Application of Electrospray Mass Spectrometry (ES-MS) for the Analysis of Monoclonal Antibody F_c Subunits

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INTRODUCTION

Recent developments in the hybridoma technology have opened up new perspectives for the commercialization of monoclonal antibodies (Mabs)[6] as pharmaceutical products. To determine the physicochemical properties and stability of therapeutic Mabs, the use of a panel of appropriate analytical methods is required (1–4). Recently, ES-MS has been introduced as a powerful technique for the determination of the MW of proteins: it has proven to be a precise and accurate method with high resolution (5–8) when compared to conventional methods, such as gel permeation chromatography and SDS-PAGE. In ES-MS the obligatory ionization is achieved via electrically assisted nebulization, yielding a series of multiply charged molecular ions. The mass spectrometer separates the ions according to the ratio between mass number and number of electrical charges. Since almost no fragmentation occurs, the set of peaks obtained represents multiply protonated molecular ions with successive numbers of electrical charges.

A common feature of IgG class antibodies is the heterogeneity in the N-linked glycosylation in the C_H2 domain (9–13). The average molecular mass of entire monoclonal antibodies and conjugates thereof has been determined by laser desorption mass spectrometry (14), but this technique does not resolve the molecular masses of individual species of large proteins that exhibit carbohydrate heterogeneity (14,15). Heterogeneous carbohydrate chains of glycoproteins have been investigated by mass spectrometry, but only of enzymatic or chemical digests with molecular masses up to about 5000 Da (15). The present study shows that mass heterogeneity in relatively large (ca. 25-kDa) glycoproteins, i.e., the F_c part of two mouse IgG Mabs, MN12 and WT31, can be resolved by ES-MS. In addition, the influence of storage of MN12 under alkaline conditions on the ES-MS spectra of the F_c subunits has been investigated.

MATERIALS AND METHODS

Materials

Production of the Mabs MN12 (mouse $IgG_{2a,\kappa}$) and WT31 (mouse $IgG_{1,\kappa}$) has been described elsewhere (16). Storage of MN12 at pH 10.0 and 37°C for 0, 16, 32, and 64 days was performed as described previously (3).

Papain (2× crystallized and lyophilized powder), DTE, and iodoacetamide were obtained from Sigma (St. Louis, MO). Digoxigenin-labeled lectins, carboxypeptidase Y, transferrin, fetuin, asialofetuin, and alkaline phosphatase-labeled monoclonal antibody against digoxigenin (anti-DIG-AP) were purchased from Boehringer (Mannheim, Germany). Bovine serum albumin (BSA) was obtained from Organon Teknika (Turnhout, Belgium). Purified PIC3 protein from *Neisseria gonorrhoeae* (17) was kindly provided by Dr. G. F. A. Kersten. All chemicals used were of analytical grade. Spectra/Por molecular porous membrane tubing with a MW cutoff of 12,000–14,000 (Spectrum, Los Angeles, CA) was used for dialysis.

Preparation of F_c Subunits

Enzymatic Digestion. Solutions of 1 mg/ml purified Mabs MN12, whether previously stored at pH 10 (see above) or not, and WT31 in 0.10 *M* Tris/HCl (pH 8.0) + 2.0 m*M* EDTA (buffer A) were prepared. Papain was activated by incubation of a 1.0 mg/ml solution in 0.10 *M* Tris/HCl (pH 8.0) + 2.0 m*M* EDTA + 1.0 m*M* DTE (buffer B) for 20 min at 37°C (activated papain). Enzymatic digestion of the Mabs was carried out by incubating 20 ml of Mab solution with activated papain for 8 hr at 37°C, using a Mab/papain ratio of 100:1 (w/w). Digestion was terminated by addition of 2 ml 0.20 *M* iodoacetamide and incubation for 1 hr on melting ice.

After storage at pH 10, MN12 had become less susceptible to digestion by papain (see Results and Discussion). Therefore, the pH 4.5 protein A fractions of the papain digests (see below) of the MN12 samples stored at pH 10 (for 0, 16, 32, and 64 days; see above) were extensively dialyzed against buffer B and incubated again with activated papain for 4 hr at 37°C, using a Mab/papain ratio of 100:1 (w/w).

