The antigenicity of the carbohydrate moiety of an insect glycoprotein, honey-bee (*Apis mellifera*) venom phospholipase A₂

The role of α 1,3-fucosylation of the asparagine-bound *N*-acetylglucosamine

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A rabbit polyclonal antiserum raised against honey-bee (*Apis mellifera*) venom phospholipase A₂ (PLA₂) contains antibodies that react exclusively with its glycosylated variants and cross-react with plant glycoproteins. The interaction of anti-(horseradish peroxidase) antiserum with PLA₂ suggests the existence of a carbohydrate determinant common to both glycoproteins. E.l.i.s.a. binding and inhibition experiments, employing glycoproteins and glycopeptides of plant and animal origin with known *N*-glycan structures, in combination with chemical and enzymic deglycosylation, identified α 1,3-fucosylation of the asparagine-bound *N*-acetylglucosamine as the antigenic determinant. This fucose residue is present in the *N*-glycan of PLA₂ and is frequently found in plant glycoproteins, whereas mammalian glycoproteins lack this modification.

INTRODUCTION

Insect cells are gaining increasing attention as expression systems for recombinant glycoproteins (Luckow & Summers, 1988). However, the potential of these cell lines to create oligosaccharide structures which are immunogenic in man has not yet been elucidated. Recent data suggest the existence of antibodies in sera of bee-sting-allergic patients (Weber *et al.*, 1987), which are directed against the highly heterogeneous *N*-glycan of the glycosylated variant of honey-bee (*Apis mellifera*) venom phospholipase A₂ (PLA₂). Structural analysis of the PLA₂-oligosaccharides identified truncated pentasaccharide core units with fucose residues α 1,3- and/or α 1,6-linked to the asparagine-bound *N*-acetylglucosamine (Staudacher *et al.*, 1992). Internal α 1,6-fucosylation of *N*-linked oligosaccharide core units is a common feature of mammalian *N*-glycans, whereas α 1,3-linked fucose residues have been proposed to be characteristic of plant glycoproteins [for a review, see Faye *et al.* (1989)].

Antisera against plant glycoproteins were shown to contain significant concentrations of anti-carbohydrate antibodies (Kaladas *et al.*, 1983; Faye & Chrispeels, 1988). The antigenicity of these oligosaccharides has been attributed to the presence of β -xylose (Laurière *et al.*, 1989), terminal α 1,6-linked mannose groups (Kurosaka *et al.*, 1991) or α 1,3-linked fucose residues (Ramirez-Soto & Poretz, 1991). Some of these antibodies react with extracts of various insect tissues (Faye & Chrispeels, 1988; Katz *et al.*, 1988). The epitope which accounts for this cross-reaction has not yet been characterized.

Here we report on the identification of the antigenic epitope of the *N*-glycan of PLA₂ and demonstrate that plant glycoproteins share this immunogenic carbohydrate determinant.

MATERIALS AND METHODS

Materials

Pooled freeze-dried honey-bee (*Apis mellifera*) venom was

purchased from Nectarcop (Sofia, Bulgaria). 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), phenylmethane-sulphonyl fluoride (PMSF), leupeptin, pepsin (from porcine stomach mucosa), trypsin [from bovine pancreas, tosylphenyl-alanylchloromethane ('TPCK')-treated], β -galactosidase from bovine testis, α -mannosidase and β -*N*-acetylhexosaminidase from *Canavalia ensiformis* (jack bean), phytohaemagglutinin from *Phaseolus vulgaris* (French bean), chicken ovalbumin (Grade V), pineapple (*Ananas comosus*) stem bromelain, BSA (Type V), horseradish peroxidase (Type VI) and rabbit anti-(horseradish peroxidase) antiserum were purchased from Sigma (St. Louis, MO, U.S.A.). Complete and incomplete Freund's adjuvant were from Difco (Detroit, MI, U.S.A.). Sephadex G-15, sulphopropyl (SP)-Sephadex C-50, CH- and CNBr-activated Sepharose were from Pharmacia-LKB (Uppsala, Sweden). Pronase and peptide:*N*-glycosidase F (PNGase F) were obtained from Boehringer-Mannheim (Mannheim, Germany). Goat anti-rabbit IgG immunoglobulins conjugated to horseradish peroxidase were purchased from Accurate (Westbury, NY, U.S.A.). Phaseolin from *Phaseolus vulgaris* and peptide:*N*-glycosidase A (PNGase A) from sweet almonds (*Amygdalus communis*) were a gift of Ms. V. Tretter, asialo-biantennary glycopeptide from human IgG was kindly provided by Dr. E. Staudacher (both from our department). *Sambucus nigra* (elder) agglutinin-I (SNA-I) and rabbit antiserum against SNA-I were available from previous studies (Mach *et al.*, 1991). SNA-I was coupled to CNBr-activated Sepharose as suggested by the manufacturer to a density of 1.5 mg/ml of gel. Elder-fruit haemagglutinin was prepared as described by Mach *et al.* (1991).

