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Microheterogeneity of the oligosaccharides carried by the recombinant bovine lactoferrin expressed in *Mamestra brassicae* cells

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The development of therapeutic glycoprotein production using the baculovirus expression system depends on the ability of insect cell lines to reproduce site specific mammalian-like N-glycans. A combination of ^{1}H-NMR and mass spectrometry techniques (MALD-MS, ES-MS, and CID-MS-MS) allowed us to elucidate the N-linked oligosaccharides microheterogeneity on three different N-glycosylation sites, Asn_{233}, Asn_{476}, and Asn_{545}, of a baculovirus-expressed recombinant bovine lactoferrin produced in *Mamestra brassicae*. Two families of N-glycan structures have been found: first, oligomannosidic glycans ($Man_{9-5}GlcNAc_2$) and secondly, short truncated partially fucosylated glycans ($Man_{3-2}[Fuc_{0-1}]GlcNAc_2$). These results indicate that *Mamestra brassicae* cell line is not able to synthesize complex N-glycans, even if an α1,6-linked fucose residue is frequently present on the asparagine-bound N-acetylglucosamine residue of short truncated structures. Nevertheless, we have shown that *Mamestra brassicae* ensures the same N-glycosylation pattern as found on natural bovine lactoferrin showing the same distribution between complex and high-mannose type glycans on the different glycosylation sites. Sites which are naturally occupied by high-mannose glycans (Asn_{233} and Asn_{545}) are substituted essentially by the same type of N-glycans in the recombinant counterpart, and the site Asn476,which carries sialylated complex type chains in the natural glycoprotein, is substituted by short, truncated, partially fucosylated chains in *Mamestra brassicae*–expressed bovine lactoferrin. These various results lead us to the conclusion that bovine lactoferrin is an interesting model to determine the potential of glycosylation of the baculovirus/insect cell expression systems.

Key words: baculovirus vector/bovine lactoferrin/insect cell/*Mamestra brassicae*/N-glycosylation

Introduction

In recent years, the development of the therapeutic glycoprotein production using the baculovirus expression system in insect cells (for reviews see: Kang, 1988; Luckow and Summers, 1988; Miller, 1988; O'Reilly *et al.*, 1994) has stimulated concerns to determine the posttranslational modifications capacity and in particular the glycosylation potential, of insect cells. In fact, it is now well established that protein bound carbohydrate side chains play important roles on the physicochemical properties and functions of glycoproteins such as antigenicity, proper folding, solubility, and biological half-life (Klenk, 1990; Takeuchi and Kobata, 1991; Munk *et al.*, 1992). Oligosaccharides play also a role in biological recognition processes such as secretion, targeting of glycoproteins to different subcellular compartments, cell–cell adhesion, tissue targeting, and tissue organization (Olden *et al.*, 1982; Elbein, 1987; Rademacher *et al.*, 1988; Branley *et al.*, 1990; Jarvis *et al.*, 1990; Springer and Lasley, 1991; Kobata, 1992; Lis and Sharon, 1993; Varki, 1993; Wojczyk *et al.*, 1995).

Nowadays, the N-glycans biosynthesis pathway in mammalian cells is well defined (Kornfeld and Kornfeld, 1985; Schachter, 1991). The biosynthesis of N-linked oligosaccharides begins with the synthesis of a lipid linked oligosaccharide moiety ($Glc_3Man_9GlcNAc_2PPDol$) that is transferred onto the nascent polypeptide chain in the rough endoplasmic reticulum (RER). The oligosaccharidic chain is transferred onto an asparagine residue at the tripeptide recognition sequence Asn-X-Ser/Th. A series of trimming reactions is then catalyzed by glucosidases and mannosidases in the RER and in the Golgi apparatus that generate N-glycans of high mannose type ($Man_{9-5}GlcNAc_2$). The N-glycan biosynthesis proceeds in the Golgi apparatus with the action of glycosyltransferases (N-acetylglucosaminyl-, fucosyl-, galactosyl-, sialyltransferases) producing N-glycans of hybrid and complex types. A number of glycoproteins which contain more than one N-glycan site carry different N-glycan structures at each recognition sequence. In addition to the glycoprotein heterogeneity, it often appears a microheterogeneity of the oligosaccharide structures attached to each glycosylation site which depends mainly on the origin of the cell line (Rademacher *et al.*, 1988; Goochee *et al.*, 1991; Furukawa and Kobata, 1992; Lis and Sharon, 1993; Jenkins, 1995.)

Compared to the information available on the structure and biosynthesis of mammalian N-glycans, our knowledge on those from insect cells appears somewhat fragmentary. Studies of N-oligosaccharide structures of recombinant glycoproteins expressed by lepidopteran cells using baculovirus vector have revealed that the N-linked oligosaccharides found are essentially of high-mannose type and short truncated structures frequently with a fucose residue α1,6-linked to the asparagine-bound N-acetylglucosamine residue (Kuroda *et al.*, 1986, 1989, 1990, 1991; Jarvis and Summers, 1989; Chen *et al.*,

1991; Wathen *et al.*, 1991, Williams *et al.*, 1991; Noteborn *et al.*, 1992; Grabenhorst *et al.*, 1993; Veit *et al.*, 1993; Voss *et al.*, 1993; Yeh *et al.*, 1993; Manneberg *et al.*, 1994; Ponimaskin *et al.*, 1994). However, a small proportion of hybrid structures with one terminal N-acetylglucosamine residue has been found in mouse recombinant interleukin-3 produced in *Bombix mori* larvae (Hogeland and Deinzer, 1994), and recent data have even suggested that an insect cell line *Estigmena acrea* is able to synthesize hybrid or complex N-glycans with N-acetylglucosamine and galactose residues at the N-oligosaccharidic sites of the recombinant human interferon γ (Ogonah *et al.*, 1996). In contrast, only one group has reported that recombinant human plasminogen carries complex type N-glycans with terminal sialic acid residues when expressed by a baculovirus vector in *Spodoptera frugiperda* 21 or *Mamestra brassicae* (Davidson *et al.*, 1990; Davidson and Castellino, 1991a,b).

Endogenous N-glycan structures found in insect cell glycoproteins have no complex type glycans (Butters and Hughes, 1981; Hsieh and Robbins, 1984; Williams *et al.*, 1991) but small amount of oligosaccharides have terminal N-acetylglucosamine or N-acetylgalactosamine residues (Hard *et al.*, 1993; Kubelka *et al.*, 1993, 1994). Moreover, it has been possible to detect low level of β1,2-N-acetylglucosaminyltransferase I activity (Altmann *et al.*, 1993; Velardo *et al.*, 1993) and α-mannosidase II activities (Altmann and März, 1995) in *Bombix mori, Mamestra brassicae* and *Spodoptera frugiperda 21* and *9*, and recently, *Trichoplusia ni, Spodoptera frugiperda,* and *Mamestra brassicae* were shown to have a β1,4-N-acetylgalactosaminyltransferase enzymatic activity catalyzing the transfer of GalNAc residues to oligosaccharides carrying a terminal β-linked GlcNAc residue (Van Die *et al.*, 1996). N-Glycans from honeybee phospholipase A_2, as well as membrane bound glycoproteins of *Spodoptera frugiperda 21, Mamestra brassicae,* and *Bombix mori,* were shown to contain fucose residues α1,6- or α1,3-linked to the asparagine-bound N-acetylglucosamine residue. In addition, some of these glycans are difucosylated in α1,6- and α1,3-linkages onto the same asparagine-bound N-acetylglucosamine residue. Remarkably, glycoproteins isolated from *Mamestra brassicae* cells contain α1,3-fucosylated glycans, predominantly in difucosylated form (Staudacher *et al.*, 1992a; Kubelka *et al.*, 1993, 1994). Finally, data have revealed distinct fucosylation potentials in the three different cell lines: *Mamestra brassicae* is able to transfer fucose into α1,6- and α1,3-linkages whereas *Bombix mori* and *Spodoptera frugiperda* 9 transfer fucose residues only in α1,6-linkage (Staudacher *et al.*, 1992b).

In summary, insect cell lines are able to carry out N-glycosylation of recombinant glycoproteins but N-glycan structures found at each glycosylation site are often different from those occurring in the corresponding natural glycoprotein. These data raise several questions: (1) Do insect cell lines synthesize complex N-glycans, as it has been shown by Davidson *et al.* on recombinant human plasminogen expressed in *Spodoptera frugiperda*-9 and *Mamestra brassicae*? (2) Where is the N-glycosylation pathway aborted during the expression of the recombinant glycoprotein? (3) How does an insect cell line process the differently N-glycosylation sites of the same recombinant glycoprotein? In order to answer these questions, we have determined the nature of the N-glycans chains occurring at each glycosylation site of the recombinant bovine lactoferrin (rbLF) produced in the lepidopteran cell *Mamestra brassicae*. The natural bLF (nbLF), which has been extensively studied in our laboratory, has five potential sites of N-glycosylation but no O-glycosylation site. Among the potential N-glycosylation sites, only four are occupied by N-glycans. The consensus sites at Asn_{233} and Asn_{545} are substituted exclusively by high-mannose type oligosaccharides, while Asn_{476} carries predominantly complex type glycans and the site Asn_{368} is occupied by both types of structure (Coddeville *et al.*, 1992; Spik *et al.*, 1993). Therefore, a simple site by site microheterogeneity comparison between both nbLF and rbLF, allowed us to know whether (4) the *Mamestra brassicae* cell line was able to reproduce on the rbLF, the N-glycosylation pattern found in nbLF, or not.

As a first step, we have studied the N-linked chains of the complete rbLF by analyzing the monosaccharides composition and molar ratios using gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS).

Secondly, glycopeptides obtained by enzymatic digestion of rbLF (The V8 protease digestion of native BLF allows isolation of only three of four glycosylated sites (Asn_{233}, Asn_{476}, and Asn_{545}; G. Spik, unpublished observations)) were purified by reversed-phase high performance liquid chromatography (HPLC) and the primary structure of oligosaccharides linked to Asn_{233}, Asn_{476}, and Asn_{545} has been determined using two methods of mass spectrometry desorption: matrix assisted laser desorption mass spectrometry (MALD-MS) and electrospray mass spectrometry (ES-MS). Moreover, we have confirmed our results by collision-induced dissociation tandem mass spectrometry (CID-MS-MS).

Finally, oligosaccharides released by hydrazinolysis from rbLF were submitted to 400 MHz ^{1}H-NMR spectroscopy in order to determine the linkage of the fucose residues.

Results

Expression of rbLF and comparison of the electrophoretic profiles of nbLF and rbLF

The expression of rbLF in *Mamestra brassicae* cells has been detailed in materials and methods section. The rbLF was purified by ion exchange chromatography from 14 liters of culture supernatant containing 10 μg/ml of the recombinant glycoprotein. The yield of the purification process was estimated to be 75%. Purification steps of rbLF was followed by immunodetection after Western blotting using a polyclonal Ab anti-bLF (data not shown). The purity of rbLF was assessed by HPLC on C_{18} stationary phase (data not shown) and by 8% SDS–PAGE analysis (Figure 1, lane 2), which showed one band at approximately 80 kDa, slightly lower than the nbLF (Figure 1, lane 1). To check whether this variation in the migration of both bLFs could be due to changes in glycosylation, both bLFs were digested with PNGase F. As shown in Figure 1 (lanes 4 and 5), both deglycosylated bLFs run similarly with an apparent molecular weight of 73 kDa. Taken together, these data indicate that the rbLF was less glycosylated than its natural counterpart.

