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Characterization of the F(ab′)$_2$ fragment of a murine monoclonal antibody using capillary isoelectric focusing and electrospray ionization mass spectrometry

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Abstract

We characterized a F(ab′)$_2$ fragment obtained by pepsin cleavage from a murine monoclonal IgG3 by means of electrospray ionization (ESI) mass spectrometry (MS), capillary isoelectric focusing (cIEF), high-performance anion-exchange chromatography–pulsed amperometric detection (HPAEC–PAD) and LC–MS peptide mapping. Separation of the fragment by cIEF under nonreducing conditions resulted in a number of distinct peaks. Using reducing conditions the heavy chain and the light chain were separated into two peaks each. Analysis by ESI-MS revealed a high mass heterogeneity of the molecule. Digestion with neuraminidase simplified both the cIEF pattern and the mass spectrum. The cIEF of the reduced molecule showed that the sialic acids were located only on the heavy chain of the F(ab′)$_2$-fragment. By incubation with *O*-glycosidase a further reduction of the complexity of the mass spectrum was achieved showing 8 different isoforms. By LC–MS peptide mapping these isoforms could be attributed to the heterogeneity of the pepsin cleavage site in the hinge region of the antibody. The sugars of the *O*-linked carbohydrate chain were identified by HPAEC–PAD as galactosyl-*N*-acetyl-galactosamine (GalNAcGal) with terminal *N*-glycolylneuraminic acid. The glycosylation site was identified by peptide mapping and amino acid sequence analysis as Ser$_{222}$.

Keywords: Isoelectric focusing; Monoclonal antibodies; Immunoglobulins

1. Introduction

Monoclonal antibodies (MAbs) have found a widespread use as diagnostic and therapeutic tools in a number of clinical indications [1]. Murine MAbs can cause the formation of an unwanted immune response in patients (human anti-mouse antibodies or HAMAs) when applied in vivo. This disadvantage can be minimized when using only the Fab or F(ab′)$_2$ fragments as the immunogenic properties of the IgG are predominantly located on the Fc part of the molecule [2]. Additionally, proteolytic fragments show improved pharmacokinetics and reduced non-specific binding compared to the whole IgG [3].

We manufacture a F(ab′)$_2$ fragment of a murine IgG3 isolated from the cellfree culture supernatant of a mouse–mouse hybridoma cell line. The F(ab′)$_2$ fragment is generated by pepsin cleavage of the whole IgG molecule. Pepsin cleaves in the hinge region of the IgG leading to the F(ab′)$_2$ and the Fc′ fragments (Fig. 1). Pepsin has a rather broad specificity towards cleavage sites but cleaves predominantly at aromatic and hydrophobic residues like Phe and Leu [4]. Due to this broad specificity a certain heterogeneity of the C-terminal end of the F(ab′)$_2$ fragment can be expected.

Murine IgGs carry one *N*-glycosylation site on the

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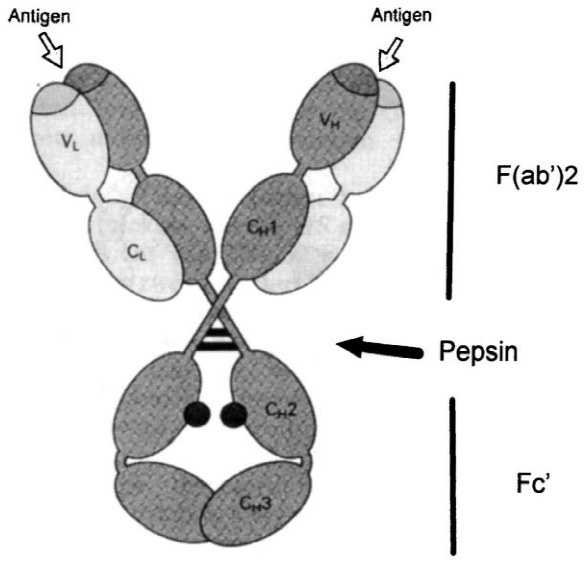

Fig. 1. Schematic structure of an IgG antibody showing the different domains of the heavy and light chains, the disulfide bonds in the hinge region and the *N*-glycosylation site in the C_H2 domain of the heavy chain. Pepsin cleaves on the C-terminal side of the disulfide bridges in the hinge region. The cleavage leads to the $F(ab')_2$ fragment consisting of the V_L, C_H1 and the hinge region. The Fc′ part is further cleaved by the action of the protease to small peptides.

