# Use of Mass Spectrometry To Rapidly Characterize the Heterogeneity of Bovine α-Lactalbumin

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From a bovine whey protein fraction the nonglycosylated and glycosylated α-lactalbumin fractions were isolated by gel-permeation chromatography followed by reversed-phase high-performance liquid chromatography. Both fractions were studied by electrospray-ionization mass spectrometry (ESI-MS). For the nonglycosylated fraction, a mass of 14 178 Da was found, which was in accordance with the known amino acid sequence of the protein. The glycosylated fraction appeared to be a mixture of components with mass values ranging from ca. 15 840 to 16 690 Da. Given the published carbohydrate composition of the whole glyco-α-lactalbumin fraction, these masses could be matched to those of 14 differently glycosylated forms of α-lactalbumin. Further support for these forms was obtained from the results of a separate mass spectrometric analysis of the mixture of oligosaccharides released from the protein by treatment with peptide-$N^4$-(*N*-acetyl-*β*-glucosaminyl)asparagine amidase F. ESI-MS was found to be a powerful tool to gain insight into the composition of the complex mixture of glycoforms of α-lactalbumin without the need of further purification of these forms or of the oligosaccharides released thereof.

**Keywords:** *α-Lactalbumin; (de-)glycosylation; size-exclusion chromatography; reversed-phase HPLC; mass spectrometry*

## INTRODUCTION

α-Lactalbumin, a major whey protein in milk of various species, plays an important role in the biosynthesis of lactose (Brodbeck and Ebner, 1966; Ebner et al., 1966; Brew and Grobler, 1992). On the basis of the most recent sequence data (Wang et al., 1989), the molecular mass of bovine α-lactalbumin is 14 178 Da. However, in fresh, nonprocessed milk, a small part of the native protein occurs in the glycosylated form (glyco-α-lactalbumin) (Barman, 1970). By monosaccharide analysis of glyco-α-lactalbumin, varying amounts of *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), mannose (Man), galactose (Gal), fucose (Fuc), and *N*-acetylneuraminic acid (NeuAc) were found, the quantities of Gal, Fuc, and NeuAc being relatively low (Barman, 1970; Hindle and Wheelock, 1971). In bovine α-lactalbumin, Asn-45 was reported to be the single point of attachment of carbohydrate groups (Hopper and McKenzie, 1973; Hill and Brew, 1975), although according to the usual consensus sequence for *N*-linked carbohydrate chains (Asn-X-Thr/Ser) one more potential glycosylation site is present at Asn-74. It is noteworthy that the carbohydrate moiety does not seem to be involved in the role of α-lactalbumin in lactose biosynthesis, since the glycosylated and nonglycosylated forms are equally active as lactose synthase specifier protein (Barman, 1970).

Hopper and McKenzie (1973) found that glyco- and nonglyco-α-lactalbumin forms could be separated by Sephadex G-75 chromatography. Further purification by DEAE ion-exchange chromatography resulted in two electrophoretically distinguishable glyco-α-lactalbumin fractions ($S_1$ and $S_2$) and a main and minor component (M and F) which both were nonglycosylated.

No further data on the structure of the carbohydrate moiety of glyco-α-lactalbumin were reported until the publication of a symposium abstract by Tilley et al. (1991). In this note not only were the carbohydrate composition and single glycosylation site of glyco-α-lactalbumin confirmed, but also the oligosaccharide structure of a main glycoform of α-lactalbumin was shown.

For the determination of one or more glycosylation sites and the detailed structure of attached carbohydrate chains it is usual to proteolytically digest a glycoprotein and fractionate the digest to fragments each containing only one glycosylated position. The glycopeptides obtained or the oligosaccharides released thereof are purified and then analyzed by, for instance, NMR spectrometry (Vliegenthart et al., 1983, 1991; van Halbeek, 1989; Vliegenthart and Montreuil, 1995). Recent developments in mass spectrometric techniques have opened new ways into the study of glycoproteins (Vliegenthart and Montreuil, 1995; Rademaker and Thomas-Oates, 1996; Harvey, 1996; Settineri and Burlingame, 1996; Green et al., 1996).

In the present paper we describe the isolation and purification of the nonglycosylated and glycosylated bovine α-lactalbumin fractions as well as their characterization by electrospray-ionization mass spectrometry (ESI-MS). On the basis of our results and the published carbohydrate composition of total glyco-α-lactalbumin, the compositions of 14 glycoforms of α-lactalbumin are postulated. The presence of these glycoforms is supported by ESI-MS results obtained with the whole mixture of oligosaccharides enzymatically released from the protein chain by peptide-$N^4$-(*N*-acetyl-*β*-glucosaminyl)asparagine amidase (PNGase F).