Preparation of glycopeptides

Phospholipase A₂ (PLA₂) was purified as previously described (Altmann *et al.*, 1991) and exhaustively digested with Pronase (Weber *et al.*, 1986). The resulting glycopeptide pool was fractionated by h.p.l.c. on a 5 μ Shandon-Hypersil ODS-column (4 mm $\times$ 250 mm; ÖFZ, Seibersdorf, Austria) at 60 °C using a

Abbreviations used: ABTS, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid); PBS, phosphate-buffered saline (composition and pH are given in the text); PLA₂, phospholipase A₂ (EC 3.1.1.4.) from honey-bee (*Apis mellifera*) venom; PLA₂-GA, phospholipase A₂-derived glycoasparagine; PMSF, phenylmethanesulphonyl fluoride; PNGase, peptide:*N*-glycosidase [peptide-*N*⁴-(*N*-acetyl- β -glucosaminyl)asparagine amidase; EC 3.5.1.52]; SNA, *sambucus nigra* L. (elder) agglutinin; SP-, sulphopropyl.

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linear gradient (0–7.5% (v/v) in 13.5 min) of acetonitrile in 25 mM-ammonium acetate buffer, pH 6.0, at a flow rate of 1 ml/min. The absorbance of 220 nm was monitored. The predominant glycopeptide fraction eluted between 2 and 5 min was shown to contain asparagine and, to a minor extent, glycine, as the sole amino acids; it was therefore named 'PLA₂-glycoasparagine' (PLA₂-GA). Peptic glycopeptides from pineapple stem bromelain, as well as tryptic glycopeptides from carboxymethylated horseradish peroxidase and elder-fruit haemagglutinin respectively were prepared as described by Tretter *et al.* (1991).

Enzymic deglycosylation

A 5 µg portion of glycoprotein was denatured in 10 µl of 2 M-KSCN, containing 4% (w/v) 2-mercaptoethanol, for 2 h at room temperature and digested with 0.03 munits of PNGase A in 20 µl of 0.1 M-sodium acetate buffer, pH 5.0, containing 10 mM-PMSF and 10 µM-leupeptin, for 16 h at 37 °C.

For deglycosylation with PNGase F, 5 µg of glycoprotein was denatured for 5 min at 95 °C in 10 µl of 0.5% (w/v) SDS containing 0.1 M-2-mercaptoethanol. The samples were then incubated with 0.1 munit of enzyme in 35 µl of 0.1 M-Tris/HCl buffer, pH 8.0, containing 10 mM-EDTA, 10 mM-PMSF, 10 µM-leupeptin and 0.2% (w/v) Nonidet P40, for 16 h at 37 °C.

Exoglycosidase treatments

A 500 nmol portion of bromelain glycopeptide was digested with jack-bean α -mannosidase (1.5 units) in 50 mM-sodium acetate buffer, pH 4.5, containing 0.1 mM-ZnCl₂. Asialo-IgG glycopeptide (1 µmol) was treated with β -galactosidase from bovine testis (0.6 unit) and jack-bean β -N-acetylhexosaminidase (0.7 unit) in 50 mM-sodium citrate buffer, pH 5.2, containing 0.02% (w/v) NaN₃.

Incubations were carried out for 48 h at 37 °C; the glycopeptides were then recovered by gel filtration on a Bio-Gel P-30 column (1.0 cm × 50 cm), 50 mM-ammonium acetate buffer, pH 5.0, being used as eluent.

Chemical defucosylation

For defucosylation, 2 µmol of bromelain glycopeptide was dissolved in 1 ml of trifluoroacetic acid and kept at 20 °C for 20 h (Yasuda *et al.*, 1970). The product was desalted over a column (1.0 cm × 50 cm) of Sephadex G-15 with 50 mM-ammonium acetate buffer, pH 5.0, as eluent.