Purification and characterization of glycopeptides

In order to compare the glycopeptide sites of natural and recombinant bLF, both were digested by the V8 protease, and the peptides and glycopeptides obtained were subsequently fractionated by reversed-phase-HPLC. More than 30 fractions were separated as shown in the HPLC-C18 profile of the V8 protease digestion of the rbLF (Figure 2). The different frac-

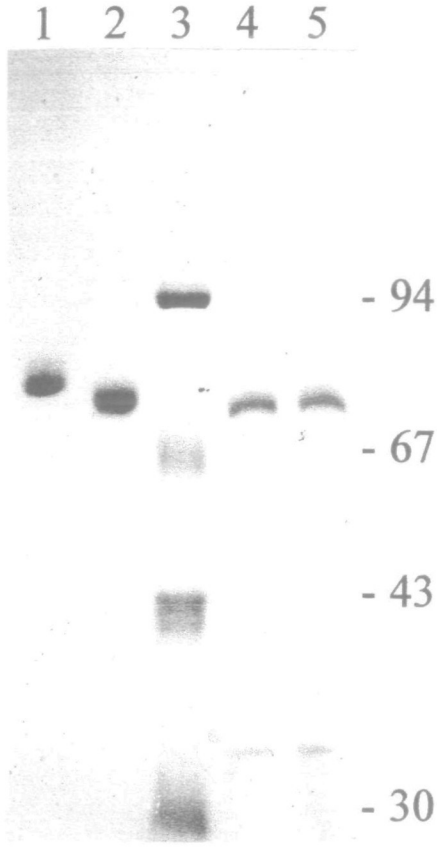


Fig. 1. SDS–PAGE analysis of nbLF and rbLF expressed in *Mb* and visualized by Coomassie blue staining. Lane 1, reduced nbLF; lane 2, reduced rbLF; lane 3, prestained SDS–PAGE molecular weight standards; lane 4, nbLF after PNGase F digestion; lane 5, rbLF after PNGase F digestion. The molecular weights of the standards are indicated (in kDa) on the right side of the figure.

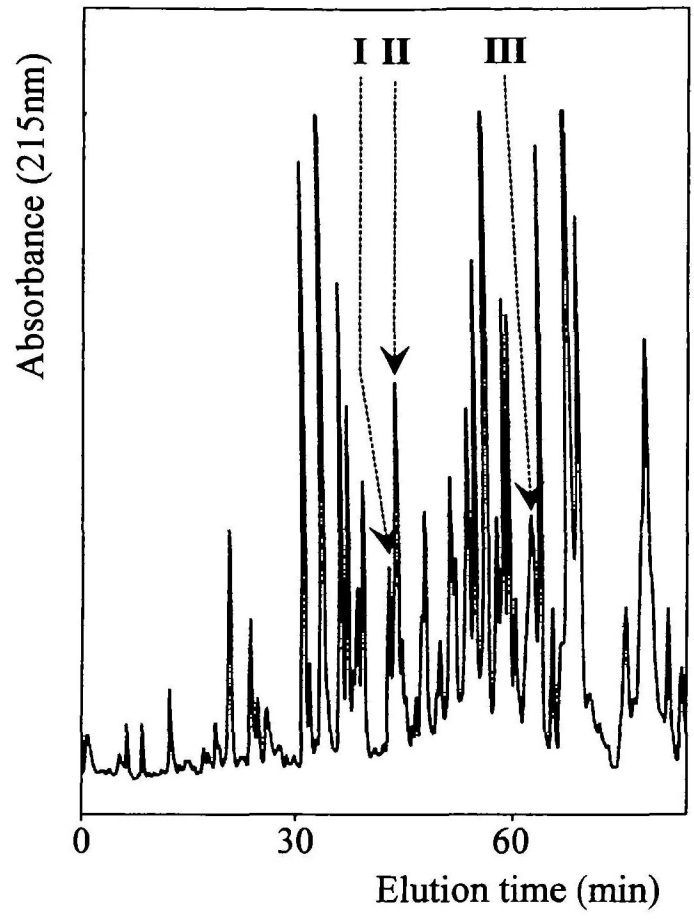


Fig. 2. HPLC-C18 profile of the V8 protease digestion of the rbLF expressed in *Mb*. The glycopeptides containing peaks I, II, and III are indicated by arrows.

tions were then analyzed by MALD-MS, and three peaks containing glycopeptides (named peaks I, II, and III, respectively, in Figure 2) were detected in the rbLF as well as in the nbLF digestion.

To identify the different glycosylation sites, Edman degradation was carried out for each glycopeptide and the molecular mass of each PNGase F deglycosylated peptide was determined by MALD-MS. Data obtained for each glycopeptide (Table I) indicate that the peaks I, II, and III, correspond, respectively, to Asn_{233}, Asn_{545}, and Asn_{476} sites. From the MALD-MS and amino acid sequence data, the Asn_{545} glycosylation site (peak II) appears to be contaminated by glycoforms of the Asn_{233} glycosylation site.

Monosaccharides composition

The results of the GC analysis of the trimethylsilylated monosaccharides obtained from nbLF, rbLF, and V8 protease digestions of peaks I, II, and III, are summarized in Table II and have been confirmed by GC-MS (data not shown). As previously observed on the electrophoretic profile, shown in Figure 1, the native rbLF is less glycosylated (5.0%) than its natural counterpart (9.0%). The presence of N-acetylglucosamine and mannose residues, which are the two major monosaccharides, indicates that all the oligosaccharidic chains of rbLF are N-glycosidically linked to the protein backbone and their molar ratio show that glycans belong mainly to the oligomannosidic family. The presence of fucose and of a trace of galactose residues on the native rbLF and in peaks I, II, and III indicates that some oligosaccharidic chains have been partially processed via the complex type chains pathway. N-acetylgalactosamine residues were observed in both nbLF and rbLF, but not in glycopeptides generated from rbLF, whereas sialic acid residues were observed only on nbLF.

Mass spectrometry analysis of glycan structures

V8 glycopeptide peak I. From the glycan mass analysis (Figure 3, Table III) and the molar carbohydrate composition (Table II), it can be inferred that Asn_{233} linked-glycans are of high mannose type (from $Man_9GlcNAc_2$ to $Man_6GlcNAc_2$). Centered spectrum shows that Asn_{233} is predominantly glycosylated by $Man_8GlcNAc_2$ (39%) and $Man_7GlcNAc_2$ (44%). To confirm the oligosaccharidic glycan structures, CID-MS-MS analysis was carried out on selected predominant glycoforms (Figure 6A)

V8 glycopeptide peak II. Assignment of the glycoforms was made difficult due to the mixture of glycopeptides belonging to both Asn_{545} and Asn_{233} glycosylation sites (Figure 4, Table IV). To overcome this problem, CID-MS-MS analysis was performed. This mass spectrometry technique allows selection of a precursor ion of a given molecular mass $[M+H]^+$ in the

Table I. Amino acid sequence of the glycopeptides present in the peaks I, II, and III from the V8 protease digestion of the rbLF expressed in *Mb* and, determination of the molecular mass by MALD-MS analysis of each peptide backbone present in the PNGase F deglycosylated peaks I, II and III

Peak	Inclusive numbers	Amino acid sequence	Calculated peptide mass[a] (average) [M]	Observed peptide mass by MALD-MS[b] $[M + H]^+$	Corresponding glycosylation site
I	229–244	LLCLNNSRAPVDAFKE	1847.1	1849.0	Asn_{233}
II	536–550	DVGDVAFVKNDTVWE	1693.8	1696.0	Asn_{545}
	229–244	LLCLNNSRAPVDAFKE	1847.1	1849.0	Asn_{233}
III	445–485	GLTWNSLKDKKSCHTAVDRTAGWNIPMGLIVNQTGSCAFDE	4580.1	4582.0	Asn_{476}

The N-glycosylation site of each peptide is indicated in bold characters.
[a]The cysteine residues have been alkylated by the iodoacetamide.
[b]The PNGase F cleavage transforms the asparagine glycan linked residue on aspartic residue.

first mass spectrometer. This parent ion is then fragmented by a collision gas in a second step, and the different product ions are scanned in a third stage. By choosing an appropriate collision energy it is possible to fragment the glycopeptide only at its glycan moiety. The sequential release of the monosaccharides allows, first, verification of the glycan assignment of the selected glycoform and, second, identification of the glycan containing peptide. Thus, considering a selected glycoform, it is possible to discriminate between a mixture of glycopeptides belonging to different glycosylation sites.

The Asn_{545} linked-glycans are of high-mannose type (from $Man_9GlcNAc_2$ to $Man_6GlcNAc_2$), being predominantly $Man_8GlcNAc_2$ (49%) and $Man_7GlcNAc_2$ (27%), as proven by CID-MS-MS (Figure 6B). Moreover, glycoform J (Figure 4, Table IV) could be of the hybrid type $Man_4GlcNAc_3$ (9%), but this assumption has not been confirmed by CID-MS-MS.

The Asn_{233} glycopeptides, which coelute with Asn_{545} glycopeptides in peak II (Figure 4 and Table IV), are substituted by $Man_5GlcNAc_2$ (24%), short truncated partially fucosylated glycans $Man_3[Fuc_{0-1}]GlcNAc_2$ (fucosylated 13%, nonfucosylated 28%) and a more degraded structure $Man_2[Fuc]GlcNAc_2$ (26%). The presence of fucose residues is confirmed by the carbohydrate composition presented in Table II. H^1-NMR data further indicate that the fucose residue is α-1,6 linked to the GlcNAc-1 residue (Table VI). We also observe on the ES-MS spectrum the presence of three smaller peaks (B, C, D) corresponding to the glycoforms previously described in peak I (Figure 3, Table III).

V8 glycopeptide peak III. Putative N-glycan structures of the Asn_{476} site inferred from mass spectrometry data (Figure 5, Table V) are in agreement with the monosaccharides content which indicates the presence of N-acetylglucosamine, mannose and fucose in a molar ratio 2.0:3.3:0.4 (see Table II). Asn_{476} linked-N-glycans are constituted by the partially fucosylated trimannosyl core $Man_3[Fuc_{0-1}]GlcNAc_2$ (fucosylated 13%, nonfucosylated 41%) and by more degraded structures $Man_2[Fuc_{0-1}]GlcNAc_2$ (fucosylated 19%, nonfucosylated 21%), as shown in the CID-MS-MS profile (Figure 7C,D). In addition, the glycoform N could be of the hybrid type $Man_3GlcNAc_3$ (6%), although this assumption has not been confirmed by CID-MS-MS.

^{1}H-NMR spectroscopic analysis of the pool of oligosaccharides

The oligosaccharides from rbLF released by hydrazinolysis were submitted to 400 MHz ^{1}H-NMR spectroscopy. The 1D and 2D ^{1}H-NMR spectra are shown in Figure 8 and spectrum data are summarized in Table VI. The isolated glycans correspond to a mixture of oligomannosidic type oligosaccharides.