C_H2 domain of the heavy chain which is removed with the Fc′ part by pepsin cleavage. However, *N*- as well as *O*-glycosylation have also been reported to occur in the Fab part of IgGs [2]. Thus, heterogeneity due to glycosylation has to be considered for $F(ab')_2$ antibody fragments, too. For a therapeutic antibody consistency of production batches is an essential prerequisite. We therefore developed a set of methods to investigate and characterize a possible heterogeneity of the $F(ab')_2$ fragment.

Capillary isoelectric focusing (cIEF) and electrospray ionization mass spectrometry (ESI-MS) are increasingly used for the characterization of monoclonal antibodies and recombinant proteins. Compared to classical isoelectric focusing on polyacrylamide gels cIEF offers a number of advantages: shorter separation times, higher resolution and an easier and more precise quantitation. Since cIEF can resolve proteins with only tiny differences in isoelectric point (p*I*), it is a very suitable method for the separation of isoforms with modifications that lead to changes in the number of charged groups [5–12]. Complementary to cIEF, which separates proteins according to their charge, ESI-MS allows the determination of molecular masses with very high accuracy. Thus, even minor modifications in the primary structure of a molecule can be detected. ESI-MS was reported in several characterization studies of intact humanized antibodies [13–15] or recombinant engineered fragments [16].

Besides cIEF and ESI-MS, LC–MS peptide mapping and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) were also used to study the microheterogeneity of the $F(ab')_2$ fragment of the murine IgG3 antibody described in this paper.

2. Experimental

2.1. CE instrumentation and methods

cIEF was performed on a P/ACE 5500 (Beckman, Fullerton, CA, USA) equipped with a UV detector [5]. The capillary was thermostated at 25°C. The absorbance of the focused proteins was detected at 280 nm. Samples were diluted to a concentration of 300–500 ng/μl and applied by filling the whole capillary with a 1:1 mixture of ampholyte and sample. Since migration times were influenced e.g. by the salt content of the sample, internal p*I* markers were used for easier peak assignment. Therefore cIEF was performed twice for each sample with and without the addition of p*I* markers to the sample/ampholyte mixture. In between runs the capillary was rinsed with 20 mmol/l H_3PO_4.

cIEF under nondenaturing conditions was performed in a coated capillary (μ-Sil DB-1, 27 cm×50 μm I.D., J&W Scientific, Folsom, CA, USA). To further reduce the electroosmotic flow (EOF) methylcellulose was added to the ampholyte mixture [4% Pharmalyte 3–10, 1.0% *N,N,N′,N′*,-tetramethylethylenediamine (TEMED)] using a 1% stock solution to give a final concentration of 0.8%. The catholyte consisted of 20 mmol/l NaOH and the anolyte of 20 mmol/l H_3PO_4. Samples were first focused for 5 min at 25 kV and were then mobilized

chemically by changing the composition of the catholyte to 30 mmol/l NaCl+20 mmol/l NaOH. To allow the assignment of peaks in different samples cytochrome *c* and myoglobin were used as internal p*I* markers.

For cIEF under denaturing and reducing conditions a one-step focusing method was applied using the residual EOF for mobilization. Samples were diluted in 8 mol/l urea and 2% dithiothreitol (DTT) and mixed in a 1:1 ratio with the ampholyte (4% Pharmalyte 3–10, 1.5% TEMED in 8 mol/l urea, 2% DTT). Focusing with simultaneous mobilization took place at 15 kV using reversed polarity in the shorter part of the capillary (37 cm×50 μm I.D., eCAP Neutral Capillary, Beckman). The catholyte consisted of 20 mmol/l NaOH and the anolyte of 20 mmol/l H_3PO_4. As internal p*I* markers cytochrome *c* and β-lactoglobulin A were used.

2.2. Mass spectrometry

ESI-MS of the antibody fragment was performed using the API 100 (PE-Sciex, Toronto, Canada) quadrupole mass spectrometer in the positive ion mode. Samples were delivered by a pump at 5 μl/min using a 100 μl syringe directly to the stainless steel sprayer held at +4 kV. The orifice potential was 40 V. Data acquisition was from *m/z* 1740 to *m/z* 1915 in 0.2 u steps and 1.0 ms dwell time per step.

Sample preparation for ESI-MS: 1 mg of the antibody preparation was desalted on a fast desalting column (HR10/10; Pharmacia, Uppsala, Sweden) equilibrated with 40% acetonitrile, 1% formic acid. The eluate with a protein concentration of about 1 mg/ml was directly used for ESI-MS.