Immunization of a rabbit with honey-bee venom PLA₂

The antiserum against PLA₂ was raised in a New Zealand White Rabbit by intradermal injection of a solution of 1 mg of enzyme in distilled water, emulsified with an equal volume of complete Freund's adjuvant. Subsequent injections (after 20 and 30 days) were done with 0.5 mg of antigen and incomplete Freund's adjuvant respectively. Blood samples were collected at 2-week intervals and the corresponding sera were pooled.

Purification of carbohydrate-specific antibodies

An immunoaffinity resin was prepared by coupling PLA₂-GA to activated CH-Sepharose according to the manufacturer's instructions. The coupling yield was 3 mg/ml of gel. Immunoaffinity chromatography of anti-PLA₂ antiserum was done using 3 M-KSCN in 0.5 M-NH₄Cl buffer, pH 10.0, as eluent (Johnstone & Thorpe, 1982).

Immunoblotting

After separation by SDS/14%-(w/v)-PAGE (Laemmli, 1970; Hasilik & Neufeld, 1980), polypeptides were transferred to nitrocellulose sheets (Towbin *et al.*, 1979), followed by incubation

with the respective antisera and detection with goat anti-rabbit IgG-immunoglobulins conjugated to horseradish peroxidase (Theisen *et al.*, 1986).

E.I.s.a. and inhibition experiments

All incubations were carried out at 37 °C for 90 min unless otherwise stated. Microtitre plates (Nunc, Roskilde, Denmark) were coated with 0.5 µg of antigen/well. Residual protein-binding sites were blocked with 3% (w/v) BSA in PBS (1.5 mM-KH₂PO₄/10 mM-Na₂HPO₄/3 mM-KCl/140 mM-NaCl, pH 7.4) containing 0.05% (w/v) Tween 20. A 50 µl portion of antibody solution, appropriately diluted with blocking solution, was added to the wells after preincubation with or without the different inhibitors for 16 h at 4 °C. Bound antibodies were detected by using goat anti-rabbit IgG-immunoglobulins conjugated to horseradish peroxidase. Peroxidase activity was determined using ABTS and H₂O₂, measuring the increase of absorbance at 405 nm against 492 nm.

Other methods

Determinations of amino acid and monosaccharide composition as well as methylation analyses were done as previously described (Tretter *et al.*, 1991).

Affinity purification of antibodies on nitrocellulose-bound polypeptides was performed as described by Smith & Fisher (1984).

RESULTS

Preparation and characterization of carbohydrate-specific antibodies against PLA₂

An antiserum was raised in a rabbit against honey-bee venom PLA₂. Immunoblot experiments demonstrated significant cross-reaction with several plant glycoproteins, such as pineapple stem bromelain and horseradish peroxidase (Fig. 1a). Very similar reactions were found with phaseolin and phytohaemagglutinin from *Phaseolus vulgaris*, *Sambucus nigra* agglutinin I (SNA-I)

Fig. 1. Cross-reactivity of anti-PLA₂ and anti-(horseradish peroxidase) antisera with insect and plant glycoproteins

A 1 µg portion of PLA₂ (1), of bromelain (2) and of horseradish peroxidase (3) were separated by SDS/PAGE and analysed by immunoblotting using anti-PLA₂ (a) and anti-(horseradish peroxidase) antiserum (b) respectively as described in the Materials and methods section. The M_r standards used were ovalbumin (45000), bovine carbonic anhydrase (29000), bovine β -lactoglobulin (18400) and hen's-egg lysozyme (14300).

Fig. 2. Sensitivity of the cross-reacting epitope to endoglycosidase treatment

Two 5 µg portions of horseradish peroxidase were separately incubated in the absence (–) or presence of PNGase A (A) or PNGase F (F) as described in the Materials and methods section. Aliquots (1 µg) were analysed by SDS/PAGE and immunoblotting with anti-PLA₂ antiserum as outlined in the legend to Fig. 1. The arrow marks the position of deglycosylated horseradish peroxidase.

Table 1. Carbohydrate of elder-fruit lectin and various glycopeptides

Lectin or glycopeptide	Molar ratios* of:			
	Man	Xyl	Fuc	GlcNAc
Elder-fruit lectin	3.4	0.8	0.9	2.0
Glycopeptide from:				
PLA ₂ (= PLA ₂ -GA)	3.5	n.d.†	0.5	2.0
Horseradish peroxidase	3.2	0.9	1.0	2.0
Bromelain	2.0	1.2	0.8	2.0
Defucosylated	1.9	1.0	0.1	2.0
Demannosylated	1.5	1.2	0.7	2.0
IgG, modified‡	3.3	n.d.	0.7	2.0

* Values are expressed as molar ratios relative to GlcNAc = 2.0.