It may be inferred from ^{1}H-NMR data that oligosaccharides have in common, at least in part, Man residues 3, 4, 4′, A, B, and the di-N-acetylchitobiose unit (the monosaccharide nomenclature used to describe the glycan structures is according to Vliegenthart *et al.* (1983)). Thus, ^{1}H-NMR signals reflect the heterogeneity of the oligosaccharide moiety. The anomeric proton of the GlcNAc-1 (δ H-1α) gives rise to two doublets at δ 5.190 and 5.186 ppm, assessing the completeness of the hydrazinolysis reaction (Van Halbeek *et al.*, 1993). The N-acetyl signals of GlcNAc-1 (δ 2.026 and δ 2.007 ppm) and GlcNAc-2 (δ 2.053 and δ 2.064 ppm) are both split into two singlets. The chemical shifts of the N-acetyl signal of GlcNAc-2 (δ 2.053 and δ 2.064 ppm) are typical of the extension of the pentasaccharide core with Man residues. Moreover, each H-1 atom of mannose residues gives two signals, both of them being a singlet representing the addition of each characteristic doublet.

The set of chemical shifts of the correlated H-1 and H-2 atoms of Man-4′ gives two signals. The coordinates of the first signal, H-1 δ 4.868 and H-2 δ 4.152 ppm, are indicative of a

Table II. GC analysis of the nbLF, rbLF and of peaks I, II, and III from the V8 protease digestion of the rbLF expressed in *Mb*

	Fucose	Mannose	Galactose	GalNAc	GlcNAc[a]	Sialic acid	Glycan (%)
nbLF	trace	4.1	0.3	0.2	**2.0**	0.2	9.0
rbLF	0.2	4.7	Trace	trace	**2.0**	n.d.[b]	5.0
Peak I	n.d.	7.7	Trace	n.d.	**2.0**	n.d.	
Peak II	0.6	6.3	Trace	n.d.	**2.0**	n.d.	
Peak III	0.4	3.3	Trace	n.d.	**2.0**	n.d.	

[a]The molar ratio have been calculated on the basis of two GlcNAc residues. This value takes into account the lost of the asparagine-bound N-acetylglucosamine residue during the methanolysis.
[b]Not detected.

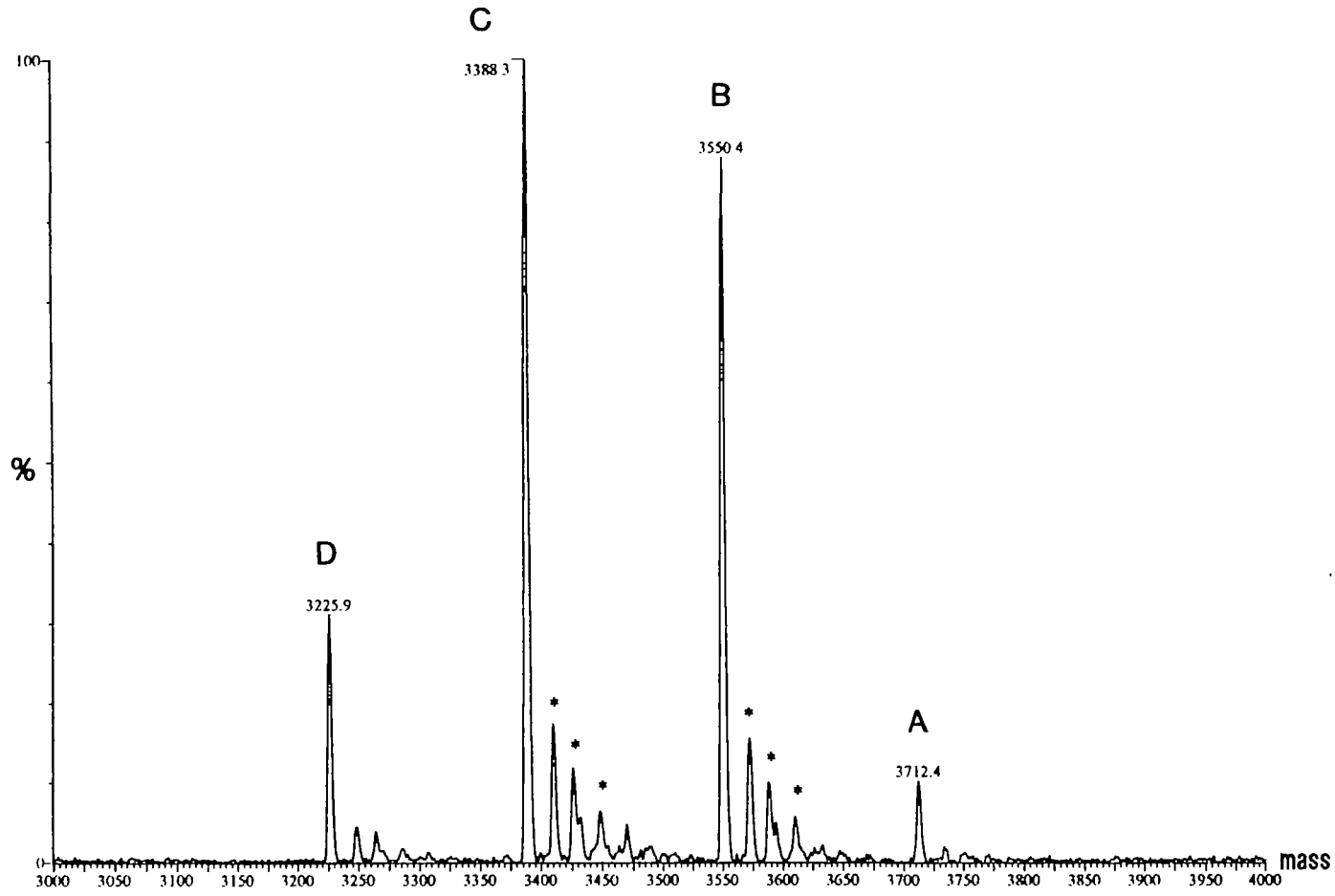


Fig. 3. Deconvoluted ES-MS spectrum of the peak I isolated from the V8 protease digestion of the rbLF expressed in *Mb*. The characteristics of the different Asn_{233} glycoforms A, B, C, and D are summarized in Table III. *, Recalculated mass of corresponding $M+Na^+$, $M+K^+$, or $M+Na^++K^+$ ion.

disubstitution of Man-4′ at O-3 and O-6 by Man-A and Man-B, whereas the second signal, H-1 δ 4.915 and H-2 δ 3.983 ppm, corresponds to a Man-4′ in a terminal position.

The H-1 and H-2 atoms of Man-B give rise also two signals. At first the signal H-1 δ 4.907 and H-2 δ 3.983 ppm, corresponds to a Man-B at terminal position in the oligomannosidic chain. Secondly the signal H-1 δ 5.144 and H-2 δ 4.030 ppm, indicates a substitution of the Man-B residue with an α1,2-linked Man-D_3 (δ H-1 5.042 ppm).

The H-1 and H-2 atoms of Man-A also give two signals. The signal at H-1 δ 5.092 and H-2 δ 4.07 ppm resonates in an area that is characteristic of a terminal nonreducing position of Man-A. The signal at H-1 δ 5.402 and H-2 δ 4.10 ppm indicates that Man-A residue is substituted with an α1,2-linked Man-D_2 (H-1 δ 5.056 ppm).

For H-1 and H-2 atoms of Man-4 in the upper branch, two signals are observed at H-1 δ 5.102 and H-2 δ 4.077 ppm, when Man-4 occupies a terminal position, and at H-1 δ 5.346 and H-2 δ 4.106 ppm indicative of the presence of an α1,2-Man-C linked to the Man-4 residue. One signal at δ 5.306 ppm assigned to the H-1 of Man-C shows that Man-C can have a O-2 substituted Man-D_1 (δ 5.042 ppm added with Man-D_3). Dealing with the signal of the nonsubstituted Man-C, the H-1 and H-2 resonances are found theoretically in the same area of the respective signals of the Man-D_2; then, it is impossible to distinguish between Man-D_2 and the nonsubstituted Man-C signals.

The α1,6-fucose linked to GlcNAc-1 is evidenced for the structural reported group signals H-1 δ 4.894; H-2 δ 3.792; H5 δ 4.099; H-6 δ 1.210 and 1.220 ppm. Two N-acetyl signals are

Table III. Analysis of ES-MS spectrum presented in Figure 3 of the peak I isolated from the V8 protease digestion of the rbLF expressed in *Mb*

Glycosylation site and calculated peptide mass ($M_{cal.}$)	Glycoform	Calculated mass (average)	Observed mass	%[a]	Glycan mass[b]	Glycan assignment
Asn_{233} ($M_{cal.}$ 1847.1)	A	3712.6	3712.4	4	1866.3	$Hex_5HexNAc_2$
	B	3550.5	3550.4	39	1704.3	$Hex_4HexNAc_2$
	C	3388.3	3388.3	44	1542.2	$Hex_3HexNAc_2$
	D	3226.2	3225.9	13	1379.8	$Hex_2HexNAc_2$

[a]Percentage of Asn_{233} glycoforms present in the peak I.
[b]Glycan mass = glycopeptide mass observed − (peptide mass − 1).

