Amino Acids (2010) 38:1043–1049
DOI 10.1007/s00726-009-0312-1

ORIGINAL ARTICLE

Complete sequencing of the recombinant granulocyte-colony stimulating factor (filgrastim) and detection of biotinylation by mass spectrometry

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Received: 10 February 2009 / Accepted: 30 May 2009 / Published online: 13 June 2009

Abstract Granulocyte-colony stimulating factor stimulates production and antibacterial function of neutrophiles. Therapy using the recombinant protein drug represents a major step forward in oncology. The protein has not been, however, completely sequenced at the protein level and this formed the rationale of the current study. Recombinant G-CSF (filgrastim) was run on two-dimensional gel electrophoresis (2DE), the protein was in-gel digested with trypsin and chymotrypsin, and peptides were analysed on Nano-ESI-LC–MS/MS (high performance ion trap, HCT). Bioinformatic tools used were Mascot v2.2 and Modiro^{TM} v1.1 softwares. A single spot was detected on 2DE and peptides resulting from in-gel digestion were unambiguously identified by the MS/MS approach leading to complete sequencing when both searching engines were applied. N-terminal methionine loss, N-terminal methionine oxidation and amidination were observed. Both softwares identified modifications. Complete sequencing by a non-sophisticated and rapid gel-based mass spectrometry approach confirmed the primary structure predicted from nucleic acid sequences. A chemical modification of glutamine 26 with the interim name PentylamineBiotin (Unimod accession number #800) compatible with biotinylation with 5-(biotinamido) pentylamine by the producer was detected by both softwares. Although there is some evidence that biotinylated G-CSF analogues are active, it remains open whether this modification may be responsible for the side effects observed or lead to changes of antigenicity.

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Keywords Two-dimensional electrophoresis · Filgrastim · PentylamineBiotin · Complete sequencing · Mascot · Modiro

Introduction

Recombinant granulocyte-colony stimulating factor (G-CSF) is an inevitable therapy for granulopenia and representing a major step forwards in oncology. Molecular cloning and expression of cDNA for the human G-CSF has been described (Nagata et al. 1986; Souza et al. 1986). The mature human G-CSF is an 18.8-kDa protein of 174 amino acids cleaved from a precursor of 207 amino acids. It contains two intra-molecular disulphide bonds and one free cysteine 17 (Lu et al. 1989). Native hG-CSF has a single O-glycosylation site at Thr133 protecting the protein from aggregation although not being relevant for biological activity (Oh-eda et al. 1990). A short and a long isoform have been reported, the longer one represents the canonical sequence (P09919-1).

The recombinant human G-CSF (rhG-CSF) from *E. coli* has identical biological activity and only differs in that it contains an N-terminal methionine residue and is of course not glycosylated (Vanz et al. 2008).

Chemical modification of rhG-CSF by polyethylene glycol resulted in decreased renal clearance of the protein in rats (Yang et al. 2004) and biotinylation analogues were proposed to show no differences in biological activity (Angelotti et al. 1991). rhG-CSF from *E. coli* was reported to be oxidised by Leon et al. (1999). One mono-, one di-, and two trioxidation products of methionine were described. An oxidative folding pathway of rhG-CSF was reported by Lu et al. (1992) showing that disulfide bond formation is crucial for maintaining the molecule in a

properly folded and biologically active form. Incomplete sequencing of rhG-CSF was published by Jones et al. (1994) by peptide map analysis.

Knowledge of the complete sequence is, however, mandatory because only the full primary structure allows determination of splice variants, posttranslational modifications and, more specifically, generation of antibodies. Moreover, changes of primary structure are required for understanding antigenicity of rhG-CSF that may be responsible for probable side effects generated by the protein drug.

Materials and methods

Sample preparation

NEUPOGEN® filgrastim 5 mL (0.3 mg protein/mL) (Amgen Inc., Thousand Oaks, CA) was added to 2 mL of sample buffer consisting of 8 M urea (Merck, Darmstadt, Germany), 4% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate) (Sigma, St. Louis, MO), 10 mM 1,4-dithioerythritol (Merck, Germany) and 0.5% carrier ampholytes "Resolyte" 3,5–10 (BDH Laboratory Supplies, Electran®, UK). The suspension was transferred into Ultrafree-4 centrifugal filter units (Millipore, Bedford, MA) for desalting and concentrating proteins. The protein content of the supernatant was quantified by the Bradford protein assay system (Bradford 1976). The standard curve was generated using bovine serum albumin and absorbance was measured at 595 nm.