† n.d., not detectable.

‡ Sialic acid, Gal and GlcNAc removed.

Table 2. Potential of various glycopeptides to inhibit the binding of carbohydrate-specific anti-PLA₂ antibodies to elder-fruit lectin

Source of glycopeptide	Concentration required for 50% inhibition (µM)
PLA ₂ (= PLA ₂ -GA)	0.4
Horseradish peroxidase	0.2
Bromelain	0.4
Defucosylated	8.9
Demannosylated	0.6
IgG, modified*	> 100.0

* Sialic acid, Gal and GlcNAc removed.

and elder-fruit haemagglutinin, which all contain β-xylosylated oligomannose-type N-glycans with fucose α1,3-linked to the asparagine-bound N-acetylglucosamine, but not with chicken ovalbumin (result not shown). On the other hand, rabbit anti-(horseradish peroxidase) antiserum reacted not only with other

plant glycoproteins, but also with the glycosylated fraction of PLA₂ (Fig. 1b). If anti-PLA₂ antibodies bound to nitrocellulose-immobilized horseradish peroxidase were eluted from the membrane, they recognized exclusively the glycosylated PLA₂ variant and retained their affinity towards other plant glycoproteins (results not shown).

Furthermore, an antiserum raised against another plant glycoprotein, SNA-I, showed a strong carbohydrate-dependent interaction with elderberry lectin (results not shown). Immobilized PLA₂-GA could effectively bind the carbohydrate-directed antibody fraction of anti-SNA-I antiserum. *Vice versa*, preadsorption of anti-PLA₂ antiserum on SNA-I-Sepharose abolished its reaction with elder-fruit haemagglutinin. The remaining anti-PLA₂-directed antibodies did not bind to the PLA₂-GA matrix (results not shown).

Identification of the carbohydrate moiety as the cross-reacting determinant

Enzymic deglycosylation of horseradish peroxidase with PNGase A significantly reduced its reactivity with anti-PLA₂ antiserum (Fig. 2). On the other hand, PNGase F failed to produce a similar effect. The action of this endoglycosidase to release glycoprotein N-glycans was recently shown to be inhibited by the α1,3-fucosylation of the asparagine-bound N-acetylglucosamine (Tretter *et al.*, 1991).

Characterization of the carbohydrate epitope

The carbohydrate-specific antibody fraction of anti-PLA₂ antiserum was isolated by affinity chromatography on immobilized PLA₂-GA, accounting for approx. 0.5% of the total anti-PLA₂ antibodies. Subsequently, elderberry lectin was used instead of horseradish peroxidase as the immunoadsorbent in e.l.i.s.a. experiments. This lectin contains one relatively homogeneous N-glycosidically bound carbohydrate unit (for analysis, see Table 1). Methylation analysis confirmed that fucose occurs exclusively in α1,3-linkage to the asparagine-bound N-acetylglucosamine (results not shown). The binding of the anti-glycan antibodies to this antigen could be inhibited by PLA₂-GA and by glycopeptides prepared from pineapple stem bromelain and horseradish peroxidase. An α1,6-fucosylated biantennary asialo-glycopeptide from human IgG did not act as an inhibitor in this system (result not shown). This effect remained unchanged after enzymic removal of the terminal N-acetyl-lactosamine units (Table 2). These results indicated that the α1,3-fucosylation, but not the α1,6-fucosylation, of the asparagine-bound N-acetylglucosamine plays a key role in the specificity of the carbohydrate-directed anti-PLA₂ antibodies.

Evidence that α1,3-linked fucose is sufficient to create an immunogenic epitope

Pineapple stem bromelain glycopeptide was available in quantities sufficient to allow chemical and enzymic modification (Table 1). Treatment with trifluoroacetic acid removed approx. 90% of the total fucose of the otherwise unchanged glycopeptide, as confirmed by methylation analysis (results not shown), and resulted in an approx. 22-fold reduction of its inhibitory potency. Partial demannosylation did not significantly affect the inhibitory activity (Table 2).