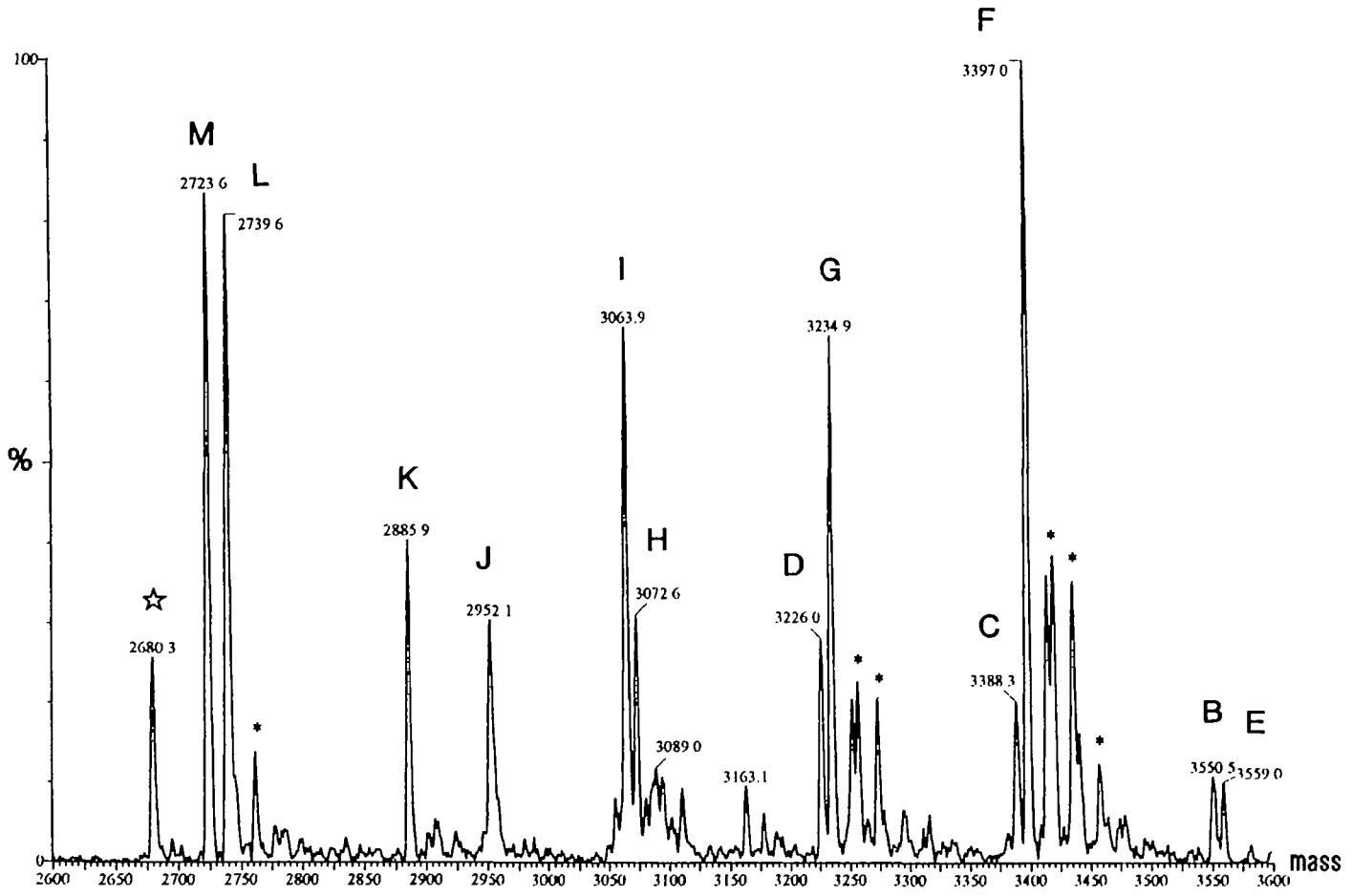


Fig. 4. Deconvoluted ES-MS spectrum of the peak II isolated from the V8 protease digestion of the rbLF expressed in *Mb.* The characteristics of the different glycoforms from Asn_{233} (B, C, D, I, K, L, M, and N) and from Asn_{545} (E, F, G, H, and J) are summarized in Table IV. *, Recalculated mass of corresponding $M+Na^+$, $M+K^+$, or $M+Na^++K^+$ ion. Star, Contaminant peptide resulting from the V8 protease digestion of rbLF.

specific for the fucosylated di-N-acetylchitobiose unit, one singlet for GlcNAc-1 at δ 2.026 ppm, and another at δ 2.092 ppm for GlcNAc-2. In summary, MS studies have demonstrated the presence of short N-glycan fucosylated structures, and ^{1}H-NMR data have proven that the fucose residues were linked to the reducing terminal N-acetylglucosamine via an α1,6-linkage.

No ^{1}H-NMR signal has been observed for the H-1 atom of the Gal or GalNAc residue probably due to the low quantity of these two monosaccharides in the glycan sample. No signal at δ 2.074 ppm, that is usually observed when the fucosylated GlcNAc-1 belongs to an hybrid or a complex type oligosaccharide is detected.

Discussion

This present report details the N-glycosylation pattern of the recombinant bLF glycoprotein expressed in the lepidopteran cell line *Mamestra brassicae* using the baculovirus as an expression vector. Preliminary studies on the native rbLF, using GC and GC-MS, allowed us to establish its monosaccharide composition. Two main monosaccharides are present on the glycoprotein: mannose and N-acetylglucosamine, associated with little amount of fucose (Table II). ^{1}H-NMR data obtained with oligosaccharides released from rbLF (Table VI), as well as monosaccharide ratio, have confirmed the presence of a mixture of various oligomannosidic structures. Furthermore, we have determined the microheterogeneity of the three isolated N-glycosylation sites Asn_{233}, Asn_{476}, and Asn_{545} by MS analysis (MALD-MS, ES-MS, and CID-MS-MS). The Asn_{233} site presents the greatest microheterogeneity since it carries eight different N-glycan structures (Table III) grouped into two families of oligomannosidic type and short-truncated N-glycans partially fucosylated ($Man_{3-2}[Fuc_{0-1}]GlcNAc_2$). Both Asn_{545} and Asn_{476} sites are substituted by five different glycoforms (Tables IV and V). The glycosylated Asn_{545} site is predominantly substituted by four oligomannosidic structures ($Man_{9-6}GlcNAc_2$) and one unique hybrid structure ($Man_4GlcNAc_3$). Besides, the Asn_{476} site carries almost exclusively short-truncated partially fucosylated N-glycans ($Man_{3-2}[Fuc_{0-1}]GlcNAc_2$), and a small proportion of one hybrid structure ($Man_3GlcNAc_3$) (see Figure 9).

The choice of bLF as a recombinant glycoprotein model to determine the glycosylation potential in *Mamestra brassicae* cells was due to the fact that the nbLF N-glycosylation sites have been extensively studied in our laboratory (Coddeville *et al.*, 1992; Spik *et al.*, 1993). On the nbLF, the two sites Asn_{233} and Asn_{545} carry exclusively oligomannosidic glycans ranging from $Man_9GlcNAc_2$ to $Man_6GlcNAc_2$ with $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ as major glycoforms (Figure 9). As in nbLF, both rbLF Asn_{233} and Asn_{545} sites are substituted by oligomannosidic glycans with predominantly $Man_8GlcNAc_2$ and

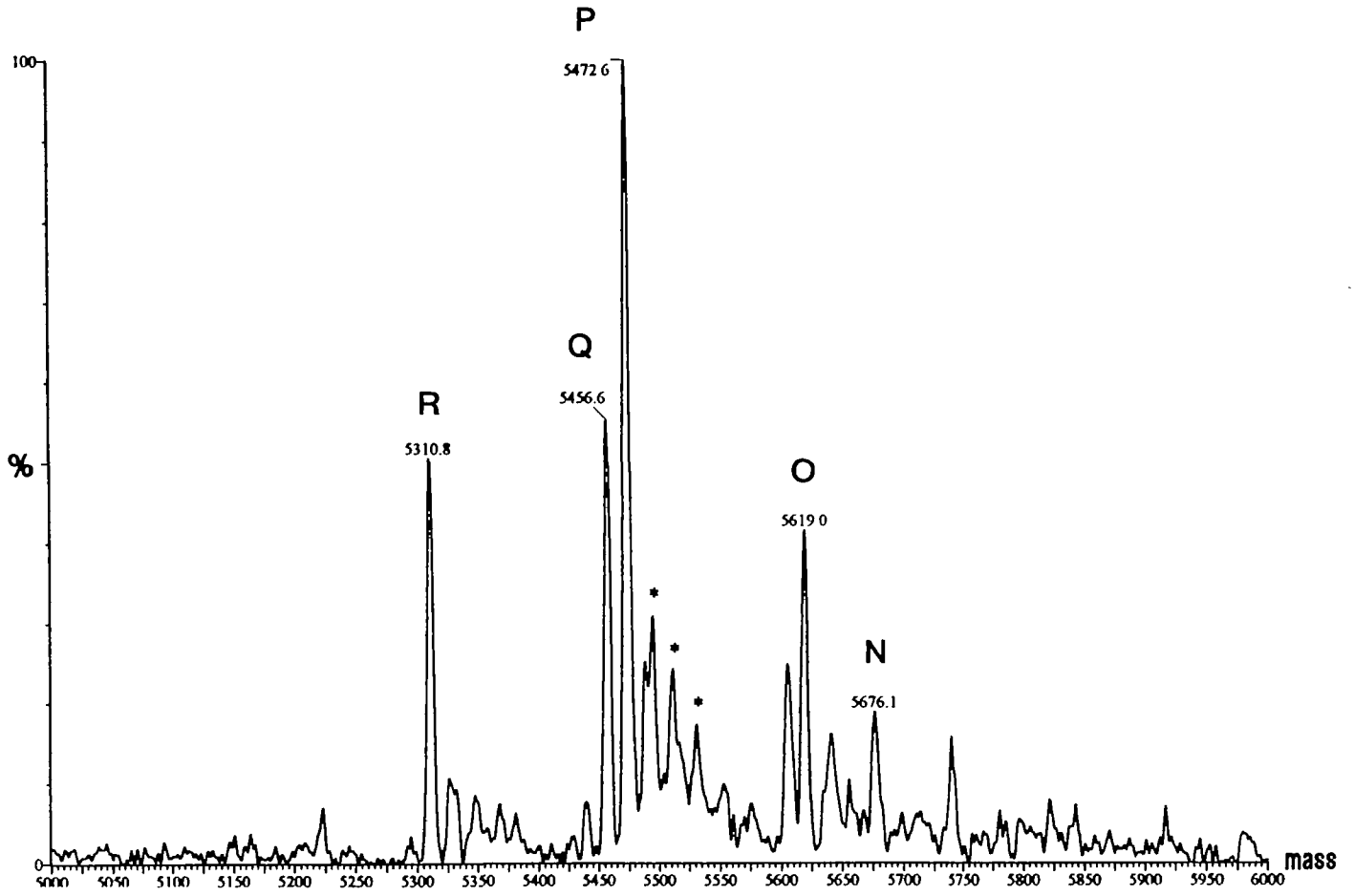


Fig. 5. Deconvoluted ES-MS spectrum of the peak III from the V8 protease digestion of the rbLF expressed in *Mb.* The characteristics of the different Asn_{545} glycoforms N, O, P, Q, and R are summarized in Table V. *, Recalculated mass of corresponding $M+Na^+$, $M+K^+$, or $M+Na^++K^+$ ion.

$Man_7GlcNAc_2$ glycoforms. In addition, we found, associated with oligomannosidic type glycans of the recombinant Asn_{233} site, short-truncated partially fucosylated N-glycans ($Man_{3-2}[Fuc_{0-1}]GlcNAc_2$). The third N-glycosylation site Asn_{476}, which carries partially sialylated complex N-glycans in the nbLF, is on the rbLF predominantly substituted by short-truncated partially fucosylated glycans. Moreover, the micro-heterogeneity is less important in the recombinant Asn_{476} site than in its natural counterpart.

In summary, each glycosylation site of rbLF produced by *Mamestra brassicae* cells is differently substituted, and it appears that this insect cell line reproduces the differences observed on the nbLF. Firstly, the site Asn_{545} appears to be processed identically in both recombinant and natural bLF. Secondly, the site Asn_{233} is substituted by oligomannosidic sugar chains as in the nbLF, but also by short truncated fucosylated structures.

