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# *N*-Linked Glycan Chains on *S*-Allele-Associated Glycoproteins from *Nicotiana alata*

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**The products of the self-incompatibility locus of flowering plants are glycoproteins. The specificity of different alleles at this locus might be expressed through differences in either amino acid sequences or by the glycan substituents. We have investigated the numbers of *N*-linked glycan chains on the *S*-glycoproteins and obtained information on their structure by enzymic cleavage with *N*-glycanase and endo-β-*N*-acetylglucosaminidase H. In addition to there being variation in the numbers of chains on the *S*-glycoproteins, each glycoprotein appears to consist of a spectrum of "glycoforms" bearing chains of differing type and fine structure. This microheterogeneity in *N*-linked glycan chains may be functionally significant.**

## INTRODUCTION

In the preceding three papers, information relating to the cDNA sequences, the purification, and the biological activity of some *S*-allele-associated style glycoproteins of *Nicotiana alata* has been presented (Anderson et al., 1989; Jahnen et al., 1989a; Jahnen, Lush, and Clarke, 1989b). Amino acid sequence data show that, while there is extensive homology between these *S*-glycoproteins, and both the amino and carboxy termini are highly conserved, there are hypervariable regions within the main body of the polypeptide. Furthermore, the occurrences of the consensus triplets, Asn-*X*-Thr/Ser, denoting sites of potential *N*-glycosylation, are also conserved, although new sites also arise (Anderson et al., 1989). The molecular basis for self-incompatibility in flowering plants is not yet understood. It is possible that differences between the amino acid sequences of the *S*-glycoproteins account for allelic specificity; however, in other biological systems, the glycan substituents on glycoproteins are important in recognition and for biological activity. In the mouse model system used to study mammalian fertilization, *O*-linked glycans on glycoprotein ZP3 in the egg extracellular coat, the zona pellucida, mediate species-specific binding by sperm (Wassarman, 1988), and, in a similar manner, some microorganisms recognize and bind to carbohydrate on the surface of host tissues. For example, *Escherichia coli* bind to epithelial cells using a mannose-specific receptor on fimbriae (Sharon, 1987). Glycan chains on glycoproteins and glycolipids are important antigenic determinants on erythrocytes (Makita and Taniguchi, 1985) and surface signatures on many cell types (Feizi and Childs, 1985).

It is thus clear from studies in animals that glycan chains on glycoproteins and glycolipids are also important for many biological interactions. Although less information is available for plant systems, experiments in plant pathology and physiology have demonstrated that oligosaccharides have a crucial role to play in recognition events (Samson et al., 1987), as defense signals (Darvill and Albersheim, 1984; Ryan, 1984; Davis, Darvill, and Albersheim, 1986), and signals for growth and development (McNeil et al., 1984; Tran Thanh Van et al., 1985). The interaction between gametes of opposite mating types of the algae *Chlamydomonas* spp is mediated by glycoproteins on the flagellar surface; the glycan substituents on these sexual agglutinins are necessary for biological activity, and it has been suggested that they may be also responsible for specificity (Samson et al., 1987). Although the process by which oligosaccharide chains interact with target tissues is not known, there is preliminary evidence for a high-affinity receptor for β-glucan elicitors in membrane fractions of soybean roots (Schmidt and Ebel, 1987).

The oligosaccharide chains of the *S*-allele-associated glycoproteins may therefore be involved in the incompatible response. The experiments described in this paper define the number of chains and provide some information as to the type of structure present.

## RESULTS

An SDS-polyacrylamide gel of the products from the *S*-glycoproteins after a time course hydrolysis with peptide: *N*-glycosidase F (*N*-glycanase) is shown in Figure 1. This enzyme releases from glycoproteins intact glycan chains

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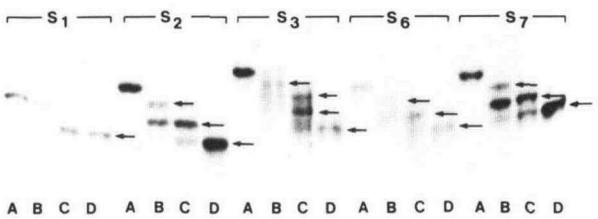


**Figure 1.** An SDS-Polyacrylamide Gel of the Products Formed during a Time Course Hydrolysis of *S*-Glycoproteins by *N*-Glycanase.

From the time of commencement of hydrolysis: (A) $t = 0.0$ hr; (B) $t = 0.5$ hr; (C) $t = 1.0$ hr; (D) $t = 8.0$ hr.

of the complex, hybrid and high-mannose type by cleaving the *N*-glycosidic linkage between the glycan chain and the polypeptide backbone (Tarentino, Gomez, and Plummer, 1985). Single molecular species of similar molecular weights remain from each *S*-glycoprotein after *N*-glycanase digestion. Three chains are present on the $S_2$-, $S_6$-, and $S_7$-glycoproteins, and the $S_1$- and $S_3$-glycoproteins have one and four chains, respectively. Hydrolysis by *N*-glycanase is considered to have removed all glycan chains from $S_2$-, $S_3$-, and $S_6$-glycoproteins since the mobility of the final product is consistent with that of a trifluoromethane sulfonic acid deglycosylated $S_2$-glycoprotein and with molecular weights calculated from amino acid sequences (Anderson et al., 1986, 1989). The molecular weights of the *N*-glycanase-treated $S_1$- and $S_7$-glycoproteins are similar, and although sequence data are not yet available, it is likely that the molecular weight differences between the five native *S*-glycoproteins examined is mostly accounted for by glycan substituents.

