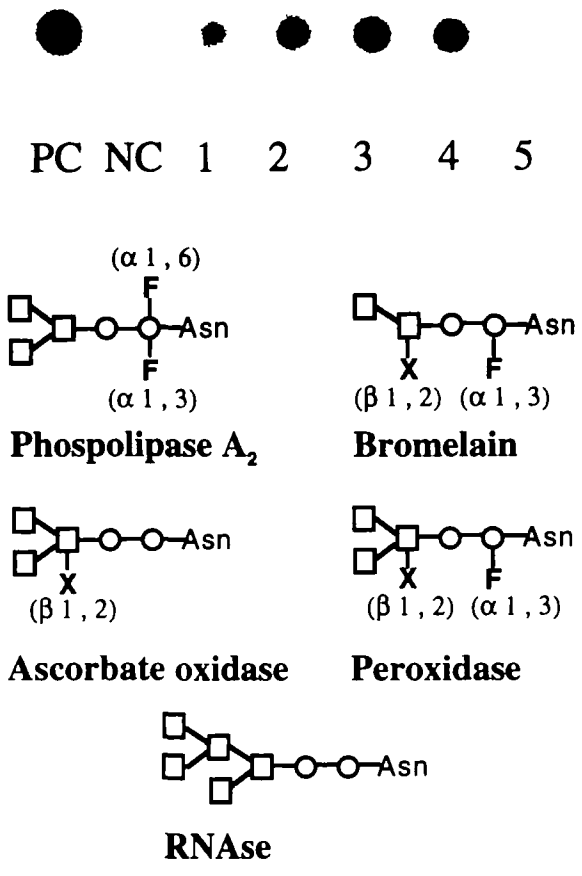

Fig. 6. Immunodetection of dot-blotted glycoproteins with hypersensitive sera. Samples: (1) honeybee venom phospholipase A_2, (2) pineapple bromelain, (3) cucumber ascorbate oxidase, (4) horseradish peroxidase, (5) bovine pancreatic ribonuclease. Membranes were treated with hypersensitive sera and then with ^{125}I-labeled anti-human IgE antibodies. Wheat allergen WTAI-CM16* and its nonglycosylated form WTAI-CM16 were included as positive (PC) and negative (NC) controls, respectively. The structure of the glycan moieties of these proteins is shown (□, mannose; ○, GlcNAc, X, xylose; F, fucose).

1992; Batanero *et al.*, 1994; Hijikata *et al.*, 1994). Nonetheless, our results indicate that this treatment can result in dramatic losses of reactivity probably caused by alterations in the peptide backbone itself, as shown for the unglycosylated barley allergen BDAI-1 (Figure 3). The relatively high reactivity of BDAI-1 (Sanchez-Monge *et al.*, 1992; Armentia *et al.*, 1993), along with the recent isolation of two nonglycosylated allergens from this protein family in rye (Garcia-Casado *et al.*, 1995, 1996), reveal that conformational and/or sequence epitopes also play an important role in the allergenicity of at least some cereal α-amylase inhibitors.