Despite previous reports showing by ion exchange chromatography that the human plasminogen expressed in *Spodoptera frugiperda 21* or *Mamestra brassicae* contained complex oligosaccharides with terminal neuraminic acid (Davidson, 1990; Davidson and Castellino, 1991a,b), we found no evidence for sialylation of the N-glycans carried by the rbLF expressed in *Mamestra brassicae.* Our results show that N-glycosylation in *Mamestra brassicae* cell line aborts at different oligomannosidic stages. Although the N-glycan sites of rbLF are substituted predominantly by high mannose type oligosaccharides and short-truncated partially fucosylated N-glycans, some data lead us to think that insect cells would be able to build complex N-glycan oligosaccharides. Firstly, a significant amount of hybrid type structures $Man_{4-3}GlcNAc_3$ have been found. In vertebrate cells, the addition of an N-acetylglucosamine residue by the N-acetylglucosaminyltransferase I is the key step in the pathway leading to complex type glycans. Presence of hybrid N-glycans is in agreement with previous published work showing that insect cells contain N-acetylglucosaminyltransferase I activity (Altmann *et al.*, 1993; Velardo *et al.*, 1993). Secondly, monosaccharide residues such as galactose, galactosamine, and fucose residues, that are usually associated to complex carbohydrates have been identified by CG and CG-MS.

Fucosylated glycan structures in rbLF are exclusively short truncated structures ($Man_{3-2}[Fuc_{0-1}]GlcNAc_2$), mainly found on the Asn_{476} site which shows complex N-glycans in nbLF (Figure 9). ^{1}H-NMR spectroscopy data conducted onto rbLF fucosylated structures, have demonstrated that the fucose residue is α1,6-linked to the reducing terminal N-acetylglucosamine. In mammalian cells, the α1,6-fucosyltransferase acceptor substrate is a complex N-glycan with two terminal N-acetylglucosamine residues resulting from the action of N-acetylglucosaminyltransferases I and II at the nonreducing end of the oligosaccharidic chain (Kornfeld and Kornfeld, 1985). Assuming that the N-glycosylation pathway in insect cells is identical to the mammalian pathway and that the insect α1,6-fucosyltransferase exhibits the same acceptor substrate

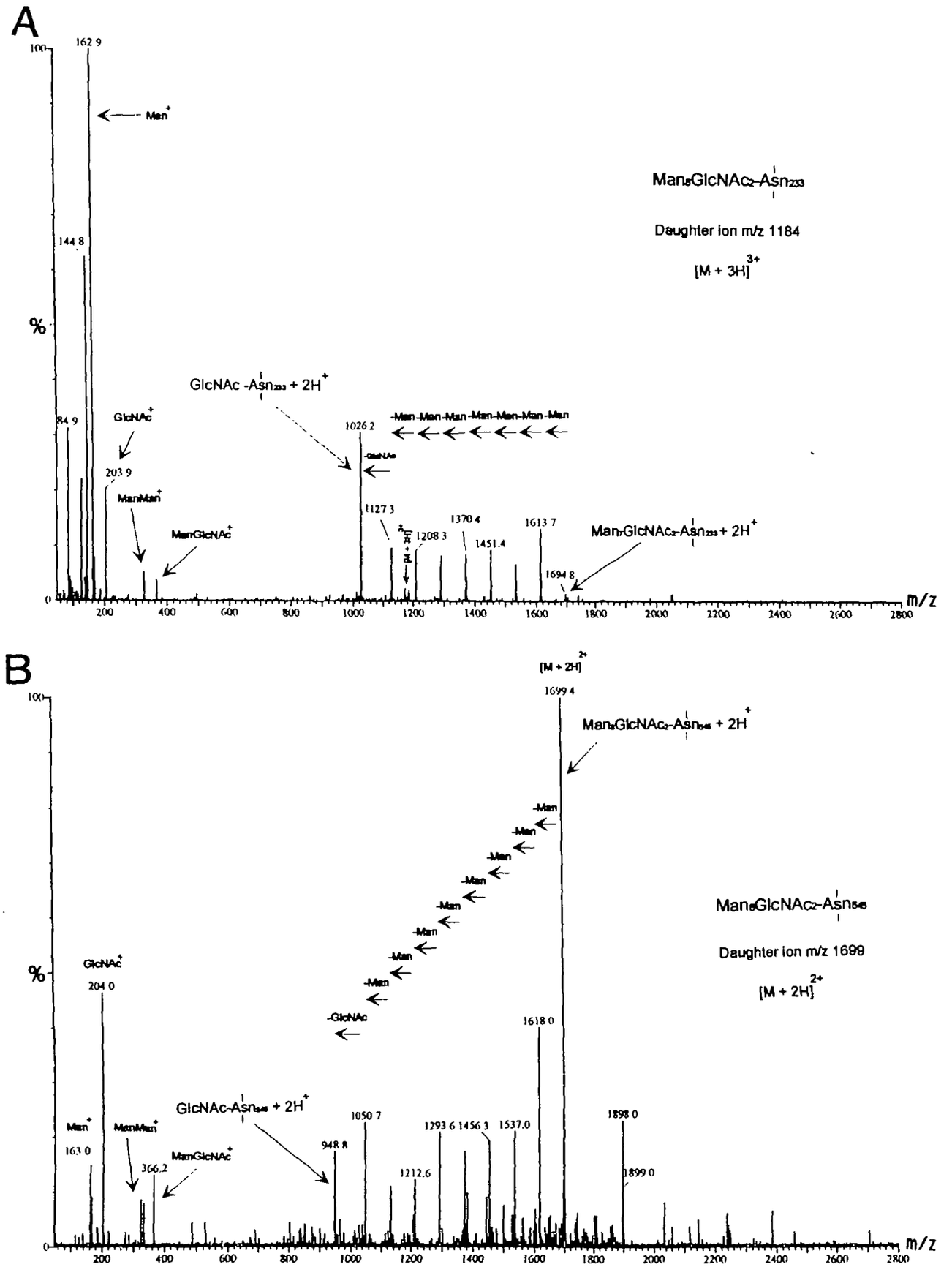


Fig. 6. Full-scan CID-MS-MS spectra of glycopeptides from the V8 protease digestion of the rbLF. (A) CID-MS-MS spectrum of the triply charged precursor ion at m/z 1184 for the Asn_{233} glycopeptide of 3550.4 Da (peak I/glycoform B). (B) CID-MS-MS spectrum of the doubly charged precursor ion at m/z 1699 for the Asn_{545} glycopeptide of 3397.0 Da (peak II/glycoform F).

Table IV. Analysis of ES-MS spectrum presented in Figure 4 of the peak II isolated from the V8 protease digestion of the rbLF expressed in *Mb*

Glycosylation site and calculated peptide mass ($M_{cal.}$)	Glycoform	Calculated mass (average)	Observed mass	%[a]	Glycan mass[b]	Glycan assignment
Asn_{233} ($M_{cal.}$ 1847.1)	B	3550.5	3550.5	C.I[c]	1704.4	$Hex_8HexNAc_2$
	C	3388.3	3388.3	C.I	1542.2	$Hex_7HexNAc_2$
	D	3226.2	3226.0	9	1379.8	$Hex_6HexNAc_2$
	I	3064.1	3063.9	24	1217.8	$Hex_5HexNAc_2$
	K	2885.9	2885.9	13	1039.8	$Fuc_1Hex_3HexNAc_2$
	L	2739.8	2739.6	28	893.5	$Hex_3HexNAc_2$
	M	2723.8	2723.6	26	877.5	$Fuc_1Hex_2HexNAc_2$
Asn_{545} ($M_{cal.}$ 1693.8)	E	3559.6	3559.0	3	1866.2	$Hex_9HexNAc_2$
	F	3397.5	3397.0	49	1704.2	$Hex_8HexNAc_2$
	G	3235.3	3234.9	27	1542.1	$Hex_7HexNAc_2$
	H	3073.2	3072.6	12	1379.8	$Hex_6HexNAc_2$
	J	2952.1	2952.1	9	1259.3	$Hex_4HexNAc_3$

[a]Percentage of Asn_{233} and Asn_{545} glycoforms present in peak II.
[b]Glycan mass = glycopeptide mass observed – (peptide mass – 1).
[c]Contaminating glycoforms from peak I.

specificity, the insect short truncated fucosylated structures would originate also from complex N-glycans, at least $GlcNAc_2Man_3[Fuc_1]GlcNAc_2$, whose terminal residues would have been removed by hexosaminidases and α-mannosidases. Accordingly, the recombinant Asn_{476} site would be substituted by complex glycans, as in the natural protein, but in the insect cell these complex structures would be degraded. Several reports support this hypothesis: (1) the presence of different hexoglycosidases and, particularly, of β-N-acetylglucosaminidase in insect cell lines (Licari *et al.*, 1993; Altmann *et al.*, 1995) and, (2) the presence in *Mamestra brassicae* cells of both α1,3- and α1,6-fucosyltransferase activities which used $GlcNAc_2Man_3GlcNAc_2$ as acceptor substrate (Staudacher *et al.*, 1992b).

Additional structural information on the insect glycans can be deduced from the ^{1}H-NMR data in conjunction with the MS data. Considering that the ES-MS glycopeptide signal is mainly due to the peptide fraction (Tables III, IV, V), we have correlated for each glycosylation site the height of each centered ES-MS signal with the relative proportion of the corresponding glycoform. In addition, it was possible to establish a correlation between the relative intensity of the 1D H-1 signals of each monosaccharide residue and their molar ratio. Our ^{1}H-NMR data show that the most represented oligosaccharides possess substituted Man-B or terminal Man-D_1 and Man-D_3, but also that most of them have terminal Man-4 or Man-4'. Thus, these two approaches allowed us to determine the most represented isomers among the two glycan families: high-mannose type and short truncated glycans (Figure 10). Concerning the high-mannose type family, there is a small proportion of the unique possible isomer of $Man_9GlcNAc_2$ (Figure 10, #1), the predominant high-mannose glycans being of the $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ type. Among the three possible isomers of $Man_8GlcNAc_2$, the main form has a non substituted Man-A (Figure 10, #3). The presence of this $Man_8GlcNAc_2$ isomer, which arises from the N-glycan biosynthesis pathway in mammalian cells (Kornfeld and Kornfeld, 1985), is in accordance with the ^{1}H-NMR data showing that the associated signal of the Man-D_1 and Man-D_3 is stronger than the Man-D_2 signal. However, the occurrence of the two other isomers $Man_8GlcNAc_2$ (Figure 10, #2 and #4) cannot be completely excluded. The signal of the nonsubstituted Man-B is in favor of the presence of the $Man_8GlcNAc_2$ isomer (Figure 10, #2), which arises from the lysosomal catabolism of oligomannosidic oligosaccharides (for review, see Michalski, 1996). In contrast, among the six different possible isomers of the $Man_7GlcNAc_2$ structure, no predominant form is found. Since terminals Man-A and Man-B give a weak signal in ^{1}H-NMR, the $Man_7GlcNAc_2$ isomer lacking Man-D_2 and Man-D_3 is present in the mixture (Figure 10, #5), although the five other isomers may be present (not represented in Figure 10). Finally, $Man_6GlcNAc_2$ and $Man_5GlcNAc_2$ structures (Figure 10, #6 and #7) could originate from the N-glycan biosynthesis pathway but also to a lesser extent from the lysosomal catabolism pathway.