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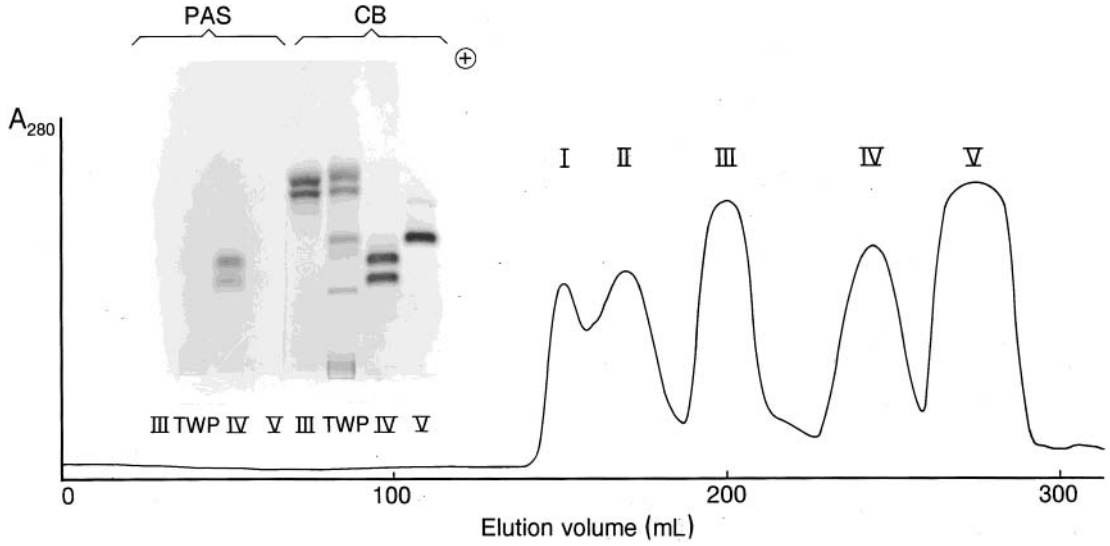



**Figure 1.** Separation of a whey protein fraction (1 g) on a 140 × 2.6 cm Sephadex G-75 Superfine column (for experimental conditions, see text). The rounding of peak summits is indicative of "overconcentration" in the photocell. Material from peaks III ($\beta$-lactoglobulin), IV (glyco-α-lactalbumin) and V (nonglyco-α-lactalbumin) was analyzed by native PAGE followed by staining with periodic acid-Schiff reagent (PAS) or Coomassie Blue G-250 (CB), as illustrated by the inset. TWP = total whey protein.

## MATERIALS AND METHODS

**Materials.** A bovine whey protein fraction, α-lactalbumin-enriched byproduct ("fraction P2") of the classical $\beta$-lactoglobulin purification according to Aschaffenburg and Drewry (1957), was used as starting material for the isolation of the main (nonglycosylated) α-lactalbumin fraction and of the minor fraction containing glyco-α-lactalbumin. For comparative purposes, an α-lactalbumin fraction prepared on a technical scale from concentrated cheese whey by ion-exchange chromatography on DEAE-Sepharose (Schmidt and van Markwijk, 1993) was employed.

PNGase F was purchased from Boehringer-Mannheim, Germany. Trifluoroacetic anhydride was a product of Sigma (St. Louis, MO). All other chemicals were of analytical grade.

**Chromatographic Isolation and Gel-Electrophoretic Characterization.** The isolation of α-lactalbumin was performed essentially as described by Davies (1974) with some modifications. The α-lactalbumin-enriched whey protein fraction (1 g dissolved in 3 mL of elution buffer, 0.01 M imidazole·HCl/0.2 M NaCl/0.02% $NaN_3$, pH 6.65) was carefully loaded onto the top of a 140 × 2.6 cm Sephadex G-75 Superfine column (Pharmacia, Uppsala, Sweden) and eluted with elution buffer at room temperature and at a flow rate of 30 mL $h^{-1}$. Absorbance was continuously measured at 280 nm using a Uvicord absorptiometer, type 8303A (LKB, Bromma, Sweden). Peak fractions were collected, dialyzed against distilled water at 4 °C, and freeze-dried.

Polyacrylamide gel electrophoresis (PAGE) at pH 8.6 was carried out in a vertical slab gel apparatus (E−C Corporation, St. Petersburg, FL) as described by Davies (1974). Staining for proteins was with Coomassie Blue G-250 and for glycoproteins with periodic acid-Schiff reagent (PAS) (Kapitany and Zebrowski, 1973).

Further purification of α-lactalbumin fractions was performed by semipreparative reversed-phase (RP)-HPLC using a water−acetonitrile gradient elution, essentially as described elsewhere (Visser et al., 1991).

**Enzymatic Deglycosylation of α-Lactalbumin and Acetylation of Released Oligosaccharides.** Deglycosylation of glyco-α-lactalbumin was carried out with PNGase F as described by Rademaker and Thomas-Oates (1996). The glycoprotein (0.33 mg dissolved in 200 $\mu$L of 50 mM ammonium bicarbonate buffer, pH 8.4, containing 1 drop of toluene) was incubated with 5 $\mu$L (1 unit) of PNGase F solution at 37 °C for 16 h. The resulting mixture of deglycosylated α-lactalbumin and released oligosaccharides was freeze-dried, redissolved in 500 $\mu$L of 5% acetic acid, and separated on a Sep-Pak Plus C18 cartridge (Waters, Milford, MA) which had been prewetted with methanol and equilibrated with 5% acetic acid. The oligosaccharides were eluted with 5% acetic acid in the first eluate. The vacuum-dried (SpeedVac vacuum centrifuge, Savant Instruments, Farmingdale, NY) eluate was peracetylated using a mixture of trifluoroacetic anhydride and acetic acid (together forming a mixed anhydride). After another evaporation step, the dried product was dissolved in dichloromethane and washed several times with distilled water (Rademaker and Thomas-Oates, 1996). The vacuum-dried mixture of oligosaccharides was then ready for mass spectrometric analysis. The deglycosylated glyco-α-lactalbumin was obtained from a second eluate fraction (20−40% 1-propanol in 5% acetic acid) during the Sep-Pak Plus C18 separation.