LC–MS runs were performed on an RP C_{18} column (250×1 mm, 120 Å, 3 μm; YMC, Schermbeck, Germany), with a 170 min gradient from 5–56% acetonitrile in 0.1% trifluoroacetic acid (TFA). About 45 μg protein were loaded onto the column. The HPLC system (120A series, PE-ABI, Forster City, CA, USA) was equipped with a 120 nl flow cell (LC Packings, San Francisco, CA, USA). After the column the flow of 50 μl/min was split and the eluting peptides were delivered to the spraying tip with 10 μl/min. The orifice potential was 40 V. Data were collected from *m/z* 300 to *m/z* 2000 in 0.2 u steps and 0.5 ms dwell time per step.

2.3. Digests

Glycosidase digests: 150 μg antibody were incubated in 20 mmol/l potassium phosphate buffer, pH 7.2 with 30 mU neuraminidase and 5 mU *O*-glycosidase for 18 h at 37°C. Glycerin present as stabilizing agent in the commercial preparation of the *O*-glycosidase was removed by diafiltration in a filtration unit (M_r 30 000 membrane) prior to use.

Protease digest: The antibody was denatured, reduced and carboxymethylated using a standard protocol [17] and was then transferred into the digestion buffer using a Pharmacia fast desalting column (HR10/10). Digestion with endoproteinase Lys C sequencing grade was in 25 mmol/l Tris, 1 mmol/l EDTA, pH 8.5 for 18 h at 37°C. The ratio of protease to antibody was 1:25 (w/w). The reaction was stopped by acidifying the incubation mixture with TFA.

2.4. Carbohydrate analysis

HPAEC–PAD analysis of the released sugars was performed on a DX-300 Series HPLC-system (Dionex, Sunnyvale, CA, USA) equipped with a pulsed amperometric detector and a Carbo-Pac PA-100 column (Dionex). Carbohydrates were separated at a flow-rate of 1 ml/min using a biphasic acetate gradient in 0.1 mol/l NaOH (2 to 10 min from 0 to 20 mmol/l sodium acetate, 10–25 min from 20 mmol/l to 250 mmol/l sodium acetate).

2.5. Reagents and materials

As ampholyte for cIEF Pharmalyte 3–10 (Pharmacia) was used. TEMED (research grade) was obtained from Boehringer Ingelheim (Ingelheim, Heidelberg, Germany) and DTT from Boehringer Mannheim (Mannheim, Germany). Methylcellulose (4000 cps) and the p*I* markers were purchased from Sigma (Deisenhofen, Germany). The enzymes neuraminidase (*A. ureafaciens*), *O*-glycosidase (*D. pneumoniae*) and endoproteinase LysC sequencing grade were obtained from Boehringer Mannheim. All other chemicals used were of the highest purity available.

3. Results and discussion

Charge heterogeneity of the $F(ab')_2$ antibody was shown by cIEF under nondenaturing conditions (Fig. 2(a)). Five groups of peaks are well resolved with partial resolution within the groups. Digestion with neuraminidase led to a reduction of the number of acidic groups and thus to a shift of the desialylated isoforms to more basic values. Fig. 2(b) shows the cIEF pattern after removal of the sialic acids. The number of peaks is reduced but still three different isoforms can be observed suggesting a further modification of the antibody.

The isoform pattern could be simplified by performing the cIEF under denaturing and reducing conditions. As the disulfide bridges are cleaved the isoforms of heavy and light chains are focused separately, the more basic being heavy chain isoforms and the more acidic being light chain isoforms (Fig. 3(a)). After neuraminidase treatment the more acidic isoform (HC 2) of the heavy chain is largely reduced due to the removal of sialic acid whereas the more basic one (HC 1) increases (Fig. 3(b)): The sum of the peak areas of HC 1 and HC 2 remains constant and the peak areas of LC 1 and LC 2 remain unchanged. Further digestion with *O*-glycosidase does not result in any changes in the cIEF pattern compared to that of the desialylated sample as only neutral carbohydrates are released and the number of charged groups remains unaltered.

This allows the conclusion that a glycosylation site is located on the heavy chain and a further site for modification is located on the light chain. Since the two isoforms of the light chain are not influenced by the neuraminidase treatment glycosylation can be excluded as a source of the observed modification of the light chain. Deamidation, leading to a shift to more acidic p*I* due to the generation of an additional acidic group, is often another reason for charge heterogeneity of proteins [18]. It occurs preferentially at an asparaginyl residue with glycine or serine on the C-terminal side [19]. As the sequence of the