Hydrolysis of the *S*-glycoproteins by endo-$\beta$-*N*-acetylglucosaminidase H (endo H) is shown in Figure 2. This endoglycosidase hydrolyzes the glycosidic linkage between the core *N*-acetylglucosamine residues of *N*-linked glycan chains of high-mannose and some hybrid types (Trimble and Maley, 1984); thus, its specificity is more restricted than that of *N*-glycanase. No hydrolysis is seen for the $S_1$-glycoprotein, whereas the other glycoproteins bear some susceptible chains. The hydrolysis by the enzyme is likely to have gone to completion since no difference in products was seen after extending the reaction time from 8 hr to 24 hr, after additional enzyme had been added.

## DISCUSSION

The number of potential *N*-glycosylation sites on the $S_2$-, $S_3$-, and $S_6$-polypeptides, predicted from cDNA sequence data (Anderson et al., 1986, 1989), are indicated diagrammatically in Table 1. Four of the glycosylation sites are conserved between alleles and a new site has arisen in the $S_3$-glycoprotein. *N*-Glycanase hydrolysis (Table 1 and Figure 1) indicates that one site remains unoccupied on each polypeptide. The mechanism is not yet known by which the oligosaccharyltransferase complex in the rough endoplasmic reticulum recognizes which particular consensus sequence should be glycosylated in a newly synthesized protein; it appears, however, that a glycosylation site-binding protein is involved, having the capacity to recognize multiple polypeptide domains in the vicinity of the glycosylation site (Geetha-Habib et al., 1988). In a sporophytic incompatibility system, information is available for the disposition of *N*-glycosylation sites in three *S*-glycoproteins from *Brassica oleraceae* (Nasrallah et al., 1987) and three *S*-glycoproteins from *B. campestris* (Isogai et al., 1987), and indicates that potential sites are conserved and additional sites appear. In the case of *B. campestris*, the failure to detect PTH-asparagine residues during amino acid sequencing at six out of the nine potential sites suggests that three of these sites do not bear glycan chains (Isogai et al., 1987). *N*-Glycanase is reported to hydrolyze all classes of *N*-linked chains hitherto examined, provided both the amino and carboxyl groups of the asparagine residue to which they are attached are in peptide linkage (Tarentino, Gomez, and Plummer, 1985), but provides little information with regard to the type of chain present. Endo H, on the other hand, will cleave off high-mannose structures and some hybrid types (Trimble and Maley, 1984), and the ability of the enzyme to remove chains from the $S_2$-, $S_3$-, $S_6$-, and $S_7$-glycoproteins demonstrates that high-mannose and/or hybrid chains are present. The results of the hydrolysis of the *S*-glycoproteins are indicative of heterogeneity in chain type at a particular glycosylation site. For example, after hydrolysis of the $S_6$-glycoprotein by endo H is complete, four bands are present in the gel (see Figure 2.) The lowest molecular weight band relates to the situation where all three glycan chains on $S_6$-glycoprotein have been cleaved off by the enzyme and are thus of the hybrid/high-mannose type. In the highest molecular weight band, where no hydrolysis has taken place and all three chains remain on the glycoprotein, these chains can either be of the complex type or hybrid structures which are resistant to this enzyme.

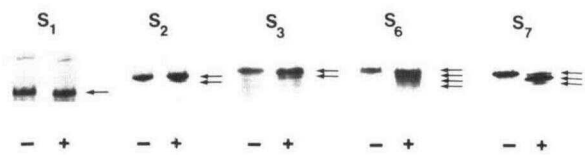


**Figure 2.** An SDS-Polyacrylamide Gel of the Endo H-Treated *S*-Glycoproteins.

Symbols indicate the presence (+) or absence (–) of endo H in digests.

**Table 1.** *N*-Linked Glycan Chains on *S*-Glycoproteins

| *S*-Glycoprotein | Potential Glycosylation Sites[a] | Number of Sites Glycosylated[b] | Endo H Susceptibility[c] |
|---|---|---|---|
| $S_1$ | Not available | 1 | – |
| $S_2$ | $NH_2$ ▼▼ ▼▼▼ COOH | 3 | + |
| $S_3$ | ▼▼ ▼▼ ▼▼ | 4 | + |
| $S_6$ | ▼▼ ▼▼▼ | 3 | + |
| $S_7$ | Not available | 3 | + |

[a] Deduced from the occurrence of Asn-*X*-Ser/Thr in amino acid sequences.
[b] Determined by *N*-glycanase hydrolysis.
[c] Indicating the presence (+) or absence (–) of hybrid/high-mannose chains.