**Mass Spectrometry.** For the analysis of the α-lactalbumin fractions, ESI mass spectra were obtained with a Quattro II triple quadrupole instrument (Micromass, Cheshire, U.K.). Samples were dissolved in 50% acetonitrile, 0.3% formic acid, and analyzed by infusion with a syringe infusion pump, type 22 (Harvard Apparatus, South Natick, MA), of the sample solution in 50% acetonitrile at 4 $\mu$L $min^{-1}$ through the electrospray interface. Nitrogen was used as a nebulizing and drying gas. The capillary was held at 3.9 kV; the cone voltage was 40 V. The acetylated carbohydrate fraction was dissolved in 50% methanol and 0.3% formic acid and analyzed by infusion as described above with the capillary held at 3.5 kV and the cone voltage at 30 V. Mass calibration was performed by multiple-ion monitoring of a NaI/CsI mixture. About 10−15 spectra were averaged to obtain an adequate signal-to-noise ratio. The raw mass spectral data were processed and transformed with the Masslynx software version 2.2 (Micromass, Cheshire, U.K.) on a Windows NT workstation.

## RESULTS AND DISCUSSION

**Chromatographic Isolation and Gel-Electrophoretic Characterization of α-Lactalbumin Fractions.** The fractionation of the whey protein fraction on a Sephadex G-75 Superfine column is illustrated by Figure 1 (numbering of peaks according to Armstrong et al., 1970), with the PAGE gel patterns of isolated fractions III ($\beta$-lactoglobulin), IV (mainly glyco-α-lactalbumin), and V (nonglyco-α-lactalbumin) shown in the inset of that figure. Fraction I (pink) and II (yellowish), both not shown on the gel, included minor whey proteins such as lactoferrin, $\gamma$-globulins, and serum albumin (Davies, 1974). Particularly when focusing on a satisfac-

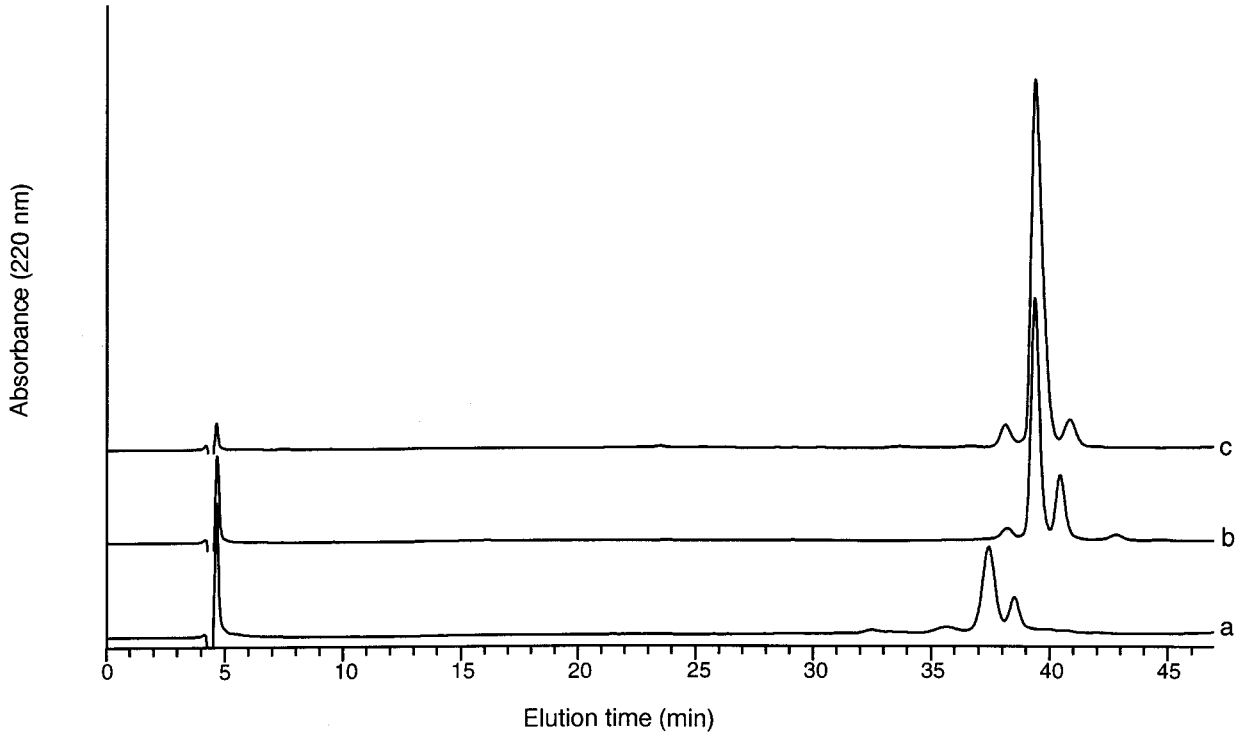



**Figure 2.** Reversed-phase HPLC patterns of (a) peak component IV of Figure 1, (b) peak component V of Figure 1, and (c) a sample of α-lactalbumin prepared by DEAE-Sepharose chromatography on a technical scale. Peak material was collected and used for mass spectrometric analysis.

tory separation between glyco-α-lactalbumin and the other fractions, we obtained better results with Sephadex G-75 than by using G-100 material. The latter had been used in the method of Davies (1974), who separated ca. 50 mg amounts of whey protein on a much shorter Sephadex G-100 column, collecting fractions IV and V together. Starting from 1 g of whey protein fraction, we obtained ca. 70 and 380 mg of dry material after dialysis and freeze-drying of fractions IV and V, respectively. The two electrophoretic glycoforms (PAS-positive) found in fraction IV (Figure 1) are the same as those reported in the literature (Barman, 1970; Hopper and McKenzie, 1973; Davies, 1974; Armstrong et al., 1970; Baumy and Fauquant, 1989) and represent the $S_1$ and $S_2$ fractions found by Hopper and McKenzie (1973) to include NeuAc-containing and NeuAc-free components, respectively.