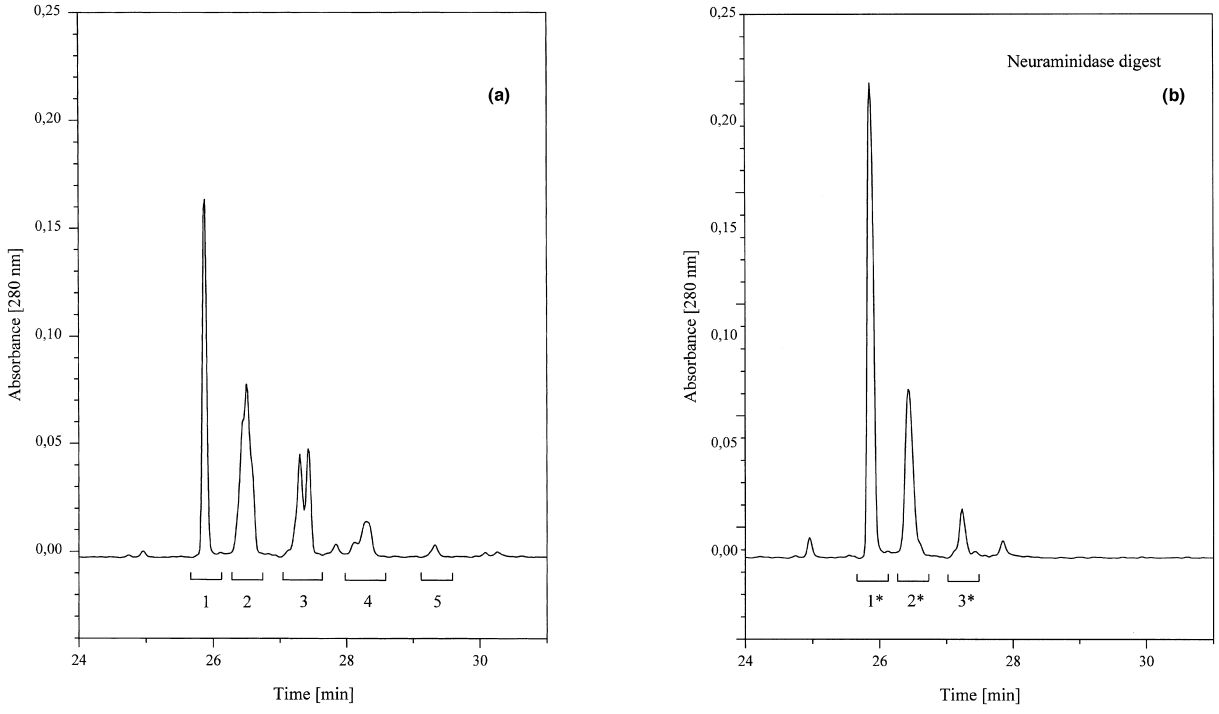

Fig. 2. cIEF with chemical mobilization under non-denaturing conditions. (a) Isoform pattern of the $F(ab')_2$ antibody showing five groups of peaks. (b) Isoform pattern after neuraminidase treatment. The number of peaks is reduced due to the removal of sialic acids. For a further interpretation of the observed heterogeneity see Table 1.