Involvement of β1→2 xylose and α1→3 fucose in IgE-mediated cross-reactions

Cross-reactions between pollen, vegetable foods, and insect venom glycoproteins due to IgE antibodies against an undetermined ubiquitous carbohydrate epitope have been reported previously (Aalberse *et al.*, 1981; Calkhoven *et al.*, 1987; van Ree and Aalberse, 1993). When heterogeneous protein mixtures from different origins were evaluated for IgE-binding from baker's asthma patients' sera, we found reactive proteins in noncereal seeds (*Phaseolus vulgaris, Glycine max,* and *Pisum sativum*) and the coleopteran insect *Tenebrio molitor,* but not in the highly allergenic house dust mite *Dermatophagoides pteronissinus* or in a protein extract of chicken heart (Figure 5A). Furthermore, most of the reactive proteins were also recognised by a serum specific for xylose-containing plant complex glycans (Laurière *et al.*, 1989), which is compatible with a major role for such moieties in the binding of specific IgE. Besides, the above cross-reactions (or their absence) are in agreement with previous reports that the complex N-linked glycans of insects, but not mammals, have similar structural features to those found in plants (Faye and Chrispeels, 1988). To investigate to what extent the observed cross-reactivities could be explained by shared conformational epitopes and/or similarities in amino acid sequences, we also analysed the IgE-binding of leaf proteins from wild-type *A.thaliana* and from the *cgl* mutant, whose glycoproteins contain only high-mannose glycans (von Schaewen *et al.*, 1993). Unlike the wild-type, no reactive bands were detected in the *cgl* mutant, which strongly supports the hypothesis that, at least in plants, the glycan moieties of the complex type constitute important epitopes for IgE antibodies from allergic patients. To further delimit which structural features of such glycans are involved in the allergenic response, unrelated glycoproteins carrying defined sugar moieties were immunoblotted with hypersensitive sera (Figure 6). It can be concluded from this experiment that the presence of a β1→2 xylosyl residue attached to the β-linked mannose of the core constitutes a key IgE-reactive determinant, and probably explains to a large extent the cross-reactivities between plant and insect glycoproteins found by us (Figure 5) and others (Aalberse *et al.*, 1981). The experiment shown in Figure 6 also suggests an important, although less relevant, role in the hypersensitive response for the α1→3 fucose bound to the proximal GlcNAc of bee phospholipase A_2, in agreement with previous findings that this structural element constitutes an important epitope for IgE from honeybee venom allergic individuals (Tretter *et al.*, 1993).

Our results indicate that, conversely to the prevalent opinion that IgE antibodies are generally directed against noncarbohydrate epitopes (Calkhoven *et al.*, 1987; Sward-Nordmo *et al.*, 1989; Mucci *et al.*, 1992), the complex glycans commonly found on plant and insect, but not mammalian, glycoproteins may play a major role in some hypersensitive reactions and probably underlie the high degree of cross-reactivity observed between a large number of vegetable food, pollen, and insect proteins. Identification of β1→2 xylosylation and, to a lesser extent, α1→3 fucosylation as the key features responsible for the high allergenicity of many proteins should help develop more reliable allergenic extracts for the diagnosis and therapy of allergic diseases. Furthermore, the results presented here are relevant concerning the use of plants or plant cell cultures to overproduce heterologously pharmacologically important proteins with low allergenicity, a serious limitation of plant biotechnology at present. The availability of a mutant plant line that lacks complex glycans but completes its normal life cycle (Von Schaewen *et al.*, 1993) and the identification of N-acetylglucosaminyl transferase I as the defective enzyme in this mutant (Von Schaewen *et al.*, 1993; Gomez and Chrispeels, 1994) should open a way to progress in such direction.

Materials and methods

Biological material

Flour from *Hordeum vulgare* (barley) cv. Bomi, *Triticum turgidum* (pasta wheat) cv. Enano de Andújar, and *Secale cereale* (rye) cv. INIA c-171/M were

used for the purification of cereal allergens. Protein extracts (see below) from seeds of *Phaseolus vulgaris* (bean) cv. Contender, *Glycine max* (soybean) cv. Willliams, and *Pisum sativum* (pea) cv. Frisson, as well as from *Arabidopsis thaliana* cv. Columbia (wild type and the *cgl* mutant), *Tenebrio molitor* (yellow mealworm) larvae, *Dermatophagoides pteronissinus* (house dust mite), and chicken heart were also used in this study.

Isolation of wheat, barley, and rye allergens

The barley monomeric α-amylase inhibitor BMAI-1 was purified by gel filtration and reverse-phase HPLC as in Mena *et al.* (1992). The tetrameric α-amylase inhibitor subunits BTAI-CMb* and BTAI-CMb (barley) and WTAI-CM16*, WTAI-CM16, and WTAI-CM3 (wheat) were purified as described in Sanchez-Monge *et al.* (1992). Rye α-amylase inhibitors RAI-1 and RAI-2 were isolated as described by Garcia-Casado *et al.* (1996).