RP-HPLC patterns of fractions IV and V (Figure 1) are seen in Figure 2 (traces a and b) together with, for comparison, the pattern of an α-lactalbumin preparation produced from cheese whey by DEAE-Sepharose chromatography (trace c) (Schmidt and van Markwijk, 1993). Glyco-α-lactalbumin (trace a) still contained a major and a minor peak, in an area ratio of about 70:30. Assuming that the minor peak represents a chemically induced α-lactalbumin derivative (see next section), we may conclude that, on the basis of the above yields of fractions IV and V, ca. 10% (m/m) of α-lactalbumin in our starting material existed in the glycosylated form. This is a significantly higher percentage than the 3% obtained by Hopper and McKenzie (1973) when separating total whey protein via a similar gel-filtration technique. Our percentage only slightly exceeds the 7% content reported by Baumy and Fauquant (1989), who used Sephadex G-50 chromatography and started from an industrial α-lactalbumin preparation. The minor component eluting just before the main one in trace *c* of Figure 2 is different from the minor component in trace *a*. It had an elution time corresponding to that of the last part of the main peak in trace *a* and, as seen in the next section, contained only part of the glycoforms of α-lactalbumin. Several peak fractions of the RP-HPLC separations were collected and dried by vacuum centrifugation (SpeedVac) before use in mass spectrometric analyses.

**Mass Spectrometry of α-Lactalbumin Components and Released Oligosaccharides.** Mass spectra and deconvoluted spectra of the main RP-HPLC components representing glyco- and nonglyco-α-lactalbumin (see Figure 2) are depicted in Figures 3−5. It was found that nonglyco-α-lactalbumin had a mass of 14 178.8 Da (Figure 3), which was in agreement with the theoretical value of 14 178 Da (Wang et al., 1989). The minor peak following the main one in the RP-HPLC pattern of Figure 2, trace *a*, cannot be ascribed to a natural glyco-α-lactalbumin form, as the corresponding mass (14 503 Da) was too low to account for any oligosaccharide chain attached to α-lactalbumin (result not shown). We have experimental evidence now that this component results from a chemical lactosylation process taking place during heat processing of milk (Slangen and Visser, unpublished). Recently, such components were also reported to occur in isolated *β*-lactoglobulin (Burr et al., 1996; Léonil et al., 1997). They can be considered as early Maillard products. A similar product, isolated from human blood serum, was found to be a reaction product of serum albumin and blood glucose (Bunk, 1997).

The mass spectrum of glyco-α-lactalbumin (Figure 4) was fairly complicated and contained, after deconvolution, at least 15 discrete peaks (Figure 5). Given the presence of GlcNAc, GalNAc, Man, Gal, Fuc, and NeuAc in glyco-α-lactalbumin (Barman, 1970; Hindle and Wheelock, 1971) and the known core structure of *N*-linked carbohydrates, we were able to match the masses of the various components shown in Figure 5 to those of 14 glycoforms of α-lactalbumin (Table 1).