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[6] Abbreviations used: AAA, *Aleuria aurantia* lectin; BSA, bovine serum albumin; DSA, *Datura stramonium* lectin; ES-MS, electrospray mass spectrometry; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GNA, *Galanthus nivalis* lectin; hex, hexose; hexNAc, *N*-acetylhexose; Mab, monoclonal antibody; Man, mannose; MW, molecular weight; NeuAc, *N*-acetylneuraminic acid; PBS, phosphate (10 m*M*)-buffered saline, pH 7.3; PNA, *Arachis hypogaea* lectin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNA, *Sambucus nigra* lectin; TBS, tris-(hydroxymethyl)-aminomethane (50 m*M*)-buffered saline, pH 7.5.

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Digestion was terminated by the addition of 0.1 vol 0.20 *M* iodoacetamide and incubation for 1 hr on melting ice.

The digests were extensively dialyzed at 4°C against binding buffer for protein A affinity chromatography, *viz.*, PBS (MN12) or 1.5 *M* glycine/NaOH (pH 8.9) + 3.0 *M* sodium chloride (WT31).

Separation of F_{ab} and F_c Fractions. The F_{ab} and F_c fractions were separated by protein A affinity chromatography, using an HR 10/10 column containing 8 ml protein A–Sepharose CL6B Fast Flow (supplied on special request by Pharmacia, Uppsala, Sweden), as described previously (16,18). Dialyzed digest was put on the column using the above-mentioned buffers to bind the F_c fraction to the column. Bound material was eluted with 0.1 *M* sodium citrate, pH 4.5, neutralized with 1 *M* Tris, and extensively dialyzed against PBS at 4°C.

Reduction and Concentration of F_c Fractions. Reduction of F_c was carried out by incubating 1 vol of dialyzed F_c fraction with 0.1 vol of 0.10 *M* DTE for 2 hr at 37°C. The reaction was terminated by the addition of 0.1 vol of 0.20 *M* iodoacetamide and incubation for 1 hr on melting ice. The reduced and alkylated samples were extensively dialyzed at 4°C against 50 m*M* ammonium carbonate (pH 7.7) + 0.01% (w/v) sodium azide. Next the samples were concentrated by ultrafiltration to a protein concentration of 3 to 5 mg/ml, using an 8MC ultrafiltration cell (Amicon, Lexington, MA) and a Diaflo PM10 ultrafiltration membrane with a MW cutoff of 10,000 (Amicon). Finally, the samples were filtered through a Minisart NML 165 34 K cellulose acetate filter with 0.2-μm pores (Sartorius, Göttingen, Germany) and kept at 4°C until used for ES-MS analysis.

ES-MS

A BIO-Q mass spectrometer (VG Instruments, Manchester, UK) was used for ES-MS. Calibration of the instrument was performed with horse heart myoglobin (average molecular mass, 16951.5 Da), using the average atomic weights of the elements: C = 12.011, H = 1.00794, N = 14.0067, O = 15.9994, and S = 32.06.

Fifty microliters of methanol and 5 μl of 90% (v/v) formic acid were added to 50 μl of sample. Ten microliters of the resulting solution was delivered to the nebulizer head by pumping (5 μl/min) through a capillary with an internal diameter of 60 μm. A potential difference of 3 kV was maintained between the capillary tip and a circular electrode placed at a distance of 2 cm. Spectra were obtained using a scan rate of 11 sec from mass/charge ratios (m/z) of 980 to 1720, and typically 50 to 80 scans were summed to obtain the final spectrum.

For each pair of peaks the molecular mass was calculated using the following equations (6,7):

$$z_2 = (m_1 - 1.00794)/(m_1 - m_2) \quad (1)$$

$$M = R_{z2} * (m_2 - 1.00794) \quad (2)$$

where z_2 is the calculated charge of a multiply charged ion at m/z m_2, m_1 and m_2 are m/z values for two adjacent peaks with $m_1 > m_2$, M is the average molecular mass, and R_{z2} is the nearest integer value to z_2.