Bands in the gel track that are between these extremes are intermediate cases. These data suggest that some of the *S*-glycoproteins consist of a number of species or "glycoforms" (Rademacher, Parekh, and Dwek, 1988) having a varying number of complex and hybrid/high-mannose type chains. Furthermore, structural studies on oligosaccharides isolated from the $S_2$-glycoprotein indicate that there is microheterogeneity in the fine structure of a particular chain type (J.R. Woodward, A. Bacic, S. Munro, D. Craik, and A.E. Clarke, manuscript in preparation). Microheterogeneity in chain structure in glycoproteins is a widespread phenomenon (Rademacher, Parekh, and Dwek, 1988), but it is unclear whether it relates to specific functions or to incomplete biosynthesis or to degradation. Isolated *N*-linked chains from ovalbumin from a single source were reproducible both quantitatively and qualitatively (Iwase, 1988), and it has been suggested that species, tissue, cellular, and environmental considerations may influence oligosaccharide synthesis (Iwase, 1988).

With the extensive variation in number, type, and fine structure of glycan chains on the *N. alata* *S*-glycoproteins, there appears to be ample potential to encode allelic specificity. The possibility of the presence of *O*-linked substituents conferring a further degree of complexity can only be ruled out in the case of the $S_2$-glycoprotein, since none have been detected in chemical studies (J.R. Woodward, A. Bacic, S. Munro, D. Craik, and A.E. Clarke, manuscript in preparation). It is not easy to discern how the apparently extensive microheterogeneity can be reconciled to the specific recognition event occurring before the rejection of self-pollen in the incompatibility response. Many glycoproteins bear glycan chains at multiple sites and it appears that, in some cases, each chain might be involved in quite different functions. Site-directed mutagenesis of the nucleotide sequence of the glycoprotein hormone erythropoeitin indicates that, whereas glycosylation at Asn-38, Asn-83, and Ser-126 is necessary for correct biosynthesis and secretion, glycosylation at Asn-24 and Asn-38 is required for biological activity (Dubé, Fisher, and Powell, 1988).

If the allelic specificity of the *S*-glycoproteins were to reside in the fine structure of the glycan chains, then an additional controlling factor must either be a glycosyltransferase to elaborate the oligosaccharide or a hydrolase to trim down a precursor. Such an enzyme would be required to be closely linked genetically with the *S*-locus. However, if the position or presence of a glycan chain on the polypeptide, and its core structure rather than fine ornamentation, is a determining factor, then the primary structure of the *S*-allele product becomes the dominant component for determining self-incompatibility, requiring no unique post-translational modification. Whether allelic specificity is determined by the polypeptide or the carbohydrate domains of the *S*-glycoproteins remains to be elucidated; however, the central role of glycan moieties in mammalian and lower plant fertilization systems provides an intriguing possibility for a similar function in higher plants that warrants further investigation.

## METHODS

### *S*-Glycoprotein Preparation

The *S*-glycoproteins were isolated from plants homozygous for the $S_1$-, $S_2$-, $S_3$-, $S_6$-, and $S_7$-alleles as described by Jahnen et al. (1989a).

### Enzymic Removal of *N*-Linked Chains

*S*-Glycoproteins to be deglycosylated were denatured for 5 min at 100°C in 20 mM β-mercaptoethanol containing 1.2 times g/g excess of SDS relative to protein. Hydrolysis by peptide:*N*-glycosidase F (*N*-glycanase) (Genzyme Corp.) (10 units/ml) was performed at 25°C in 50 mM Tris/acetate buffer, pH 8.6, containing 5 mM EDTA, 20 mM β-mercaptoethanol, and 1.75% (v/v) Nonidet P-40. Glycoproteins to be hydrolyzed by endo-β-*N*-acetylglucosaminidase H (endo H) (Du Pont-New England Nuclear) (160 milliunits/ml) were incubated at the same temperature in 50 mM MES/NaOH buffer, pH 5.5, containing 20 mM β-mercaptoethanol, 1.75% (v/v) Nonidet-P-40, and 10 μg/ml each of pepstatin, leupeptin, chymostatin, and antipain.

Further enzyme was added after 8 hr to give final concentrations of 20 units/ml and 320 milliunits/ml for *N*-glycanase and

endo H, respectively. Hydrolysis was terminated by addition of electrophoresis sample buffer and heating at 100°C for 5 min.

### SDS-Polyacrylamide Gel Electrophoresis

Hydrolysis products of *N*-glycanase digests were separated in a 10% to 18% SDS-polyacrylamide gradient gel, having a 5% stacker, essentially as described by Laemmli (1970), whereas those from endo H hydrolysis were run in 8% to 25% gradient gels using a PhastGel Separation System (Pharmacia LKB Biotechnology, Uppsala, Sweden). Gels were stained with silver (Heukeshoven and Dernick, 1988).

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