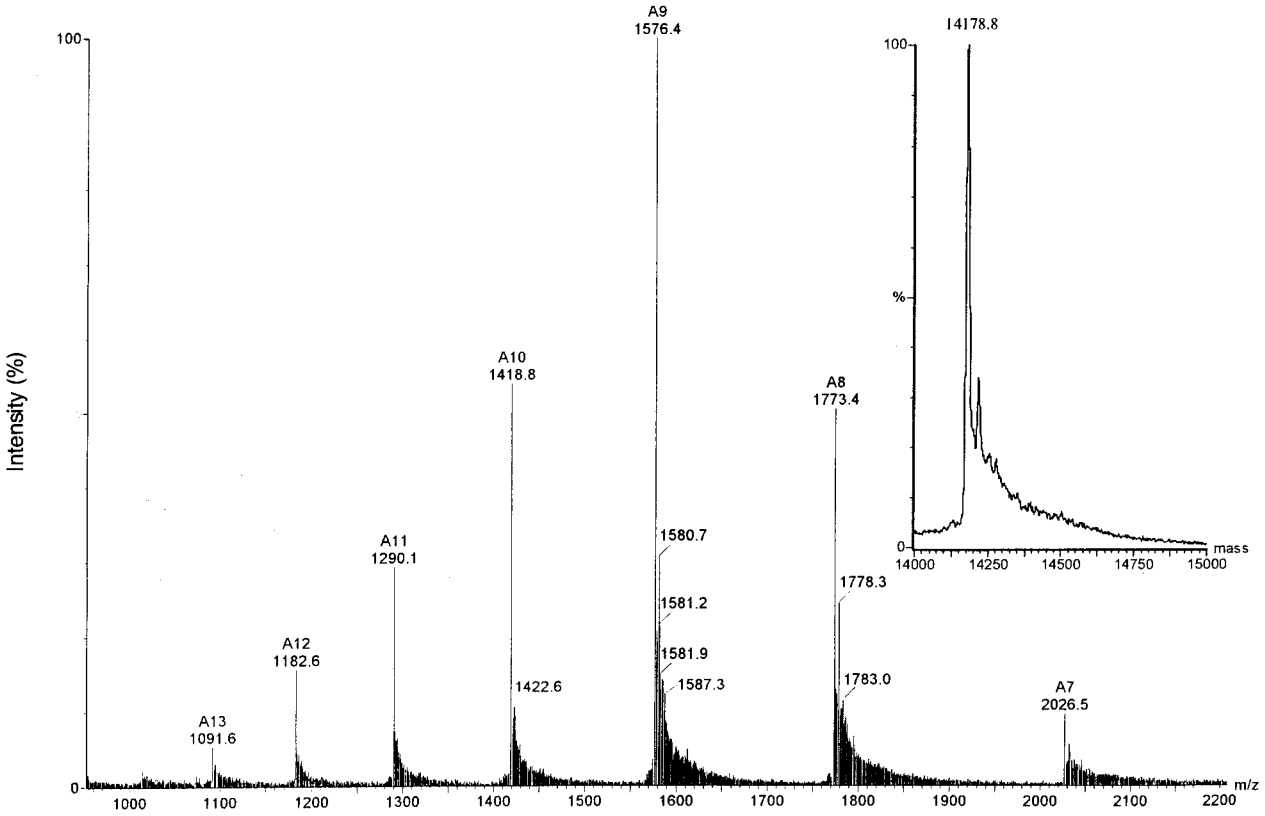



**Figure 3.** Electrospray mass spectrum of purified nonglyco-α-lactalbumin (main peak in Figure 2, trace b). Charge numbers of the molecular-ion clusters are also indicated, with a maximum mass intensity at nine charges (A9). The inset shows the deconvoluted spectrum.

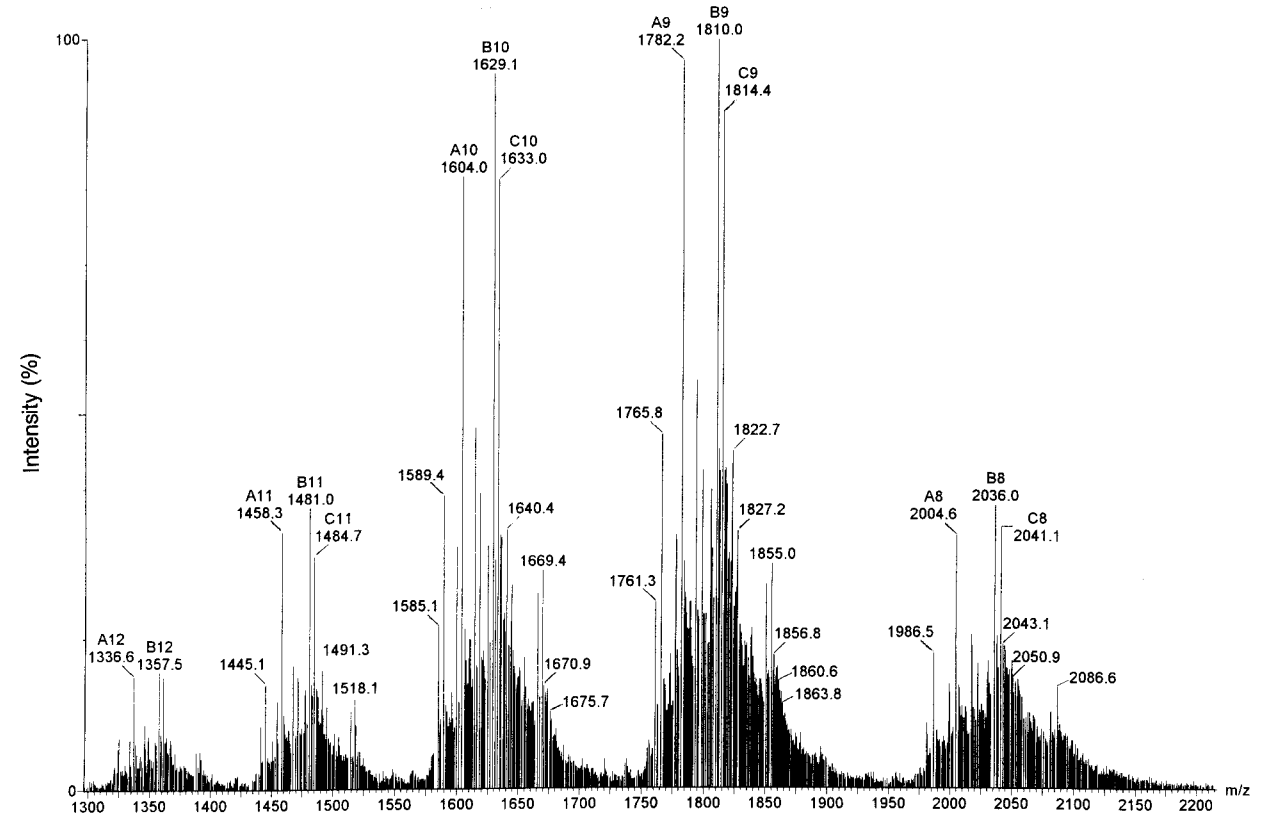



**Figure 4.** Electrospray mass spectrum of purified glyco-α-lactalbumin (main peak in Figure 2, trace a). A*x*, B*x*, and C*x* denote components of the three main envelopes of the spectrum.

Our results indicate that 8 of the 14 proposed glycoforms concern sialylated oligossacharide chains. This distinction between sialylated and nonsialylated oligosaccharides would account for the presence of the two PAS-positive bands in the PAGE-pattern of glyco-α-lactalbumin (Figure 1) (Hopper and McKenzie, 1973).