Immunodot Blots

Protein solutions (1 μl, 1 mg/ml) were applied onto nitrocellulose (BA85, 0.45 μm, Schleicher Schuell, Dassel, Germany). The blots were left to dry for 30 min at room temperature and then boiled in PBS for 15 min. Blocking of the blots was performed in PBS containing 0.05% (w/v) Tween 20 and 1% (w/v) BSA (PBS-T-BSA) for 1 hr at room temperature. Next the blots were washed four times for 10 min in TBS and then once in TBS containing 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, and 1 m*M* $MnCl_2$. Then the blots were incubated with the lectins [10 ml of AAA (1:1000), DSA (1:500), GNA (1:500), PNA (1:500), or SNA (1:100) in PBS-T-BSA] for 2.5 hr at room temperature, followed by five washes with TBS. Next the blots were incubated with anti-DIG-AP (1:1000 in TBS) for 2 hr at room temperature. After five washes with TBS, the blots were developed with 10 ml substrate solution [containing 0.4 mg/ml 4-nitro blue tetrazolium chloride + 0.2 mg/ml 5-bromo-4-chloro-3-indolyl phosphate + 0.8% (v/v) dimethylformamide in 50 m*M* Tris/HCl (pH 9.5) + 50 m*M* $MgCl_2$ + 0.1 *M* Na Cl] and after color development washed with distilled water and dried thoroughly.

SDS-PAGE

SDS-PAGE was carried out on a PhastSystem (Pharmacia), using PhastGel 12.5 gels and low molecular weight standards (Cat. No. 161-0304, Bio-Rad, Richmond, CA). Electrophoresis and subsequent silver staining of the gels were performed following the manufacturer's instructions.

RESULTS AND DISCUSSION

Analysis of F_c Subunits of MN12 and WT31

The ES-MS spectra of the reduced and alkylated F_c subunits of MN12 and WT31 are shown in Fig. 1. The calculated average molecular masses are listed in Table I. The spectra of both MN12 and WT31 display several multiply protonated components. In particular, the spectrum of MN12 F_c is complex. Two main components with molecular masses of 25,713 and 25,854 Da are observed (peaks K and L in Fig. 1a, Table I). In addition, at least 10 other components, with molecular masses ranging from 24,853 to 25,602 Da (peaks A–J in Fig. 1a), can be discerned. The WT31 F_c spectrum shows four main components, with molecular masses ranging from 25,726 to 26,017 Da (peaks W–Z in Fig. 1b, Table I).

There was a discrepancy between the apparent molecular mass of 31 kDa in SDS-PAGE (not shown) and the molecular masses observed by ES-MS (ranging from 24.8 to 25.9 kDa). This is probably due to aberrant migration of F_c in SDS-PAGE. Inefficient binding of SDS to glycoproteins has been documented (19) and may account for an overestimation of the molecular mass in SDS-PAGE. Clearly, the resolution of ES-MS is much higher than that of SDS-PAGE. Besides, the precision in the mass determinations of the individual components is high: using Eqs. (1) and (2), intraassay standard deviations were at most 3 Da per component. This is in good agreement with the coefficients of variation of 0.01–0.05% reported for ES-MS of proteins (6,8,15). Similar