Two-dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis was performed essentially as reported (Gruber-Olipitz et al. 2006; John et al. 2008; Myung et al. 2008). 50 µg of protein was applied on immobilized pH 3–10 nonlinear gradient strips in sample cups at their basic and acidic ends. Focusing was started at 200 V and the voltage was gradually increased to 8,000 V at 4 V/min and then kept constant for a further 3 h (approximately 150,000 V h totally). After the first dimension, strips (18 cm) were equilibrated for 15 min in the buffer containing 6 M urea, 20% glycerol, 2% SDS, 2% DTT and then for 15 min in the same buffer containing 2.5% iodoacetamide instead of DTT. After equilibration, strips were loaded on 10–16% gradient sodium dodecylsulfate polyacrylamide gels for second-dimensional separation. The gels (180 × 200 × 1.5 mm) were run at 40 mA per gel. Immediately after the second dimension run, gels were fixed for 12 h in 50% methanol, containing 10% acetic acid, the gels were stained with Colloidal Coomassie Blue (Gulesserian et al. 2007; Novex, San Diego, CA) for 12 h on a rocking shaker. Molecular masses were determined by running standard protein markers (Biorad Laboratories, Hercules, CA) covering the range 10–250 kDa. pI values were used as given by the supplier of the immobilized pH gradient strips (Amersham Bioscience, Uppsala, Sweden). Excess of dye was washed out from the gels with distilled water and the gels were scanned with ImageScanner (Shin et al. 2006; Amersham Bioscience, Uppsala, Sweden).

NanoLC-ESI-CID/ETD-MSMS analysis

Gel pieces of interest were cut and put into a 1.5-mL tube. They were washed with 10 mM ammonium bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate repeatedly. Addition of 100% acetonitrile resulted in gel shrinking and the shrunk gel plugs were then speedVac dried in a Speedvac Concentrator 5301 (Eppendorf, Germany). The dried gel pieces were re-swollen and in-gel digested with 40 ng/µL trypsin (Promega, Madison, WI) in digestion buffer (consisting of 5 mM octyl β-D-glucopyranoside (OGP) and 10 mM ammonium bicarbonate) and incubated over night at 37°C. Chymotrypsin digestion was performed by addition of 25 mM ammonium bicarbonate containing 25 ng/µL chymotrypsin (sequencing grade; Roche diagnostic) and incubated for 30 min at 30°C. Peptide extraction was performed with 15 µL of 1% formic acid (FA) in 5 mM OGP for 30 min, 15 µL 0.1% FA for 30 min and 15 µL 0.1% TFA in 20% acetonitrile for

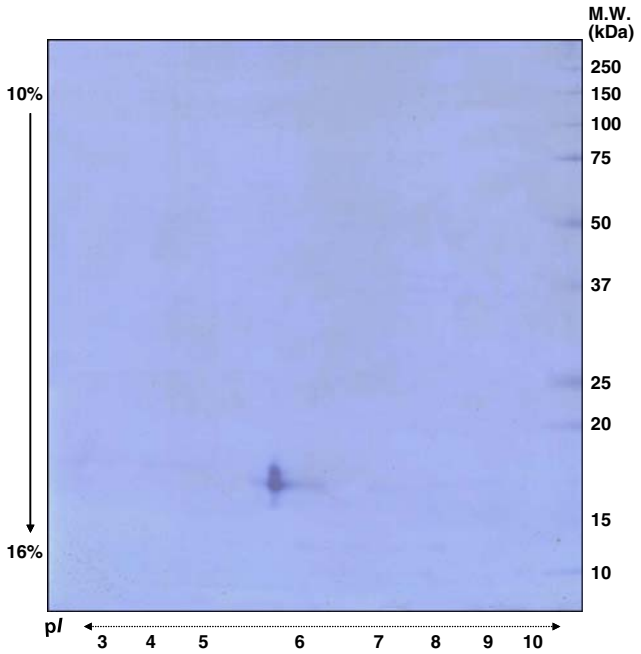

Fig. 1 Two-dimensional gel electrophoresis (2DE) of G-CSF protein. Second dimensional gel electrophoresis (2DE) with a gradient of 10–16% was applied. Experimental p*I* was 5.80/MW. 17.5 kDa, the theoretical p*I* was 5.65/MW. 18.8 kDa)