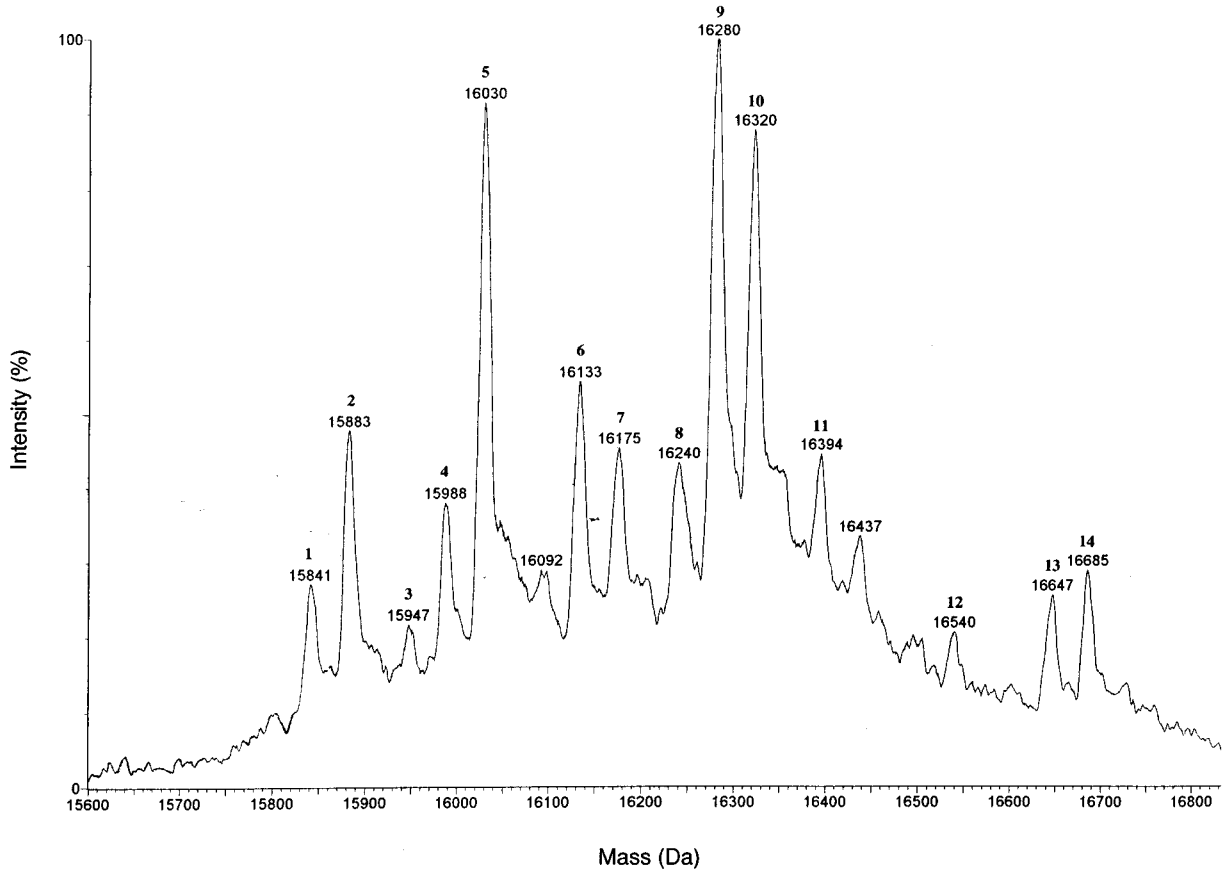


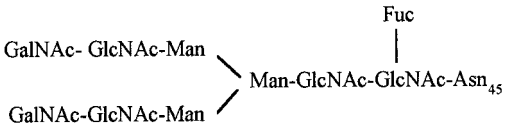

**Figure 5.** Deconvoluted mass spectrum of purified glyco-α-lactalbumin (see Figure 4). The mass components numbered 1−14 are referred to in Table 1.

**Table 1. Mass Data and Possible Monosaccharide Composition for Glycosylated Forms of Bovine α-Lactalbumin[a]**

| no. | measured mass | calcd mass | calcd residue mass of glycan | monosaccharide composition based on measured mass HexN | Fuc | Hex | NeuAc |
|---|---|---|---|---|---|---|---|
| 1 | 15841.1 | 15842.62 | 1664.54 (2691.45) | 5 | 0 | 4 | 0 |
| 2 | 15882.8 | 15883.68 | 1705.60 (2690.47) | 6 | 0 | 3 | 0 |
| 3 | 15946.7 | 15947.70 | 1769.63 (2922.66) | 4 | 1 | 5 | 0 |
| 4 | 15987.5 | 15988.76 | 1810.69 (2921.67) | 5 | 1 | 4 | 0 |
| 5 | 16029.5 | 16029.82 | 1851.74 (2920.69) | 6 | 1 | 3 | 0 |
| 6 | 16133.0 | 16133.88 | 1955.80 (3108.82) | 5 | 0 | 4 | 1 |
| 7 | 16175.3 | 16174.94 | 1996.85 (3107.84) | 6 | 0 | 3 | 1 |
| 8 | 16240.2 | 16238.96 | 2060.89 (3340.03) | 4 | 1 | 5 | 1 |
| 9 | 16280.3 | 16280.02 | 2101.95 (3339.04) | 5 | 1 | 4 | 1 |
| 10 | 16320.2 | 16321.08 | 2143.00 (3338.06) | 6 | 1 | 3 | 1 |
| 11 | 16394.3 | 16395.14 | 2217.08 (3496.21) | 7 | 1 | 4 | 0 |
| 12 | 16539.5 | 16540.28 | 2362.19 (3683.36) | 7 | 0 | 4 | 1 |
| 13 | 16646.6 | 16645.36 | 2467.28 (3914.56) | 6 | 1 | 5 | 1 |
| 14 | 16685.3 | 16686.42 | 2508.34 (3913.58) | 7 | 1 | 4 | 1 |

[a] Calculated masses of the glycoprotein components are based on the proposed monosaccharide composition in combination with a calculated average mass of 14178.06 for nonglycosylated α-lactalbumin (Wang et al., 1989). Calculated average masses of the released and fully acetylated glycan moieties, including 102.09 for terminal-group acetylation, are indicated in parentheses (experimental values to be derived from Figure 6). HexN = GalNAc/GlcNAc: average residue mass 203.20 (acetylated, 287.27). Fuc: average residue mass 146.14 (acetylated, 230.22). Hex = Gal/Man: average residue mass 162.14 (acetylated, 288.23). NeuAc: average residue mass 291.26 (acetylated, 417.37).