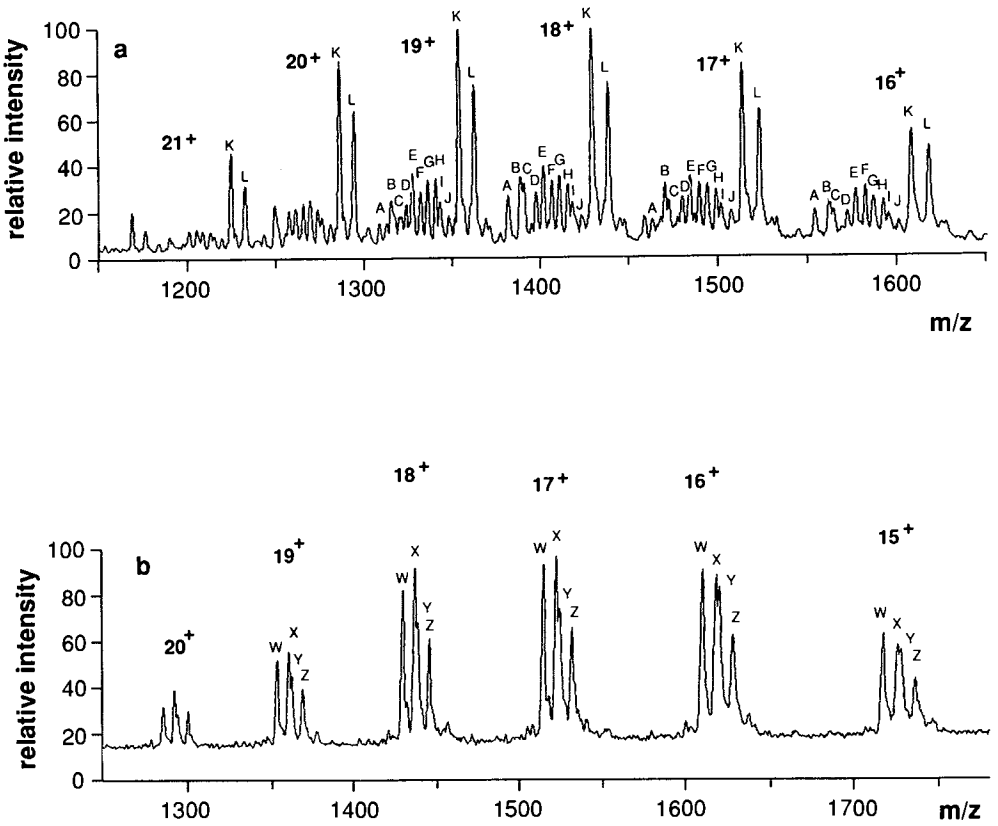
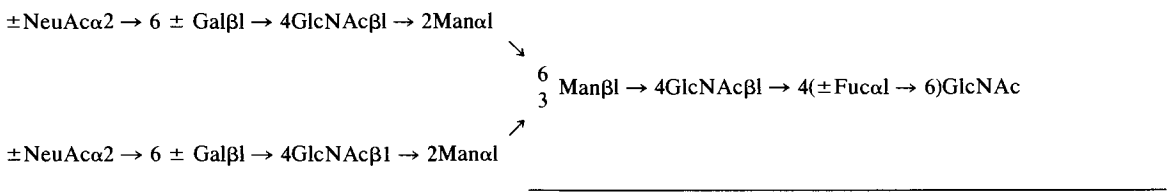


Fig. 1. ES-MS spectra of reduced and alkylated F_c fragments of MN12 (a) and WT31 (b). Capital letters and numbers mark the different components and the number of charges per component, respectively.

values were found for the variation in repeated assays of one particular sample.

The observed mass heterogeneity is likely to be due, at least partly, to glycosylation differences. Mouse IgG carbohydrate chains are composed of hexoses (mannose and galactose), *N*-acetylglucosamine, *N*-acetylneuraminic acid, and fucose (9,13,20), with average residual molecular masses of 162.142, 203.195, 291.258, and 146.143 Da, respectively. Assuming that all molecular mass differences observed by ES-MS are due to carbohydrate heterogeneity, the spectra can be well interpreted, as shown in Table I. The ES-MS spectrum of MN12 F_c can be explained by variation in the number of hexose, *N*-acetylhexose, and *N*-acetylneuraminic acid residues. In the case of WT31, hexose and *N*-acetylneuraminic acid content variability may account for the differences between the molecular masses of the four main components in the F_c spectrum.

The following structures for the biantennary asparagine-linked carbohydrate chains of mouse IgG have been reported (9,13,20):



The differences in the molecular masses in the F_c spectrum of both MN12 and WT31 cannot be ascribed to glycosylation heterogeneity if the N-linked glycosylation site in the C_H2 domain containing the above-mentioned carbohydrate structures is the only glycosylation site in the F_c subunits of the Mabs. Therefore, the F_c spectra suggest the presence of additional glycosylation sites. Additional N-linked and O-linked glycosylation sites have been reported for IgG (9,13).

In order to investigate the presence of carbohydrate heterogeneity on MN12 and its F_c fragments, immunodot-blot experiments with several lectins with specificity for different terminal carbohydrate moieties were performed. The results are summarized in Table II. The presence of terminal Fuc, Gal and/or GlcNAc, Man, and NeuAc was demonstrated on both nondigested MN12 and nondigested MN12 F_c. This shows the occurrence of carbohydrate heterogeneity in the MN12 F_c fragment. To our knowledge, the presence of terminal Man on IgG has not been reported before. Moreover, PNA (specific for O-linked terminal Gal) showed a positive

Table I. Molecular Masses M of F_c Subunits of MN12 and WT31 as Detected by ES-MS, and Comparison Between Observed Mass Heterogeneity and Calculated Mass Differences Assigned to Glycosylation Heterogeneity