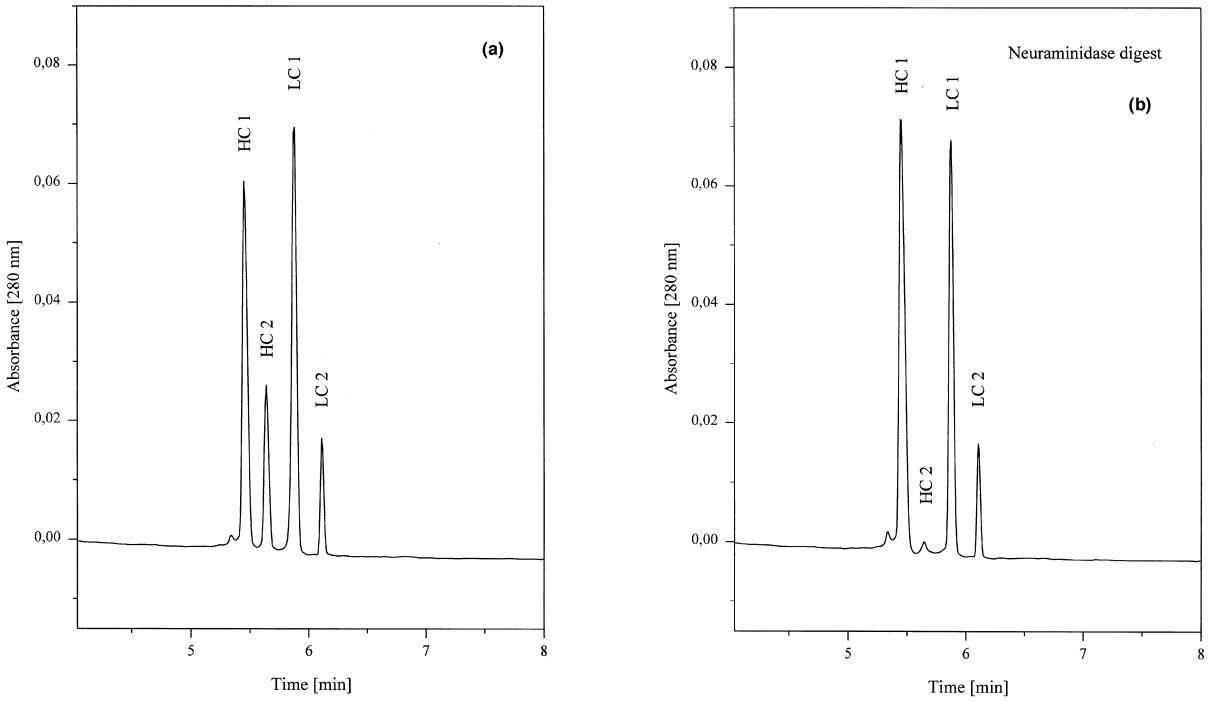

Fig. 3. cIEF under reducing conditions with EOF mobilization. (a) Isoform pattern of heavy chain (HC) and light chain (LC) of the $F(ab')_2$ antibody. (b) Isoform pattern after neuraminidase treatment. The more acidic isoform of the heavy chain HC 2 is largely reduced due to the removal of sialic acid, while HC 1 increases. The peak areas of the light chain isoforms remain unaltered.

light chain includes an Asn^{125}–Gly^{126}, deamidation is very likely the cause for the observed charge heterogeneity of the light chain. This assumption is supported by ESI-MS of the reduced $F(ab')_2$. Only a single molecular mass was found for the light chain (23 778±2) correlating within the accuracy of the

Table 1
Correlation between possible isoforms and heterogeneity observed in non-reducing cIEF in Fig. 2

Peak/ group[b]	HC 1[a]	HC 2[a] (sialylated)	LC 1[a]	LC 2[a] (deamidated)	Number of additional acidic groups
1	2	–	2	–	0
2	1	1	2	–	1
	2	–	1	1	1
3	1	1	1	1	2
	–	2	2	–	2
	2	–	–	2	2
4	1	1	–	2	3
	–	2	1	1	3
5	–	2	–	2	4
1*	2	–	2	–	0
2*	2	–	1	1	1
3*	2	–	–	2	2

[a] Number of modified or unmodified heavy chains (HC) and light chains (LC) in $F(ab')_2$ isoforms.
[b] Peak assignment as in Fig. 2.

instrument (0.01%) with the theoretical mass deduced from the protein sequence (23 777.4). To our knowledge only deamidation can cause an acidic shift of the p*I* together with a mass difference of <2.

From the cIEF pattern under reducing conditions the complex pattern in Fig. 2 can be explained. All combinations of heavy/light chains with and without a modification lead to a total of five groups of isoforms differing in one charged group each as shown in Table 1: Peak 1 corresponds to the unmodified antibody, group 2 includes isoforms with one modification, either on the light or on the heavy chain, group 3 consists of all isoforms bearing two modifications, group 4 of isoforms with three modifications and peak 5 is the isoform with a modification on each chain. Since small differences in p*I* can be expected between a sialylated and a possibly deamidated isoform, a partial resolution is observed within the groups. After neuraminidase digestion charge heterogeneity is only caused by the partial deamidation of the light chain resulting in three isoforms.

ESI-MS analysis gave a rather complex mass spectrum (Fig. 4). The deconvoluted spectrum revealed mass heterogeneity as expected for a pepsin generated glycosylated $F(ab')_2$ antibody. As shown in Table 2 eight molecular masses of the deconvoluted spectrum could be assigned to a C-terminal heterogeneity. Peaks 4 and 7 were interpreted as phosphate adducts of two isoforms. Adducts in mass spectra of proteins are widely experienced and can originate from incomplete removal of the phosphate buffer prior to spectra recording.

Pepsin shows a rather broad specificity towards peptide bonds but cleaves preferentially at aromatic and hydrophobic residues like Phe and Leu [4]. In the case of the murine IgG3 antibody pepsin cleavage occurred after Leu, Gly and Ser. To our best knowledge the cleavage sites of pepsin within the hinge region of a murine IgG3 antibody are reported here for the first time.