It should be borne in mind that the mass spectrometric results do not necessarily reflect the various glyco-α-lactalbumin forms in a quantitative manner. On the other hand, the mass of the only glyco-α-lactalbumin structure published so far (Tilley et al., 1991) and reported to represent the major glycoform




corresponds with that of component 5 in Figure 5 and Table 1; this component was also found in the present study to give a relatively high response (Figure 5). It is likely to assume that the other glycoforms shown in Table 1 represent, by analogy with recently published milk glycoprotein structures (Coddeville et al., 1992; Sato et al., 1993; Girardet et al., 1995), closely similar structures, being either fucosylated or nonfucosylated, sialylated or nonsialylated, and having in a number of cases Gal instead of GalNAc. This would also be in line with the relatively small amounts of Gal, Fuc, and NeuAc that were earlier reported to occur in glyco-α-lactalbumin (Barman, 1970; Hindle and Wheelock, 1971). The occurrence of GalNAc, earlier considered as rather unusual in *N*-linked oligosaccharides (Tilley et al., 1991; Montreuil, 1980), has recently been reported for several glycoproteins of this category (Tilley et al., 1991; Sato et al., 1993; Girardet and Linden, 1996). It was suggested to be controlled by an *N*-acetylgalactosaminyltransferase acting in competition with a galactosyltransferase on common, terminal-GlcNAc-containing substrate sites (Girardet and Linden, 1996; Neeleman et al., 1994).

Molecular masses of glyco-α-lactalbumin reported in the literature were calculated from amino acid and

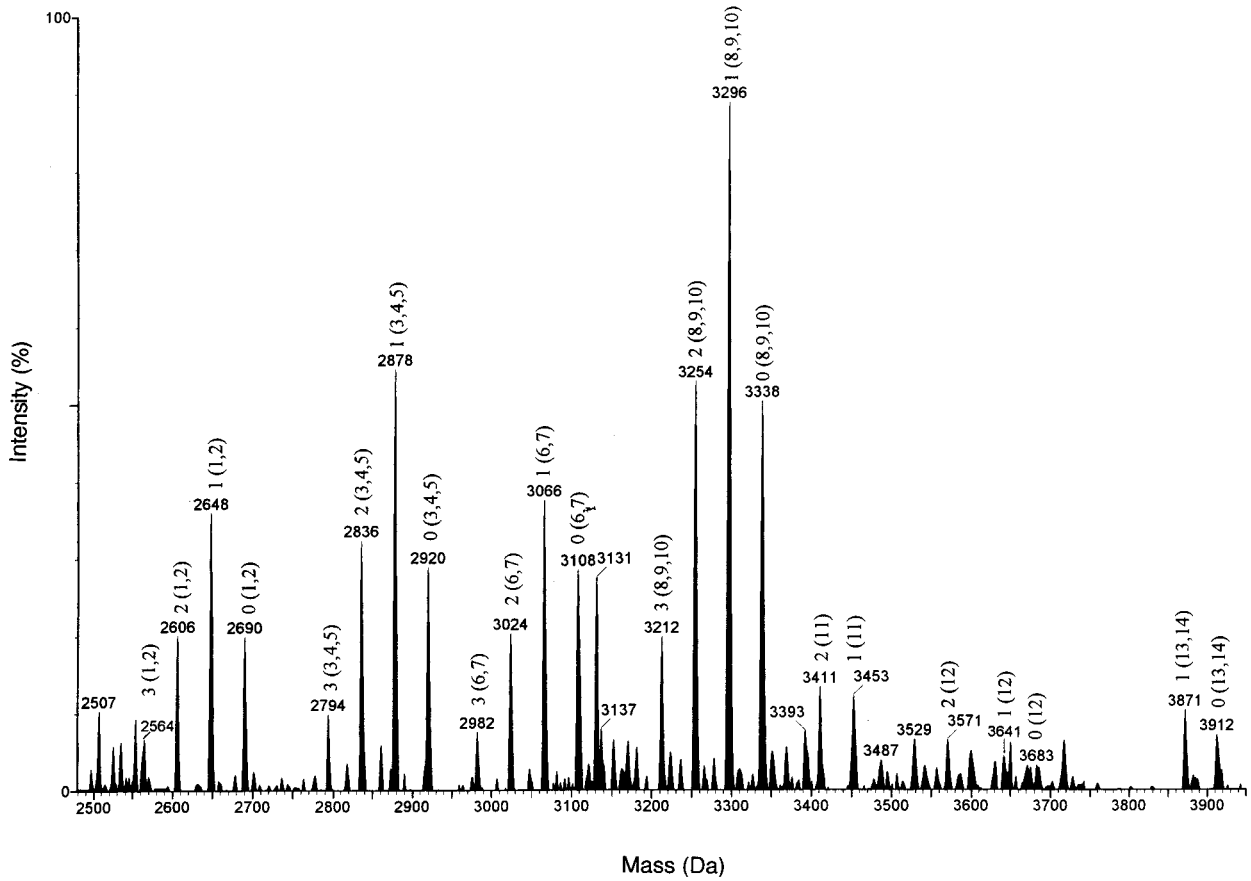



**Figure 6.** Deconvoluted mass spectrum of enzymatically released and peracetylated oligosaccharides originating from glyco-α-lactalbumin. Number of acetyl groups missing as a result of incomplete derivatization are indicated by *x* in *x*(*y*), where (*y*) corresponds to the numbering of components as used in Table 1 and Figure 5. Several oligosaccharides could not be distinguished because of a similar mass of their acetylated forms.

carbohydrate analyses or from retention behavior during gel-permeation chromatography. Values obtained were between 16 300 and 16 800 Da (Barman, 1970; Hopper and McKenzie, 1973; Baumy and Fauquant, 1989), which is within the mass range of the various individual components found in the present study (Table 1).