Sample	Component (x)[a]	M	ΔM ($M - M_{ref}$)[b]	Assignment of ΔM[c]	ΔM_{calc} ($M_{calc} - M_{ref}$)[d]	$\Delta M_{calc} - \Delta M$
MN12	A	24,853	−860	−hex − 2 hexNAc − NeuAc	−859.7	0
	B	24,980	−733	−2 hex − 2 hexNAc	−730.7	2
	C	25,012	−701	−2 hexNAc − NeuAc	−697.6	2
	D	25,141	−572	−hex − 2 hexNAc	−568.5	4
	E	25,213	−500	−hexNAc − NeuAc	−494.5	5
	F	25,301	−412	−2 hexNAc	−406.4	6
	G	25,376	−337	+hex − hexNAc − NeuAc	−332.3	5
	H	25,463	−250	−2 hexNAc + hex	−244.3	6
	I	25,507	−206	−hexNAc	−203.2	3
	J	25,602	−111	−2 hexNAc + NeuAc	−115.1	−4
	K	25,713	0	—	0	0
	L	25,874	162	+hex	162.1	0
WT31	W	25,726	−128	+hex − NeuAc	−129.1	−1
	X	25,854	0	—	0	0
	Y	25,887	33	+2 hex − NeuAc	33.0	0
	Z	26,017	163	+hex	162.1	−1

[a] Components correspond to those indicated in Fig. 1; molecular masses were calculated from the spectra [using Eqs. (1) and (2); see Materials and Methods].

[b] ΔM, observed difference between the molecular mass of component x and that of the reference; M_{ref}, observed molecular mass for reference. Components with the highest intensity were used as references (component K for MN12 and component X for WT31).

[c] hex, hexose; hexNAc, *N*-acetylhexose; NeuAc, *N*-acetylneuraminic acid.

[d] ΔM_{calc}, calculated difference between the molecular mass of component x and that of the reference; M_{calc}, calculated molecular mass for component x, on the basis of the assigned glycosylation heterogeneity pattern and assuming that M_{ref} is the true mass of the reference. Average residual molecular masses: 162.142 (hex), 203.195 (hexNAc), and 291.258 (NeuAc).

reaction, which indicates that one or more O-glycosylation sites are present (21).

The difference between the measured and the calculated molecular mass of the assigned carbohydrate residues does not exceed 6 Da (see Table I). The differences observed may be due not only to experimental error in the molecular mass determinations, but also to amino acid heterogeneity and/or substitutions, as suggested by Rothman and Warren (12).

Analysis of F_c Subunits of MN12 Stored at pH 10

It is known from previous experiments that storage of MN12 at pH 10 appears to accelerate degradation reactions that also occur under physiological conditions (3). Therefore, the MN12 antibody was stored at a high pH to ensure degradation of the molecules within a reasonably short period of time.

After storage of MN12 at pH 10.0 and 37°C for different periods of time, the Mab had become less susceptible to papain treatment. The longer the storage period, the more nondigested MN12 was still present after papain treatment. This suggests that alkali treatment of MN12 makes the hinge region less accessible to papain. Therefore, the pH 4.5 protein A eluates—which contained appreciable amounts of nondigested IgG—were digested again (see Materials and Methods). After the second digestion, only bands corre-

Table II. Results of Immunodot-Blot Experiments of MN12 IgG and MN12 F_c with Lectins

Lectin	Binding specificity for terminal carbohydrate sequence[a]	Reactivity[b]			
		MN12 IgG	MN12 Fc	Positive control[c]	Negative control[d]
AAA	L-Fuc (α 1-6) − GlcNAc −	+	+	ND[e]	−
DSA	Gal β 1-4 GlcNAc − GlcNAc (β 1-4 GlcNAc)$_2$ − GlcNAc − Ser/Thr −	+	+	+	−
GNA	Man α1-3 Man − Man α1-6 Man − Man α1-2 Man −	+	+	+	−
PNA	Gal β 1-3 GalNAc − α Gal −	+	+	+	−
SNA	NeuAc α 2-6 Gal − NeuAc α 2-6 GalNAc −	+	+	+	−

[a] Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNac, *N*-acetylglucosamine; L-Fuc, L-fucose; Man, mannose; NeuAc, *N*-acetylneuraminic acid.