The pool of short truncated glycans $Man_{3-2}[Fuc_{0-1}]GlcNAc_2$ seems to arise from the catabolism of the N-glycans (Michalski, 1996). The nonfucosylated trimannosyl core $Man_3GlcNAc_2$ comes from the catabolism of high-mannose type structures, as well as from the catabolism of complex type

Table V. Analysis of ES-MS spectrum presented in Figure 5 of the peak III isolated from the V8 protease digestion of the rbLF expressed in *Mb*

Glycosylation site and calculated peptide mass ($M_{cal.}$)	Glycoform	Calculated mass (average)	Observed mass	%[a]	Glycan mass[b]	Glycan assignment
Asn_{476} ($M_{cal.}$ 4580.1)	N	5676.0	5676.1	6	1097.0	$Hex_3HexNAc_3$
	O	5619.0	5619.0	13	1039.9	$Fuc_1Hex_3HexNAc_2$
	P	5472.8	5472.6	41	893.5	$Hex_3HexNAc_2$
	Q	5456.8	5456.6	19	877.5	$Fuc_1Hex_2HexNAc_2$
	R	5310.7	5310.8	21	731.7	$Hex_2HexNAc_2$

[a]Percentage of Asn_{476} glycoforms present in peak III.
[b]Glycan mass = glycopeptide mass observed – (peptide mass – 1).

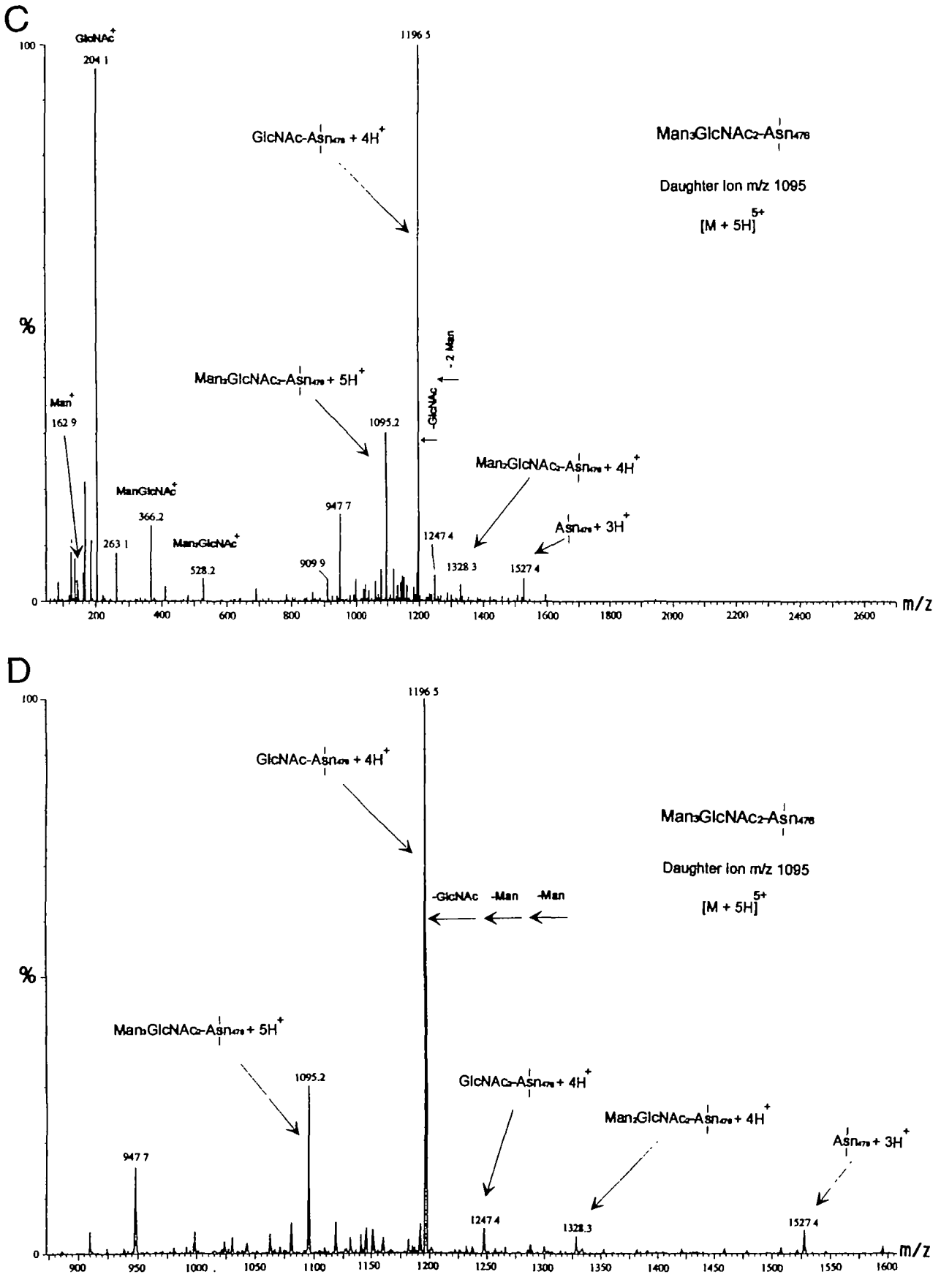


Fig. 7. CID-MS-MS spectra of glycopeptides from the V8 protease digestion of the rbLF. (C) Full-scan CID-MS-MS spectrum of the fifthly charged precursor ion at m/z 1095 for the Asn_{476} glycopeptide of 5472.6 Da (peak III/glycoform Q). (D) Enlargement of the previous CID-MS-MS spectrum around the region of m/z 1196.5 that corresponds to GlcNAc-Asn + $4H^+$.

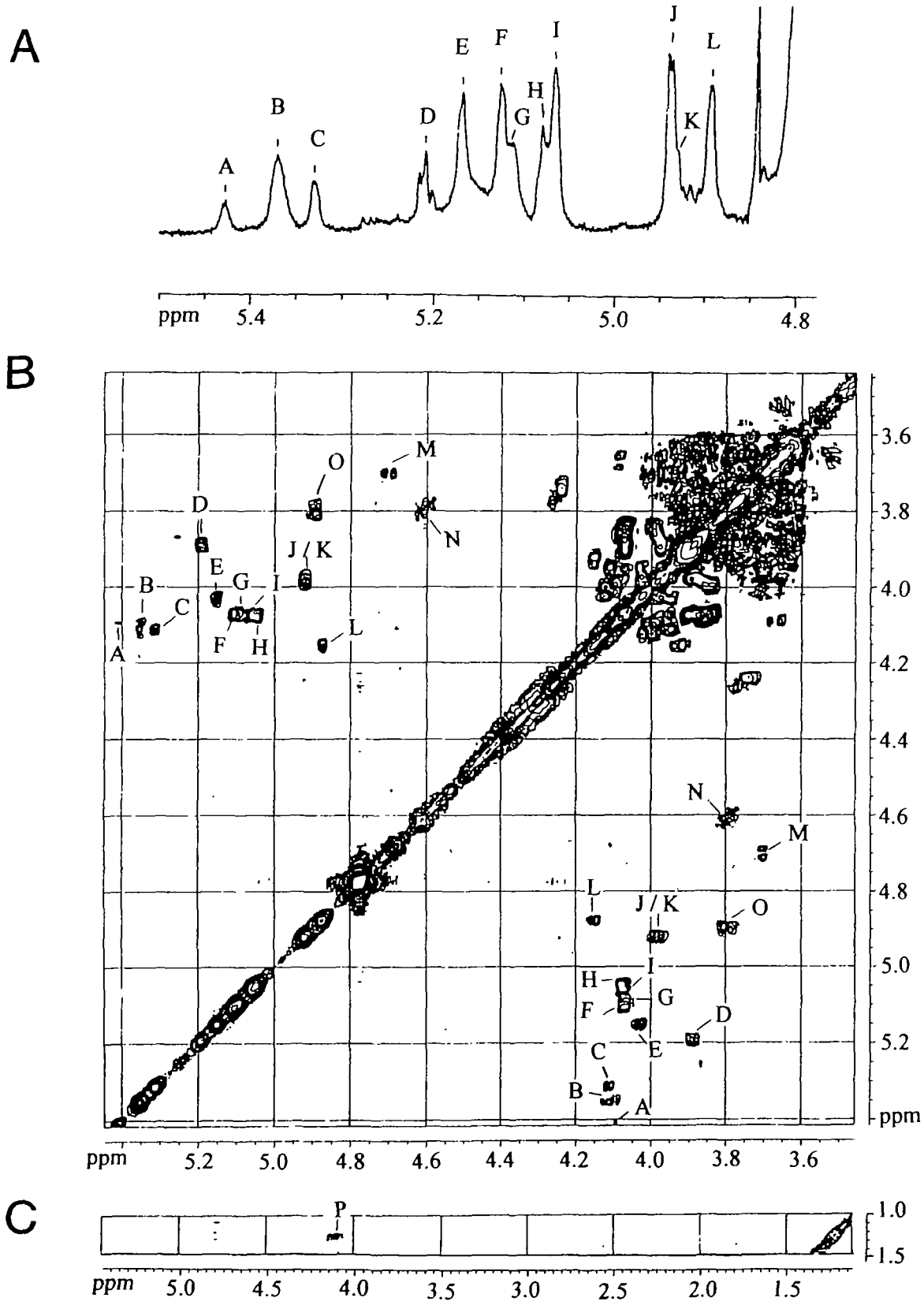


Fig. 8. ^{1}H-NMR spectra of the oligosaccharides released by hydrazynolysis from rbLF. (A) 1D spectrum focusing on the anomeric zone (4.8–5.4 ppm); (B) COSY spectrum (3.5 × 5.4 ppm); (C) COSY spectrum (1.0 × 1.5 to 1.0 × 5.5 ppm).

Table VI. ^{1}H-NMR chemical shifts in structural reporter-group of constituent monosaccharides of oligosaccharides released by hydrazinolysis from rbLF

Reporter group		Residue		Chemical shifts (ppm)		Relative signal[b] intensity
				Observed	Literature data[a]	
H-1	A	Nonterminal	Man-A	5.402	5.401	*
	B	Nonterminal	Man-4	5.346	5.345	**
	C	Nonterminal	Man-C	5.306	5.304/5.308	**
	E	Nonterminal	Man-B	5.144	5.143/5.145	***
	F	Terminal	Man-4	5.102	5.102	***
	G	Terminal	Man-A	5.092	5.09	**
	H	Terminal	Man-D_2/C	5.056	5.059	**
	I	Terminal	Man-D_1/D_3	5.042	5.042	***
	J	Terminal	Man-4′	4.915	4.915	***
	K	Terminal	Man-B	4.907	4.908	*
	L	Nonterminal	Man-4′	4.868	4.868	***
	D		GlcNAc1α	5.190	n.a.[c]	*
				5.186		
	M		β	4.700	n.a.	
	N	Nonterminal	GlcNAc-2	4.625	4.615/4.621	
				4.607	4.608	
	O	Linked to GlcNAc-1	α1-6 Fuc	4.894	4.893/4.895	*
H-2	A	Nonterminal	Man-A	4.09/10	4.10	
	B	Nonterminal	Man-4	4.106	4.10	
	C	Nonterminal	Man-C	4.11	4.10	
	E	Nonterminal	Man-B	4.030	4.02	
	F	Terminal	Man-4	4.077	4.077	
	G	Terminal	Man-A	4.07	4.07	
	H	Terminal	Man-D_2/C	4.07	4.07	
	I	Terminal	Man-D_1 /D_3	4.07	4.07	
	J/K	Terminal	Man-4′/B	3.983	3.968/3.988	
	L	Nonterminal	Man-4′	4.152	4.15	
	D		GlcNAc-1α	3.884	n.a.	
	M		β	3.698	n.a.	
	N		GlcNAc-2	3.805	n.a.	
				3.787	3.79	
	O	Linked to GlcNAc-1	α1-6 Fuc	3.792	n.a.	
H-5	P	Linked to GlcNAc-1	α1-6 Fuc α[d]	4.099	4.096/4.101	
H-6	P	Linked to GlcNAc-1	α1-6 Fuc α[d]	1.210	1.210	
			β[d]	1.220	1.220	
NAc			GlcNAc-1	2.007	2.007	***
	αFuc(1-6) linked to GlcNAc-1		GlcNAc-1	2.026	2.023	***
			GlcNAc-2	2.053	2.054	***
			GlcNAc-2	2.064	2.066	**
	αFuc(1-6) linked to GlcNAc-1		GlcNAc-2	2.092	2.094	*