The glycoforms postulated in Table 1 were supported by mass spectrometric analysis of the oligosaccharide fraction obtained after deglycosylation of glyco-α-lactalbumin with PNGase F and subsequent peracetylation. No distinction could be made between several acetylated oligosaccharides of similar mass. Acetylation was carried out in order to allow the subsequent extraction of derivatized oligosaccharides into the organic phase, which offers a more effective separation from contaminants (e.g., salts) and therefore enhances the sensitivity of detection during mass spectrometric analysis (Rademaker and Thomas-Oates, 1996). The molecular mass of the deglycosylated glyco-α-lactalbumin was found to be 14 178.5 Da (calculated: 14 179 Da for α-lactalbumin with Asn-45 converted to Asp as a result of the deglycosylation procedure). The deconvoluted mass spectrum of the released and acetylated oligosaccharides is shown in Figure 6. Taking into account the occurrence of 2−4 positive charges per molecule, a spectrum was obtained in which nearly all peaks could be assigned by assuming for each oligosaccharide moiety 0−3 acetyl residues less as a result of incomplete acetylation. Masses correlated very well with the compositions based solely on masses of total glycoprotein molecules, as postulated in Table 1. On the basis of peak intensities, the main oligosaccharides as derived from Figure 6 corresponded to the main glycoprotein components deduced from Figure 5. The fact that complete deglycosylation could be achieved with nondenatured glyco-α-lactalbumin indicates that the protein−glycan linkage is easily accessible to the enzyme. As recently reported (Pike et al., 1996), Asn-45 is indeed located in a solvent-exposed loop of the (rather compact) α-lactalbumin molecule.

For comparison, we have also analyzed the minor peak preceding the major one in trace *c* of Figure 2. It represented the glycoform of an α-lactalbumin sample prepared in a quite different way (see Materials and Methods) without the specific intention of isolating all the glycosylated forms. Indeed, this glyco-α-lactalbumin preparation appeared to contain only part of the glycoforms shown in Table 1 (mainly components 1, 6, 7, 9, and 10; result not shown). Therefore, it is likely that the number and ratio of glyco-α-lactalbumin forms found depend on the material used and, more particularly, on the mode of its isolation and purification.

In summary, it may be concluded that our mass spectrometric analysis of the nonglycosylated fraction of bovine α-lactalbumin has led to a mass value which is in perfect agreement with the amino acid sequence known for this protein (Wang et al., 1989). The glyco-α-lactalbumin fraction, although showing only two electrophoretic components on PAGE, exhibits a high degree of heterogeneity when analyzed by ESI-MS. For the most part this carbohydrate heterogeneity probably concerns closely similar structures, either fucosylated

or nonfucosylated and sialylated or nonsialylated, whereas in quite a number of cases Gal is replaced by GalNAc.

While data such as overall carbohydrate composition and single, *N*-linked glycosylation site were already known from the literature, our study demonstrates that mass spectrometry is a powerful tool to gain further insight into the structural composition of glyco-α-lactalbumin. In our case this could be achieved without any further purification of the glycoprotein fraction or of the oligosaccharides released thereof. In case any ambiguity remained concerning the composition of some glycan residue at Asn-45 after ESI-MS of the total glycoprotein fraction, the independent second approach of ESI-MS analysis of the released and peracetylated oligosaccharide fraction provided enough evidence to make the appropriate choice. For instance, the mass found for glycoprotein component 13 of Table 1 (16 646.6 Da) could also be explained by the glycan composition of component 10 plus one lactose residue resulting from lactosylation at some lysine residue (calculated mass 16 645.1 Da). However, the latter assumption becomes unlikely by considering the results of the second approach of ESI-MS analysis of the oligosaccharide fraction, i.e., the masses of 3912 and 3871 Da assigned to the acetylated glycan of component 13 (Figure 6). Moreover, no mass peaks were found which could represent any other mixed oligosaccharide/lactose-containing component.

The biological function of the glyco-α-lactalbumin fraction still remains unclear. It has been suggested (Barman, 1970) that in the Golgi region of the mammary gland cells α-lactalbumin is wholly secreted as a glycoprotein and subsequently degraded by milk glycoside hydrolases. If this would be the case, then the biological significance of the glycan moiety in α-lactalbumin might be limited to its role in the labeling of the protein for export from the cell. However, other biological functions cannot be excluded and may be a subject for further study.

## ABBREVIATIONS USED

ESI-MS, electrospray-ionization mass spectrometry; PAS, periodic acid-Schiff reagent; PNGase F, peptide-$N^4$-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; RP-HPLC, reversed-phase high-performance chromatography.

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**Supporting Information Available:** Possible glycan structures at Asn-45 of bovine glyco-α-lactalbumin components 1−14 based on the results presented in Figure 5 and Table 1 of this paper.

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