[b] +, positive reaction; −, no reaction.

[c] Asialofetuin (for DSA and PNA), carboxypeptidase Y (for GNA), fetuin (for SNA), and transferrin (for SNA).

[d] MN12 F_{ab} fragment, BSA, and PIC3 protein of *Neisseria gonorrhoeae*.

[e] Not done.

sponding to F_{ab} and F_c fragments were observed by SDS-PAGE (not shown). After reduction and alkylation of the F_c fractions, SDS-PAGE—performed under either reducing or nonreducing conditions—showed only one band with an apparent molecular mass of approximately 31 kDa for each sample.

The ES-MS spectra of the F_c subunits are presented in Fig. 2. The molecular masses of the individual components are listed in Table III. The molecular masses calculated for the F_c subunits of untreated MN12 (see Table III) are similar to those presented in Table I, indicating that the second incubation with papain did not further digest the F_c fragment.

The F_c subunits of alkali-treated MN12 exhibit additional components with higher MW (Fig. 2 and Table III, peaks N, O, and P). Moreover, peaks B-I have disappeared in the ES-MS spectrum after storage of MN12 at pH 10 and 37°C for 32 or 64 days. The longer the exposure of MN12 to

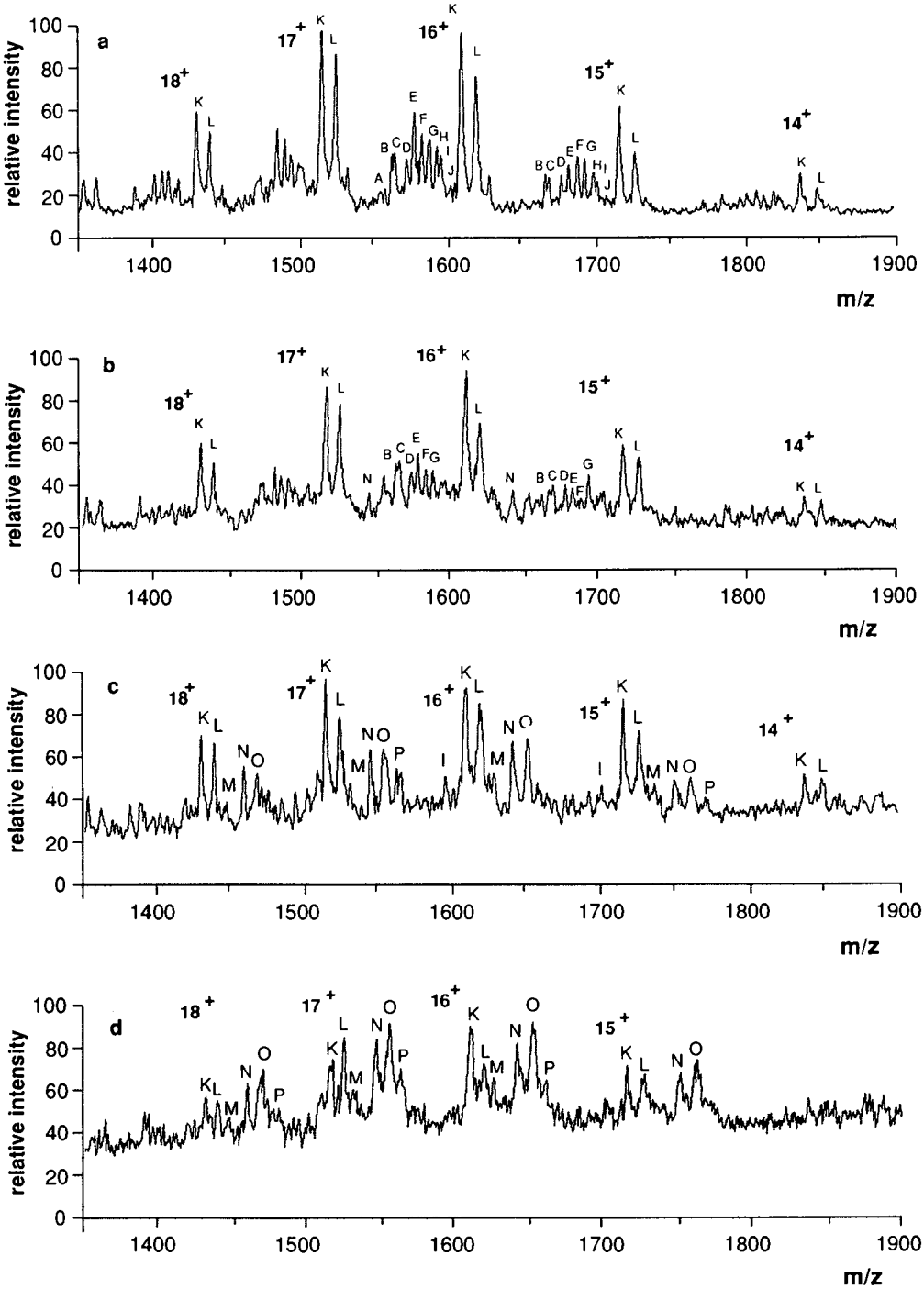


Fig. 2. ES-MS spectra of reduced and alkylated F_c fragments of MN12 after incubation of MN12 at pH 10.0 and 37°C for 0 days (a), 16 days (b), 32 days (c), and 64 days (d). Capital letters and numbers mark the different components and the numbers of charges per component, respectively.

Table III. Molecular Masses of MN12 F_c Subunits After Storage of MN12 at pH 10.0 and 37°C for 0, 16, 32, and 64 Days

Component[a]	Storage time (days)			
	0	16	32	64
A	—[b]	—	—	—
B	24,981	24,982	—	—
C	25,014	25,019	—	—
D	25,142	25,148	—	—
E	25,214	25,217	—	—
F	25,301	25,303	—	—
G	25,377	25,379	—	—
H	25,464	—	—	—
I	25,508	—	25,511	—
J	—	—	—	—
K	25,712	25,718	25,716	25,719
L	25,876	25,878	25,878	25,878
M	26,033	—	26,035	26,034
N	—	26,241	26,241	26,238
O	—	—	26,398	26,401
P	—	—	26,548	26,551

[a] Components correspond to those indicated in Fig. 2; molecular masses were calculated from the spectra [using Eqs. (1) and (2); see Materials and Methods].
[b] Not detected.

pH 10.0, the higher the relative intensities of the newly formed components (see, e.g., peaks N and O in Fig. 2).