[a]Vliegenthart *et al.*, 1983.
[b]Relative signal intensity: *, low; **, medium; ***, strong.
[c]Nonavailable.
[d]Anomerization effect.

glycans. However, the presence of a fucose residue in fucosylated structures $Man_{3-2}[Fuc]GlcNAc_2$ shows that these structures proceed from the lysosomal catabolism of complex oligosaccharides. The presence of trimannosidic structures $Man_3[Fuc_{0-1}]GlcNAc_2$ (Figure 10, #8 and #9) are responsible for the terminal Man-4′ and Man-4 ^{1}H-NMR strong signals. According to the fact that the Man-4′ signal is stronger than the Man-4 signal, we can propose that the $Man_2[Fuc_{0-1}]GlcNAc_2$ (Figure 10, #10 and #11) derived from a classical catabolism pathway (Haeuw *et al.*, 1994). However, the normal lysosomal catabolytic pathway of glycoproteins is initiated with the segregation of the intact glycan and the polypeptide moiety through the action of two endoglycosidases (Michalski, 1996). The presence of short truncated $Man_{3-2}[Fuc_{0-1}]GlcNAc_2$ structures in rbLF seems to indicate that insect cell glycosidases are able to degradate the glycan moiety of recombinant glycoproteins without the previous liberation of the oligosaccharides.

The baculovirus expression system takes advantage of the insect cell glycosylation machinery, and thus the glycosylation patterns of the expressed glycoproteins are determined to a large extent by the host. It appears that the insect glycosylation machinery can be disturbed after the baculovirus infection by the late and very high level of production of the recombinant protein under control of the p10 promoter in the insect/baculovirus system. In fact, such an overproduction causes the saturation of the secretory pathway and/or the overstepping of the catalytic activities of the different glycosyltransferases. Moreover, the lysis phenomena occurring in the insect/baculovirus system could be responsible for a decrease of the microheterogeneity of "complex" oligosaccharide structures, and for an increase of the microheterogeneity of the N-glycosylation sites substituted by high-mannose structures, both phenomena are due to the release in the culture medium of glycosidases (Licari, 1993). During the production of a recombinant glycoprotein in insect/baculovirus system there is a balance between N-glycan biosynthesis pathway and the lysosomal catabolism of these oligosaccharides. From this point of view, the present work focused on the potential of glycosyla-

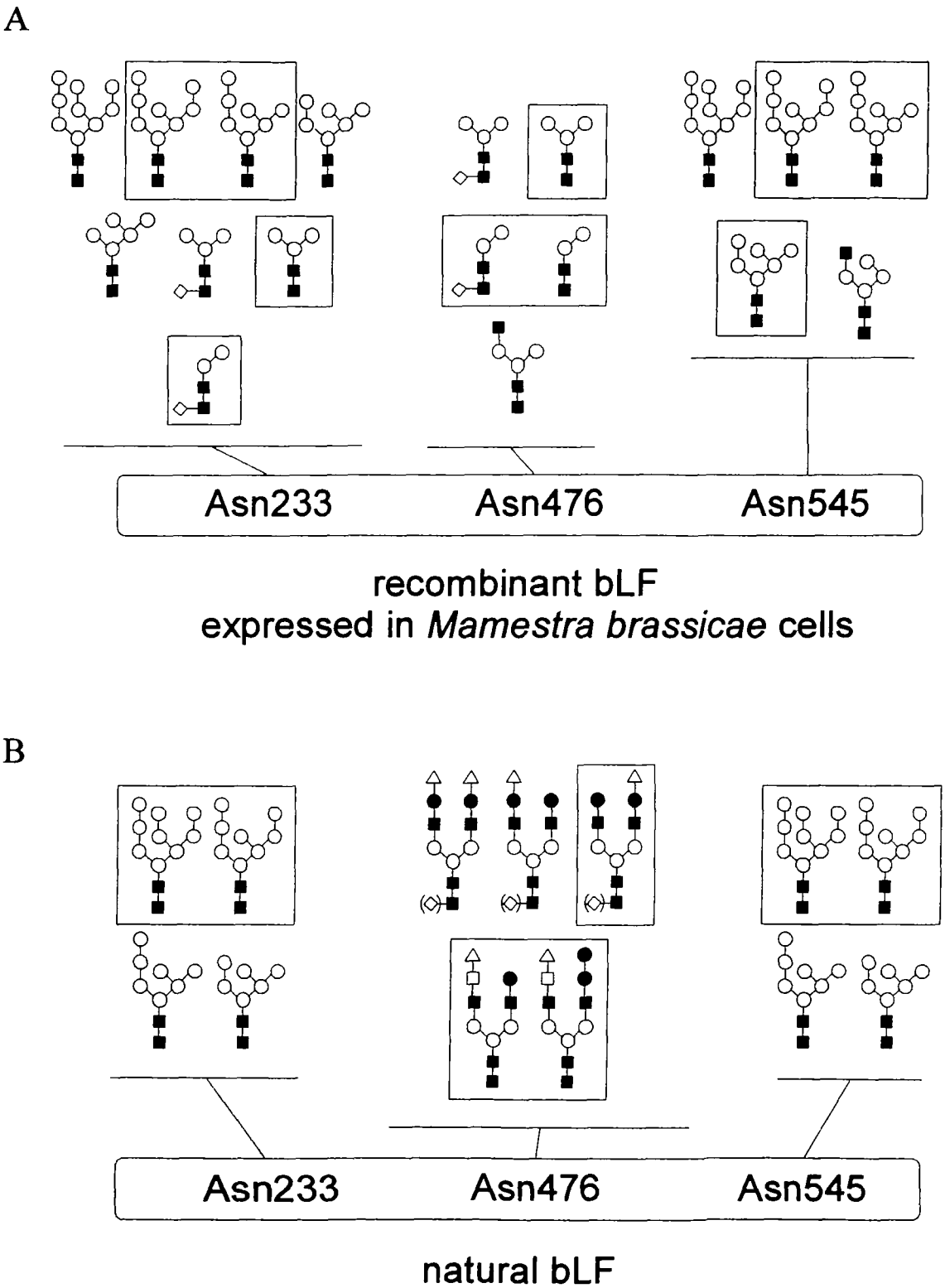
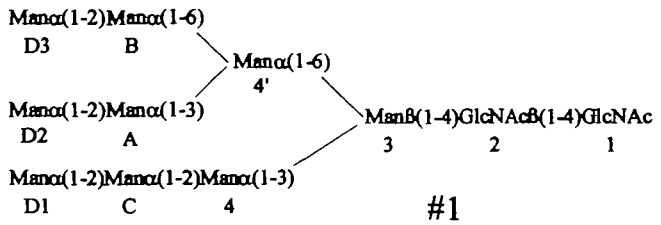
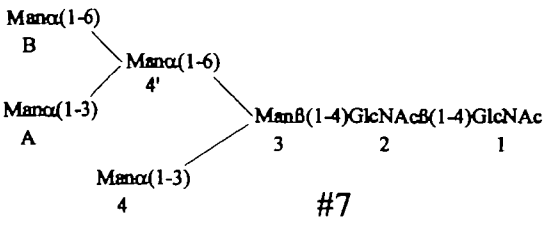
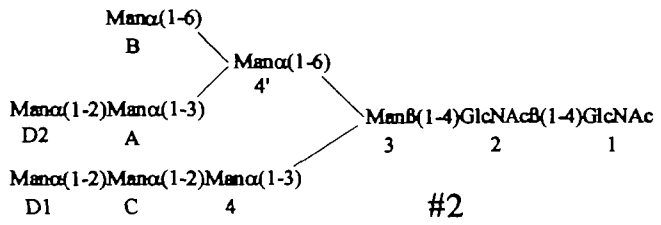
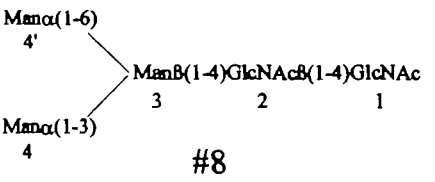


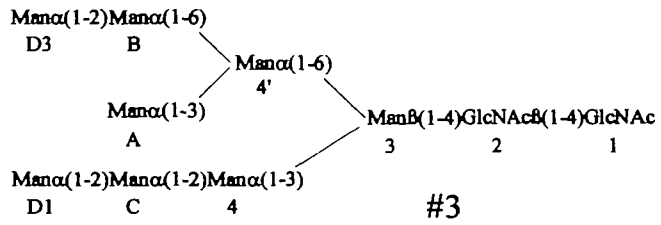
Fig. 9. Schematic representation of the main oligosaccharide structures associated with Asn_{233}, Asn_{476} and Asn_{233} glycosylation sites of (A) rbLF expressed in *Mamestra brassicae* cell line, and (B) nbLF (data compiled from Coddeville *et al.*, 1992, and Spik *et al.*, 1993). ■, GlcNAc; □, GalNAc; ○, Man; ●, Gal; ◇, Fuc; ▲, NeuAc. The monosaccharide nomenclature used for glycan structures is according to Vliegenthart *et al.* (1983). The most represented oligosaccharide structures are squared.