The further evaluation of the deconvoluted mass spectrum showed that the C-terminal isoform pattern was repeated twice at 672 higher masses each. This mass difference of 672 can be explained by the *O*-glycosylation of the $F(ab')_2$. After neuraminidase digestion a mass shift of about 307 was observed for some of the peaks, which correlates with the removal

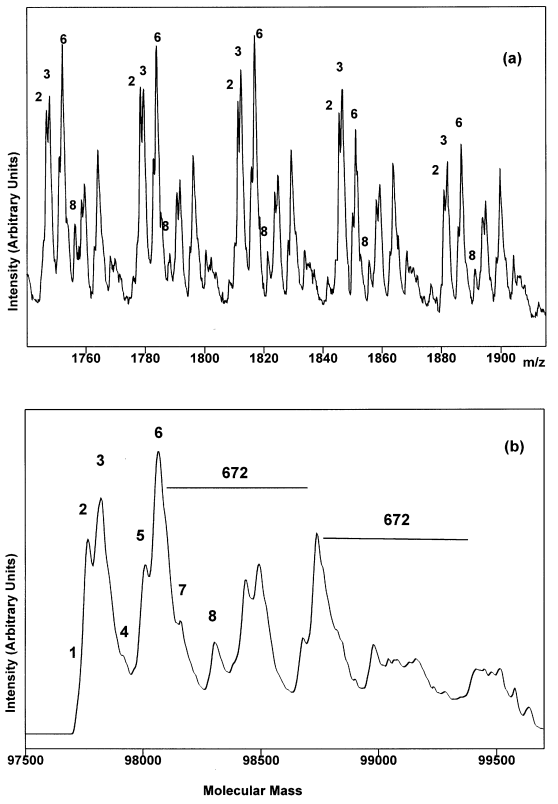

Fig. 4. ESI-MS of the $F(ab')_2$ antibody. (a) Raw spectrum recorded as described in Section 2. (b) Deconvoluted spectrum. The total m/z range was included into the calculation of the deconvoluted spectrum from the raw spectrum. Deconvolution was performend using the Sciex Bioreconstruct©software.

of one *N*-glycolyl neuraminic acid (NGNA) residue. Neuramindase cleaves the sialic acids NGNA as well as *N*-acetyl neuraminic acid (NANA) which differ in molecular mass [20]. Digestion with neuraminidase and *O*-glycosidase reduced mass heterogeneity to that of the C-terminal isoforms (Fig. 5). The additional mass shift of about 365 fitted the known substrate specificity of the *O*-glycosidase for the disaccharide galactosyl-*N*-acetyl-galactosamine (Gal-NAcGal) [21].

Although the quantitative information of mass spectra is limited, the relative intensities of the mass peaks indicate that the glycosylated isoforms predominantly carry only one *O*-glycan structure since the fully glycosylated $F(ab')_2$ gave only a weak

Table 2
C-terminal mass heterogeneity

Peak	Experimental mass	Interpretation (C-terminal sequence of the heavy chain: ... SSCPPGNILGGPS)	Theoretical mass	Delta mass
1	97 708	... NIL/ ... NIL	97 697	11
2	97 764	... NIL/ ... NILG	97 754	10
3	97 819	... NILG/ ... NILG ... NIL/ ... NILGG	97 811	8
4	97 905	3+Phosphate	97 909	4
5	98 002	... NIL/ ... NILGGPS	97 996	6
6	98 059	... NILG/ ... NILGGPS	98 052	7
7	98 150	6+Phosphate	98 150	0
8	98 304	... NILGGPS/ ... NILGGPS	98 294	10

Peak numbers correspond to the peak labels in Fig. 4. "*x*+Phosphate" indicates a phosphate adduct of the C-terminal isoform detected in peak *x*. The experimental masses were determined by calculating the corresponding hypermasses of the five most intensive *m*/*z* series shown in Fig. 4(a). Hypermasses are generally more exact than the molecular masses derived from the deconvoluted spectrum. The theoretical masses were calculated from the (DNA derived) protein sequence. For all peaks the difference between the experimental mass and the respective theoretical mass (delta mass) was within the mass accuracy (0.01%) of the mass spectrometer.

signal. These results are supported by the cIEF pattern under nonreducing conditions.

The molecular mass spectra as well as LC–MS data (see below) showed that predominantly only a single NGNA is attached to the *O*-glycan. *O*-glycosylation was reported for rabbit and mouse antibodies [22–25]. Asymmetric attachment of the carbohydrate to only one heavy chain seems to be a common feature of *O*-glycosylation as it was found for the mouse and rabbit antibodies, too. However, the *O*-glycans found in mouse IgG2b are tetrasaccharides with two sialic acids [22].

From the cIEF peak areas of the reduced $F(ab')_2$ we calculated that approximately 28% of the heavy chains are *O*-glycosylated. This is in good agreement with the estimated 40% asymmetrically glycoslyated $F(ab')_2$ found in the molecular mass spectrum.