A possible explanation for the appearance of components with higher MW may be the attack by papain at a different position in the hinge region. As mentioned before, papain digestion of alkali-treated MN12 requires a longer incubation period compared to untreated MN12. This may indicate that the conformation of the hinge region has changed. The cleavage site of papain is determined by the accessibility of amino acid residues to the enzyme rather than by a specificity of papain for particular amino acid residues or sequences (22). Thus, a conformational change in the hinge region could alter not only the sensitivity to papain digestion, but also the papain cleavage site. The appearance of components with higher molecular masses suggests that part of the alkali-treated MN12 molecules is attacked by papain at a position closer to the N-terminal position of the heavy chain. For instance, components M, N, and O in the ES-MS spectra of F_c subunits of alkali-treated MN12 may be elongated components corresponding to peaks I, K, and L.

The following hinge amino acid sequence has been reported for mouse IgG_{2a} (9):

$$^{1}\text{Glu–Pro–Arg–Gly–Pro–Thr–Ile–Lys–Pro–Cys–Cys–Pro–Pro–Lys}^{14}$$

The difference between the measured values for components M and I, N and K, and O and L varies from 520 to 529 Da (calculated from the molecular masses of these components listed in Table III). These molecular mass differences correspond with the calculated average residual molecular mass of 524.6 Da for amino acid sequence 3–7 (Arg–Gly–Pro–Thr–Ile) of the above-mentioned mouse IgG_{2a} hinge region.

Concluding Remarks

The results show that ES-MS permits the precise determination of the masses of reduced and alkylated monoclonal F_c subunits. The spectra obtained for the two Mabs are clearly different from each other, which illustrates the applicability of this technique as an identity test for Mabs preparations. The high resolution of this technique permitted the detection of several components, which could be ascribed to carbohydrate heterogeneity. We recognize, however, that further study is needed to confirm the origin of the mass heterogeneity. Not only variation in the carbohydrate content, but also amino acid substitutions could contribute to the observed mass heterogeneity. Also, the potential of ES-MS for the analysis of Mabs and Mab fragments is not yet fully explored and requires further investigation.

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