Table 1 Peptide identification by the Mascot v.2.2 software

START–END	Observed	Experimental $[M + H]^+$ ion (m/z)	Calculated $[M + H]^+$ ion (m/z)	Delta	Peptides	Ions score	Enzyme
1–14	724.79	1,447.58	1,447.70	−0.1167	- MTPLGPASSLPQSF.L	90	C
1–14	745.34	1,488.66	1,488.72	−0.0596	- MTPLGPASSLPQSF.L[a]	60	C
1–14	651.31	1,300.60	1,300.66	−0.0609	- TPLGPASSLPQSF.L[b]	30	C
1–17	901.96	1,801.92	1,801.92	−0.0074	- MTPLGPASSLPQSFLLK.C	99	C–T
2–17	828.47	1,654.94	1,654.89	0.0492	M.TPLGPASSLPQSFLLK.C	94	C–T
16–36	622.12	2,484.47	2,484.31	0.1589	L.LKCLEQVRKIQGDGAALQEKL.C	64	C
25–41	622.68	1,865.02	1,864.89	0.1226	K.IQGDGAALQEKLCATYK.L[c]	91	T
41–59	1,099.55	2,197.08	2,197.17	−0.0851	Y.KLCHPEELVLLGHSLGIPW.A	65	C
51–62	630.90	1,259.80	1,259.70	0.1018	L.LGHSLGIPWAPL.S	51	C
60–76	886.91	1,771.81	1,771.85	0.0490	W.APLSSCPSQALQLAGCL.S	106	C
63–79	910.39	1,818.77	1,818.86	−0.0854	L.SSCPSQALQLAGCLSQL.H	101	C
63–84	1,180.99	2,359.98	2,360.16	−0.1812	L.SSCPSQALQLAGCLSQLHSGLF.L	54	C
71–86	903.95	1,805.89	1,805.91	−0.0168	L.QLAGCLSQLHSGLFLY.Q	70	C
85–93	509.89	1,017.76	1,017.62	0.1458	F.LYQGLLQAL.E	28	C
91–107	877.47	1,752.93	1,752.91	0.0157	L.QALEGISPELGPTLDTL.Q	75	C
91–114	875.74	2,624.20	2,624.27	−0.0654	L.QALEGISPELGPTLDTLQLDVADF.A	37	C
94–114	1,115.48	2,228.95	2,229.10	−0.1502	L.EGISPELGPTLDTLQLDVADF.A	95	C
108–119	690.34	1,378.67	1,378.67	0.0026	L.QLDVADFATTIW.Q	51	C
115–125	691.27	1,380.53	1,380.62	−0.0925	F.ATTIWQQMEEL.G	38	C
120–145	913.41	2,737.21	2,737.25	−0.0399	W.QQMEELGMAPALQPTQGAMPAFASAF.Q	75	C
126–145	990.40	1,978.79	1,978.96	−0.1674	L.GMAPALQPTQGAMPAFASAF.Q	100	C
146–166	773.04	2,316.11	2316.23	−0.1198	F.QRRAGGVLVASHLQSFLEVSY.R	55	C
149–167	1,016.98	2,031.95	2,032.07	−0.1187	R.AGGVLVASHLQSFLEVSYR.V	130	T
154–169	616.69	1,847.05	1,846.99	0.0564	L.VASHLQSFLEVSYRVL.R	45	C

The use of this software alone lead to the generation of 96% sequence coverage

T trypsin, *C* chymotrypsin

[a] Methionine oxidation; N-terminal amidination

[b] Methionine-loss (N-terminal)

[c] PentylamineBiotin of glutamine (Q-26)

30 min. The extracted peptides were pooled for nano LC–ESI-CID/ETD-MSMS analysis.

The HPLC used was an Ultimate 3000 system (Dionex Corporation, Sunnyvale, CA) equipped with a PepMap100 C-18 trap column (300 μm × 5 mm) and PepMap100 C-18 analytic column (75 μm × 150 mm). The gradient was ($A = 0.1\%$ formic acid in water, $B = 0.08\%$ formic acid in acetonitrile) 4–30% B from 0 to 105 min, 80% B from 105 to 110 min, 4% B from 110 to 125 min. A HCT ultra ETD II (Bruker Daltonics, Bremen, Germany) was used to record peptide spectra over the mass range of m/z 350–1,500, and MS/MS spectra in information-dependent data acquisition over the mass range of m/z 100–2,800. Repeatedly, MS spectra were recorded followed by three data-dependent CID MS/MS spectra and three ETD MS/MS spectra generated from three highest intensity precursor ions. An active exclusion of 0.4 min after two spectra was used to detect low abundant peptides. The voltage between ion spray tip and spray shield was set to 1,500 V. Drying nitrogen gas was heated to 150°C and the flow rate was 10L/min. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted and peak lists were generated by DataAnalysis 3.4 (Bruker Daltonics, Bremen, Germany). Searches were done by using the Mascot 2.2 (Matrix Science, London, UK) against latest NCBI and UniprotKB database for protein identification. Searching parameters were set as follows: enzyme selected as trypsin with two maximum missing cleavage sites, species limited to human, a mass tolerance of 0.2 Da for peptide tolerance, 0.2 Da for MS/MS tolerance, fixed modification of carbamidomethyl (C) and

Table 2 Bioinformatic identification of peptides using the Modiro™ v.1.1 software