To further analyze the *O*-glycosylation we additionally separated the sugars released by glycosidase digestion using HPAEC–PAD (Fig. 6). By comparing the retention times with standard carbohydrates we could show that the sugars attached to the antibody fragment are in fact NGNA and GalNAcGal. A very small amount of NANA was also found. Masses of isoforms which carry NANA could not be assigned in the mass spectrum. However, only a very small amount of NANA isoforms was expected from the HPAEC–PAD chromatogram. Due to the complexity of the ESI-MS spectrum mass signals of NANA isoforms are probably too small to be identified. The *O*-glycan structure of the $F(ab')_2$ fragment is given in Fig. 7.

To prove the C-terminal heterogeneity as deduced from the molecular masses LC–MS peptide mapping was performed. The part of the peptide map comprising the C-terminal peptides is shown in Fig. 8. Peaks 1 and 2 contained three C-terminal peptides which were identified by their corresponding masses (Table 3). According to Table 2 four C-terminal peptides could be expected. However, the mass signal for the peptide with the C-terminal sequence ... NILGG was not detected in the peptide map. Therefore only the isoform ... NILG/ ... NILG contributes to peak 3 of the mass spectrum. The UV signal of peak 2 was about twice the signal of peak 1 and in addition the mass signal obtained for peptide ... NILG was far more intense than that of peptide ... NILGGPS. Although mass data and UV data are in general not comparable the distribution of the peak intensities indicate that the peptide ending with ... NILG is the most frequent C-terminal peptide. This is in agreement with the major isoforms of $F(ab')_2$ obtained after pepsin cleavage (peaks 2, 3, 6 in the molecular mass spectrum in Fig. 4).

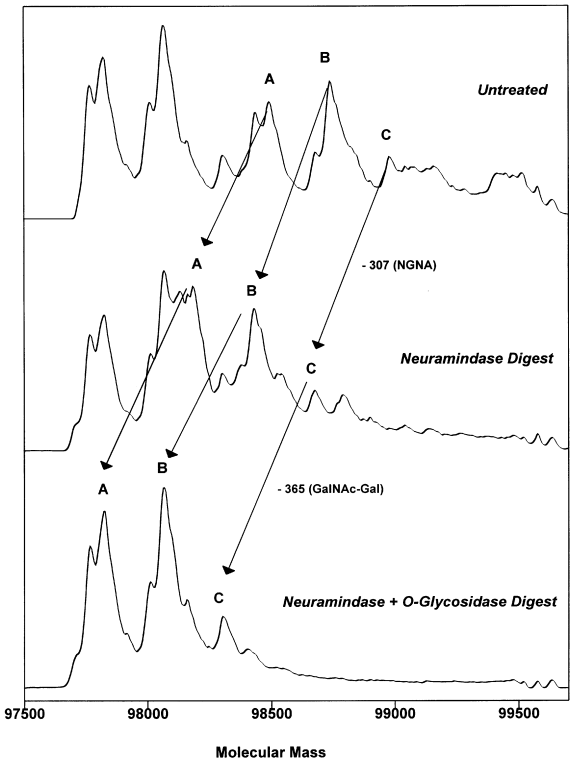

Fig. 5. Influence of glycosidase digestion on the mass spectrum. The deconvoluted spectra were calculated from the 1740–1915 m/z range recorded. A, B C indicate the mass shift observed upon glycosidase digestions. The observed difference of about 307 corresponds to the removal of NGNA, the additional reduction of about 365 to the disaccharide GalNAcGal.

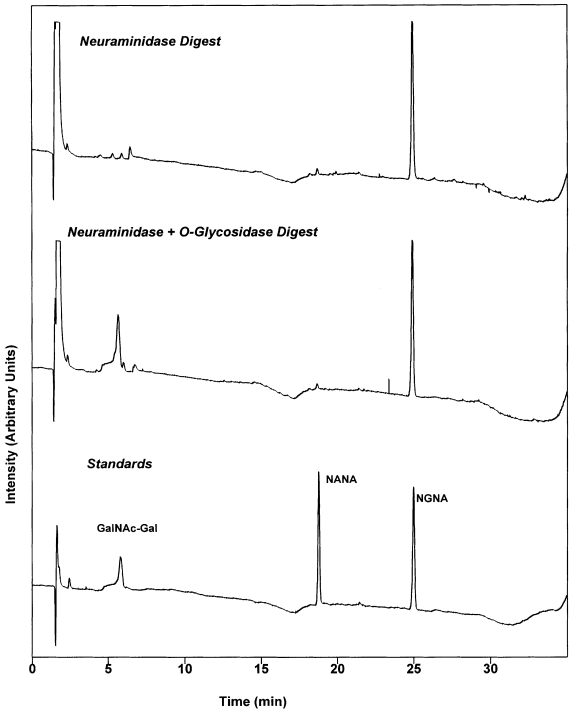

Fig. 6. HPAEC–PAD analysis of the released carbohydrates.