Protein cleavage and peptide fractionation

Enzymatic hydrolysis of purified BMAI-1 was performed by endoproteinase Lys-C (EC 3.4.99.30) during 18 h at 37°C using a 25 mM Tris–HCl (pH 8.5), 1 mM EDTA buffer. Peptides were fractionated by reverse-phase HPLC on a Nucleosil C8 column (4.6 × 250 mm), using a two-step gradient of 0.1% TFA in acetonitrile (0–28% acetonitrile in 10 min; 28–70% acetonitrile in 100 min; flow rate 1 ml/min) at 50°C. The column had previously been eluted with 0.1% TFA in water for 10 min. Lyophilized peptides were dot-blotted onto Immobilon AV Affinity membranes (Millipore) equilibrated with 0.5 M Na-phosphate (pH 7.5) buffer.

Protein extraction and immunodetection of complex glycans

Proteins from various origins were extracted by grinding the corresponding organ or organism in an ice-cold mortar with a buffer containing 0.0625 M Tris–HCl (pH 6.8), 2% SDS and 1% 2-mercaptoethanol. The supernatants obtained after centrifugation at 13,000 r.p.m. for 15 min (twice) were frozen at −20°C until further use. Appropriate quantities of the protein extracts (determined according to Smith *et al.*, 1985) were fractionated by SDS–PAGE on Bio-Rad Miniprotein II system minigels and then transferred to PVDF membranes for 60 min at 100 V on a Bio-Rad Mini Trans-Blot cell, using 50 mM Tris, 50 mM boric acid (pH 8.3) as transfer buffer. Membranes were then stained with Coomassie blue G-250 or probed with sera that react with the complex glycans of plant glycoproteins (Laurière *et al.*, 1989) or with β1→2 xylose- or α1→3 fucose-containing glycans (Faye *et al.*, 1993). We used goat anti-rabbit IgG coupled to alkaline phosphatase (Bio-Rad) as the secondary antibody. Alternatively, proteins were blotted onto PVDF membranes equilibrated with 20 mM Tris–HCl (pH 8.3), 150 mM NaCl and probed as above.

Glycoprotein assay

Glycoproteins were identified with a glycan detection kit (Boehringer) essentially as previously described (Sanchez-Monge *et al.*, 1992). Briefly, glycoproteins (1–2 μg) were oxidized and labelled with digoxigenin before separation by SDS–PAGE and electroblotting onto PVDF membranes. The incorporated digoxigenin was detected by an enzyme immunoassay using an anti-rabbit IgG–alkaline phosphatase conjugate.

Identification of IgE-binding proteins

Immunodetection of IgE-binding proteins was carried out as previously described (Sanchez-Monge *et al.*, 1992) using 1:3 dilutions of a pool of sera from five symptomatic baker's asthma patients and ^{125}I-labeled anti-human IgE. All sera were RAST 4 class when assayed with commercial wheat flour discs (Phadebas-RAST kit, Pharmacia). None of the samples tested in this study showed detectable IgE binding when assayed with pooled sera from three healthy individuals.

Chemical deglycosylation with trifluoromethylsulfonate (TFMS)

Proteins were deglycosylated with TFMS essentially as in Sojar and Bahl (1987). After being resuspended in anhydrous TFMS (150 μl of TFMS per mg of protein), protein samples were kept on ice for 2 h under a nitrogen atmosphere and then neutralized with ammonium bicarbonate.

Enzymatic deglycosylation

Glycoproteins (1 μg) were incubated for 15 min at 80°C with 10 mM Na-acetate (pH 5.1), 10 mM SDS, 4% 2-mercaptoethanol and then treated with 0.5 mU of PNGase A (EC 3.5.1.51, Boehringer) for 24 h at 37°C

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