DISCUSSION

Polyclonal antibodies against plant glycoproteins have previously been demonstrated to react with honey-bee venom PLA₂. The epitope for these cross-reactive immunoglobulins were proposed to be a xylosylated asparagine-bound carbohydrate unit (Faye & Chrispeels, 1988). However, xylose has clearly been

shown not to occur in PLA₂ (Staudacher *et al.*, 1992; V. Kubelka, F. Altmann, E. Staudacher & L. März, unpublished work).

To investigate this cross-reactivity and, at the same time, to identify the specificity of carbohydrate-directed anti-PLA₂ antibodies (Weber *et al.*, 1987), we raised a polyclonal antiserum against PLA₂. A small percentage of antibodies in the antiserum was shown to recognize specifically the carbohydrate moiety of the enzyme. Immunoblot experiments in combination with endoglycosidase treatment demonstrate that these anti-glycan antibodies account for the cross-reaction with various plant glycoproteins. *Vice versa*, antisera against two unrelated plant glycoproteins contain carbohydrate-specific immunoglobulins reacting with PLA₂, suggesting that similar structural determinants on insect and plant *N*-glycans may evoke the same immune response in other mammals. Various different monosaccharide residues have been implicated in the creation of an antigenic epitope (Kaladas *et al.*, 1983; Faye & Chrispeels, 1988; Laurière *et al.*, 1989; Kurosaka *et al.*, 1991; Ramirez-Soto & Poretz, 1991). As structural analyses of the *N*-linked oligosaccharides of honey-bee venom PLA₂ (Staudacher *et al.*, 1992; V. Kubelka, F. Altmann, E. Staudacher & L. März, unpublished work) and various plant glycoproteins (e.g. van Kuik *et al.*, 1986; Fournet *et al.*, 1987; Ashford *et al.*, 1987) respectively revealed significant differences, cross-reacting anti-glycan antibodies provided a means of identifying the determinants involved. The interaction of various glycopeptides with anti-PLA₂-glycan immunoglobulins was characterized by their inhibitory effect on the binding to a heterologous plant glycoprotein, elder fruit haemagglutinin, which was selected owing to its rather homogeneous carbohydrate moiety. This glycoprotein was originally named 'SNA-III' (Mach *et al.*, 1991), as was independently a recently described elder-seed lectin (Peumans *et al.*, 1991). However, as these two proteins appear not to be identical (L. Mach, R. Kerschbaumer & J. Glössl, unpublished work), we suggest that the fruit haemagglutinin be called 'SNA-IV'.

The reaction of plant glycopeptides with the antiserum against PLA₂ strictly depended on the presence of α 1,3-linked fucose residues linked to the asparagine-bound *N*-acetylglucosamine. In addition, we could demonstrate that this modification is the only immunogenic determinant of the carbohydrate moiety of PLA₂, as well as of at least one plant glycoprotein, *Sambucus nigra* haemagglutinin-I (SNA-I). These results are in good agreement with data for antisera raised against *Wistaria floribunda* mitogen (Ramirez-Soto & Poretz, 1991). In contrast, the antigenic epitope of horseradish peroxidase *N*-glycans was reported to involve both α 1,3-linked fucose and terminal α 1,6-linked mannose residues (Kurosaka *et al.*, 1991). It may be speculated that it is this α 1,6-linked mannose elongation that could account for the interaction of PLA₂ with an antiserum reported to be specific for β 1,2-linked xylose (Faye & Chrispeels, 1988; Laurière *et al.*, 1989).

We conclude that α 1,3-fucosylation at the reducing terminal *N*-acetylglucosamine residue of asparagine-bound oligosaccharides is a predominant immunogenic carbohydrate deter-

minant of insect and plant glycoproteins. The occurrence of this modification on recombinant glycoproteins expressed in insect or plant cells would be of significant impact if these proteins are destined for therapeutical application.

We thank Dr. E. Staudacher, Dr. F. Altmann and Ms. V. Tretter (all from our Department) for the supply of various materials and their help in determining monosaccharide compositions. We feel indebted to Ms. V. Kubelka for performing the methylation analyses and Mr. R. Kerschbaumer for the purification of SNA-IV. The expert technical assistance of Mr. T. Dalik is gratefully acknowledged. We also thank Dr. D. Kerjaschki (Department of Pathological Anatomy, School of Medicine, University of Vienna, Vienna, Austria) for the opportunity to use his laboratory facilities to raise antibodies. This work was supported in part by a grant from the Bundesministerium für Wissenschaft und Forschung.

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