The *O*-glycosylation site was also located on the C-terminal peptides. A corresponding set of C-terminal peptides (peak 3 and peak 4) was identified in the peptide map. Peak 3 eluted as the shoulder of another peptide of the map.

For the identification of the glycosylated amino acid the two glycopeptide containing peaks were sequenced. The common sequence of the C-terminal peptides is RIEPRIPKPS$_{222}$TPPGSSCPPGNIL . . . and contains 3 Ser and 1 Thr as possible glycosylation sites. In the sequencing cycle where the Ser$_{222}$ was expected no phenylthiohydantoin (PTH)-amino acid was found. For the other residues the PTH-amino acid yield was in accordance with the repetitive yield of the sequencer. These results show that only Ser$_{222}$ is glycosylated. *O*-glycosylation occurs at Ser and Thr residues but in contrast to *N*-glycosylation a consensus sequence site is not known. However, prolin-associated sequence motifs for *O*-glycosylation were determined [26]. The sequence motif Pro–Ser/Thr–X–X–Pro was identified to be preferentially but not necessarily *O*-glycosylated [27]. The *O*-glycosylation of Ser$_{222}$ fits this sequence pattern very well.

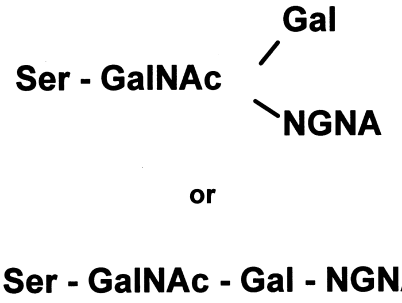

Fig. 7. Proposed *O*-glycan structure of the F(ab′)$_2$ fragment.

Table 3
C-terminal and glycosylated peptides

Peak	Experimental mass	Theoretical mass	Amino acid sequence interpretation
2	2471.3	2471.8	RIEPRIPKPSTPPGSSCPPGNIL
1	2528.3	2528.9	RIEPRIPKPSTPPGSSCPPGNILG
	2769.7	2770.1	RIEPRIPKPSTPPGSSCPPGNILGGPS
4	3144.2	3144.5	RIEPRIPKPSTPPGSSCPPGNIL-*O*-glycan
3	3200.9	3201.5	RIEPRIPKPSTPPGSSCPPGNILG-*O*-glycan
	3442.4	3442.8	RIEPRIPKPSTPPGSSCPPGNILGGPS-*O*-glycan

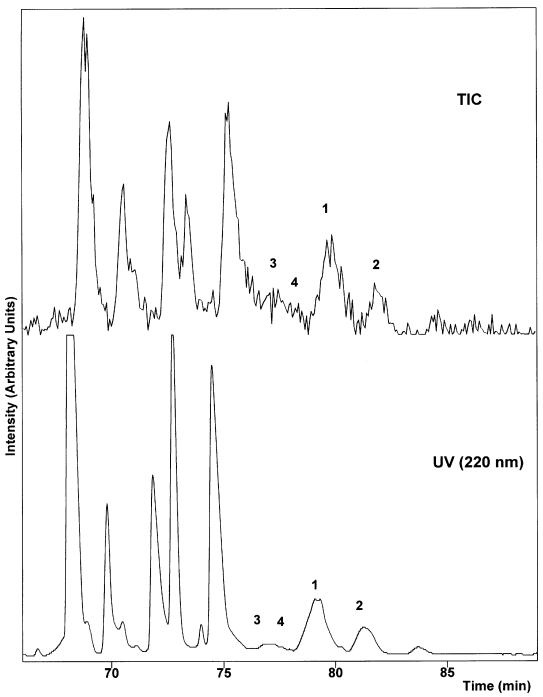

Fig. 8. LC–MS peptide mapping. Expanded view from 65 to 90 min. Peptides were generated with endoproteinase Lys C and analysed as described in Section 2. TIC: total ion current.

For one peptide of the light chain the expected mass and a mass differing by −1 were found in LC–MS (data not shown). As discussed above a possible deamidation could explain the second peak of the light chain in the cIEF pattern and is in agreement with the MS data of the reduced antibody. However, since deamidation can also occur during alkylation and protease digestion a more detailed investigation is necessary to confirm the deamidation.

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