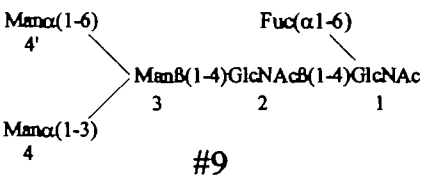


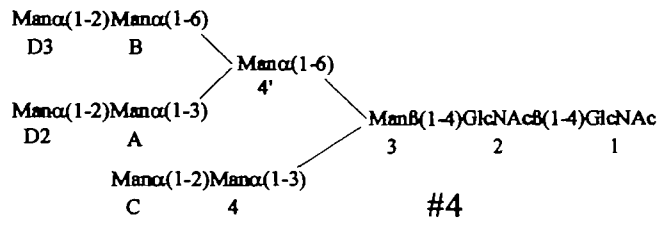


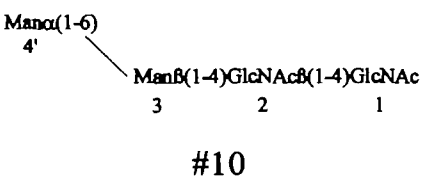


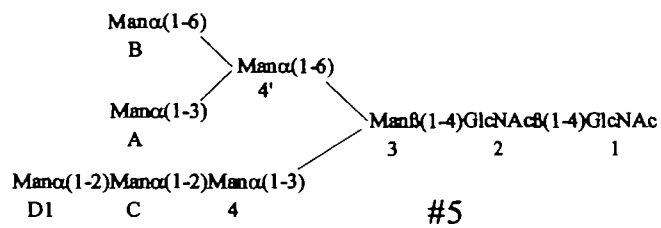


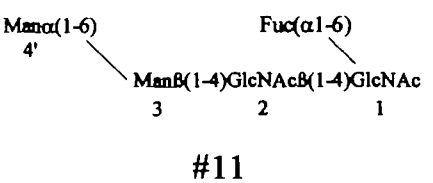












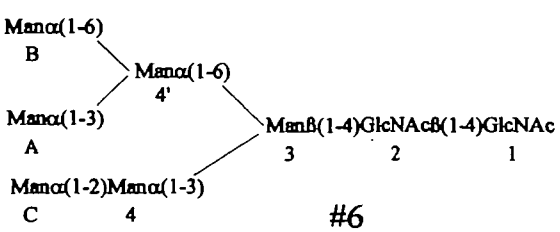


Fig. 10. Proposed structures for the oligosaccharides released by hydrazinolysis from rbLF as deduced from the ^{1}H-NMR data. GlcNAc, N-acetylglucosamine; Man, mannose; Fuc, fucose. The monosaccharide nomenclature used for glycan structures is according to Vliegenthart *et al.* (1983).

tion of a baculovirus infected insect cell line rather than on the potential of glycosylation of the *Mamestra brassicae* cells itself.

Materials and methods

Insect cells culture

The *Mamestra brassicae* clone SPCMb-92-C6 was established by J. M. Quiot at the Station de Pathologie Comparée INRA/CNRS URA n° 2209 (unpublished observations). The cell line was adapted, maintained, and cultivated at 28°C in a serum-free medium developed by J. M. Quiot at the Station de Pathologie Comparée INRA/CNRS URA n° 2209 (unpublished observations).

Recombinant baculovirus construction

cDNA sequence encoding the bLF (Pierce *et al.*, 1991) was recovered from pBSK-bLF plasmid as a Not-I fragment containing the cDNA including the whole upstream leader sequence. The bLF was inserted in the p10 locus of a baculovirus transfer vector termed pGm16 plasmid (Blanc *et al.*, 1993) initially modified by insertion of a NotI adapter at the original BglII site located downstream of the p10 promoter. *Spodoptera frugiperda* 9 cells (ATCC CRL 1711) were cotransfected with pGm16-bLF and genomic DNA of AcSLP10 baculovirus (Chaabihi *et al.*, 1993). AcSLP10 is a modified baculovirus that has only one strong late promoter p10, with the polyhedrin coding sequence inserted downstream of this promoter. The lipofection method (Felgner and Ringold, 1989) was used for this cotransfection, using the DOTAP reagent (Boehringer Mannheim, Germany). Screening and purification of recombinant baculoviruses were carried out by plaque assay as described by Summers and Smith (1987). A selected recombinant virus expressing the bLF gene under the control of p10 promoter termed AcSLP10-bLF was then amplified (5×10^7 pfu/ml) in *Mamestra brassicae* cell line cultivated in the serum-free medium developed by J. M. Quiot.

Expression of rbLF

Mamestra brassicae cells were cultivated in 15 liters of BM25 medium at 28°C using a BIOLAFITTE bioreactor. When cell density reached 0.75×10^6 cells per ml, infection with AcSLP10-bLF was performed at a multiplicity of infection equal to 5. Cell viability was estimated to be 96% by trypan blue exclusion method prior infection. The 5 days postinfection supernatant containing the rbLF was clarified by a 10 min centrifugation at $3000 \times g$ and stored at 4°C.

Purification of rbLF

Isolation of the rbLF from the culture supernatant was performed by anion exchange chromatography onto a SP-Sephadex C-50 column as described by Chéron *et al.* (1977).

During the purification procedure, rbLF was monitored by 8% SDS–PAGE (Laemmli, 1970), Western blotting, and immunodetection using a specific polyclonal antibody anti-bLF obtained in our laboratory after immunization of a rabbit by nbLF. The purity of the final rbLF pool was assessed by reversed-phase HPLC on C_{18} stationary phase and by 8% SDS–PAGE stained by Coomassie blue. The molecular weights were estimated according to high range prestained SDS–PAGE standard (Bio-Rad Laboratories).

Enzymatic digestions

PNGase F deglycosylation. Twenty micrograms of rbLF and nbLF was digested with 4000 units of recombinant peptide: N-glycosidase F (PNGase F) according to the manufacturer's procedure (Biolabs, USA). The PNGase F cleaves between the innermost N-acetylglucosamine and asparagine residues of oligomannosidic, hybrid and complex oligosaccharides from glycopeptides. In addition, the PNGase F cleavage transforms the asparagine-glycan-linked residues on aspartic residues.

V8 protease digestion. Fifteen milligrams of rbLF was reduced with dithiothreitol in 8 M urea and alkylated with iodoacetamide (Crestfield *et al.*, 1963), desalted on Biogel P2 column (1.2 cm × 50 cm) equilibrated in 50 mM ammonium acetate, pH 4, buffer and concentrated to 1 ml by using a Centriprep-30 concentrator containing an Amicon YM membrane (molecular weight cut-off 30 kDa). Under such buffer conditions, the endoproteinase Glu-C from *Staphylococcus aureus* V8 (V8 protease, Boehringer Mannheim, Germany) is known to hydrolyze only Glu-Xaa bonds (Houmard and Drapeau, 1972). Then, protein solution was digested at 37°C for 24 h by addition of 0.15 mg of V8 protease at t = 0 and at t = 12 h giving a final protease/rbLF ratio of 1/50 (w/w). Protease digestion was stopped by storing the hydrolysate at −20°C.

HPLC separation of proteolytic digests and peptide sequence analysis

Peptides generated from V8-digested rbLF were separated by reversed-phase HPLC on a Beckman C_{18} ultrasphere ODS 5 μm (4.6 × 250 mm) column eluted with a linear gradient of acetonitrile from 0 to 100% of 80% acetonitrile in 0.1% TFA for 120 min at a flow rate of 1 ml/min. Elution was monitored at 215 nm and peaks were collected, lyophilized, and stored at −20°C.

Sequencing of peptides was carried out on the gas-phase sequencer (Applied Biosystems 470A) using the 03RPTH program. Phenylthiohydantoin derivatives of amino acids were identified with an on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems 120A).

Methanolysis and trimethylsilylation

Two nanomoles of nbLF, rbLF, and each purified glycopeptide were lyophilized in a screw-cap glass tube, rapidly solubilized in 500 μl of 0.5 M HCl in anhydrous methanol and heated at 80°C for 24 h. After cooling, the solution was neutralized by addition of Ag_2CO_3 until pH 6–7 and the mixture was N-reacetylated for one night at room temperature after addition of 10 μl of acetic anhydride (Zanetta *et al.*, 1972). Then, the supernatant was dried under nitrogen flow, and the monosaccharides were derivatized using 20 μl of bis-(methylsilyl)trifluoroacetamide in the presence of 20 μl of pyridine for 2 h at room temperature (Kamerling *et al.*, 1975). Methyl trimethylsilylglycosides were analyzed by GC and GC-MS.

Gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS)

GC separation of pertrimethylsilylated methyl sugars was performed on a fused silica capillary column from SGE (0.32 mm × 25 m) using helium as carrier gas at pressure of 0.5 bar with the following temperature program (120–240°C at 2°C/min). A Varian 3400 gas chromatograph was equipped with a flame-ionization detector, while for the GC-MS analyses a Delsi DI 700 gas chromatograph was coupled with a Nermag R10–10 mass spectrometer (Rueil Malmaison, France). MS analyses were carried out in chemical ionization (C_I) mode using ammonia as reactant gas.

Matrix-assisted laser desorption mass spectrometry (MALD-MS)

MALDI mass spectra were measured on a reflectron-type Vision 2000 time-of-flight mass spectrometer (Finnigan MAT, Bremen, Germany). Samples were mounted on an x–y moveable stage allowing irradiation of selected sample areas. A nitrogen laser with an emission wavelength of 337 nm and 3 ns pulse duration was used. Spectra were recorded in the positive-ion mode and accelerated to an energy of 5 KeV before entering the flight tube. Ions were post-accelerated for detection to an energy of 5 KeV.

Samples were prepared by mixing directly on the target 1 μl of the glycopeptide solution (typically 20 pmol) and 1 μl of a 2,5 dihydroxybenzoic acid (DHB) matrix solution (12 mg/ml in CH_3OH/H_2O, 70:30). The samples were allowed to dry for 3–4 min at room temperature; 20–40 shots were accumulated for each mass spectrum.

Electrospray mass spectrometry (ES-MS and by CID-MS-MS)

Electrospray mass spectra were acquired on Micromass Quattro II triple quadripole mass spectrometer operating with an API ion source in positive ion electrospray mode. Glycopeptides samples were diluted into acetonitrile/0.2% formic acid (1:1, by volume), to a concentration of 20 pmol/μl and infused at 8 μl/min.

Mass spectra were acquired by scanning MS1 over the appropriate mass range, whilst MS-MS analyses were performed by transmitting the appropriate precursor ion through MS1 to the collision cell. The collision gas used was argon at a pressure of 4.9×10^{-3} mbar with an appropriate collision energy (between 25 eV and 45 eV). Product ions were scanned with MS2.

^{1}H-NMR spectroscopy

Oligosaccharides released and isolated from 31 mg of rbLF, according to Parekh *et al.* (1990) and Patel *et al.* (1993), were submitted to ^{1}H-NMR. Spectra of the oligosaccharide pool solution (pD = 7) in D_2O (99.95% atom D; from C.E.A., Saclay, France) (Vliegenthart, 1983) were recorded on a Brüker ASX-400-WB spectrometer operating in the pulsed Fourier-transform mode and equipped with a Brüker Aspect 3000 computer at a probe tempera-

ture of 300°K. All the data are given relative to internal acetone (δ = 2.225 ppm) with an accuracy of 0.002 ppm. The two-dimensional (2D) homonuclear COSY experiments were performed with the standard Brüker pulse sequence.

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Abbreviations

bLF, bovine lactoferrin; CID-MS-MS, collision-induced dissociation tandem mass spectrometry; 1D, one-dimensional; 2D, two-dimensional; Dol, dolichol; ES-MS, electrospray mass spectrometry; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GC, gas chromatography; GC-MS, gas chromatography–mass spectrometry; GlcNAc, N-acetylglucosamine; Hex, hexose; HexNAc, hexosamine; ^{1}H-NMR, proton nuclear magnetic resonance; HPLC, high performance liquid chromatography; MALD-MS, matrix-assisted laser desorption mass spectrometry; Man, mannose; *Mb, Mamestra brassicae;* nbLF, natural bovine lactoferrin; pfu, plaque-forming units; PNGase F, peptide:N-glycosidase F; rbLF, recombinant bovine lactoferrin; RER, rough endoplasmic reticulum.

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