Start-end	*m/z* meas. [Da]	*m/z* theor. [Da]	Error [Da]	Peptide	Score	Enzyme
1–14	745.34	744.86	0.0893	.MTPLGPASSLPQSF.L[a]	400	T–C
1–17	893.95	893.99	−0.0422	.MTPLGPASSLPQSFLLK.C	509	T–C
2–17	828.48	828.47	0.008	.TPLGPASSLPQSFLLK.C[b]	422	T
1–24	716.8	716.61	0.1802	.MTPLGPASSLPQSFLLKCLEQVRK.I	345	T
15–19	942.41	942.39	0.0144	F.LLKCL.E	73	C
17–40	1002.38	1002.50	−0.125	L.KCLEQVRKIQGDGAALQEKLCATY.K[c]	386	T–C
41–55	582.34	582.31	0.0213	Y.KLCHPEELVLLGHSL.G	400	T–C
43–59	653.02	653.00	0.0151	L.CHPEELVLLGHSLGIPW.A	295	C
49–62	736.9	736.93	−0.0348	L.VLLGHSLGIPWAPL.S	338	C
60–76	886.9	886.93	−0.0371	W.APLSSCPSQALQLAGCL.S	543	C
63–83	738.67	738.69	−0.0229	L.SSCPSQALQLAGCLSQLHSGL.F	373	C
71–84	765.82	765.89	−0.0703	L.QLAGCLSQLHSGLF.L	417	C
91–107	877.41	877.46	−0.0545	L.QALEGISPELGPTLDTL.Q	544	C
110–125	600.05	599.97	0.0787	L.DVADFATTIWQQMEEL.G	354	C
115–131	682.02	681.97	0.0473	F.ATTIWQQMEELGMAPAL.Q	242	C
117–147	860.4	860.41	−0.0124	T.TIWQQMEELGMAPALQPTQGAMPAFASAFQR.R	533	T
120–145	913.38	913.42	−0.0456	W.QQMEELGMAPALQPTQGAMPAFASAF.Q	470	C
132–153	712.32	712.29	0.0243	L.QPTQGAMPAFASAFQRRAGGVL.V	217	C
142–158	610.48	610.32	0.153	F.ASAFQRRAGGVLVASHL.Q	256	C
146–166	773.03	773.08	−0.0552	F.QRRAGGVLVASHLQSFLEVSY.R	295	C
148–170	826.75	826.81	−0.0669	R.RAGGVLVASHLQSFLEVSYRVLR.H	256	T
154–169	616.66	616.67	−0.012	L.VASHLQSFLEVSYRVL.R	397	C
171–175	565.23	565.30	−0.0793	R.HLAQP.-	114	T

Using this software 94% sequence coverage was warranted

T trypsin, *C* chymotrypsin

[a] Methionine Oxidation; N-terminal Amidination

[b] Methionine-loss (N-terminal)

[c] PentylamineBiotin of glutamine (Q-26)

variable modification of methionine oxidation and phosphorylation (Tyr, Thr, and Ser). Positive protein identifications were based on a significant MOWSE score. After protein identification, an error-tolerant search was done to detect nonspecific cleavage and unassigned modifications. Protein identification and modification information returned from MASCOT were manually inspected and filtered to obtain confirmed protein identification and modification lists of CID MS/MS and ETD MS/MS.

Modiro™ v1.1 software (Protagen, Dortmund, Germany): enzyme selected as we used with two maximum missing cleavage sites, species limited to human, a peptide mass tolerance of 0.2 Da for peptide tolerance, 0.2 Da for fragment mass tolerance, modification 1 of carbamidomethyl (C) and modification 2 of methionine oxidation. Search for unknown mass shifts, search for amino acid substitution and calculate significance were selected on advanced PTM-explorer search strategies. Positive protein identification was first of all listed by spectra view and then each identified peptide was considered its significance based on 0.2 Da delta value, ion-charge status of peptide, b- and y-ion fragmentation quality and a significant score.

Gel-free ESI-MS analysis of NEUPOGEN® filgrastim

NEUPOGEN® filgrastim 50 μL (about 50 μg) was added to 1 mL cold acetone and kept at −20°C over night to precipitate the protein. Centrifugation was done at 14,000×*g* at 12°C for 15 min. Pellets were washed again with 1 mL cold acetone, centrifuged at 14,000×*g* at 12°C for 15 min, dried in a Speedvac Concentrator 5301 (Eppendorf, Germany) for 30 min, dissolved in 400 μL 1% formic acid, 50% acetonitrile. Samples were injected to Bruker HCT ultra ETD II at a flow rate of 240 μL/h. The MS spectrum was recorded over the mass range of *m/z* 100–2,800. The voltage between ion spray tip and spray

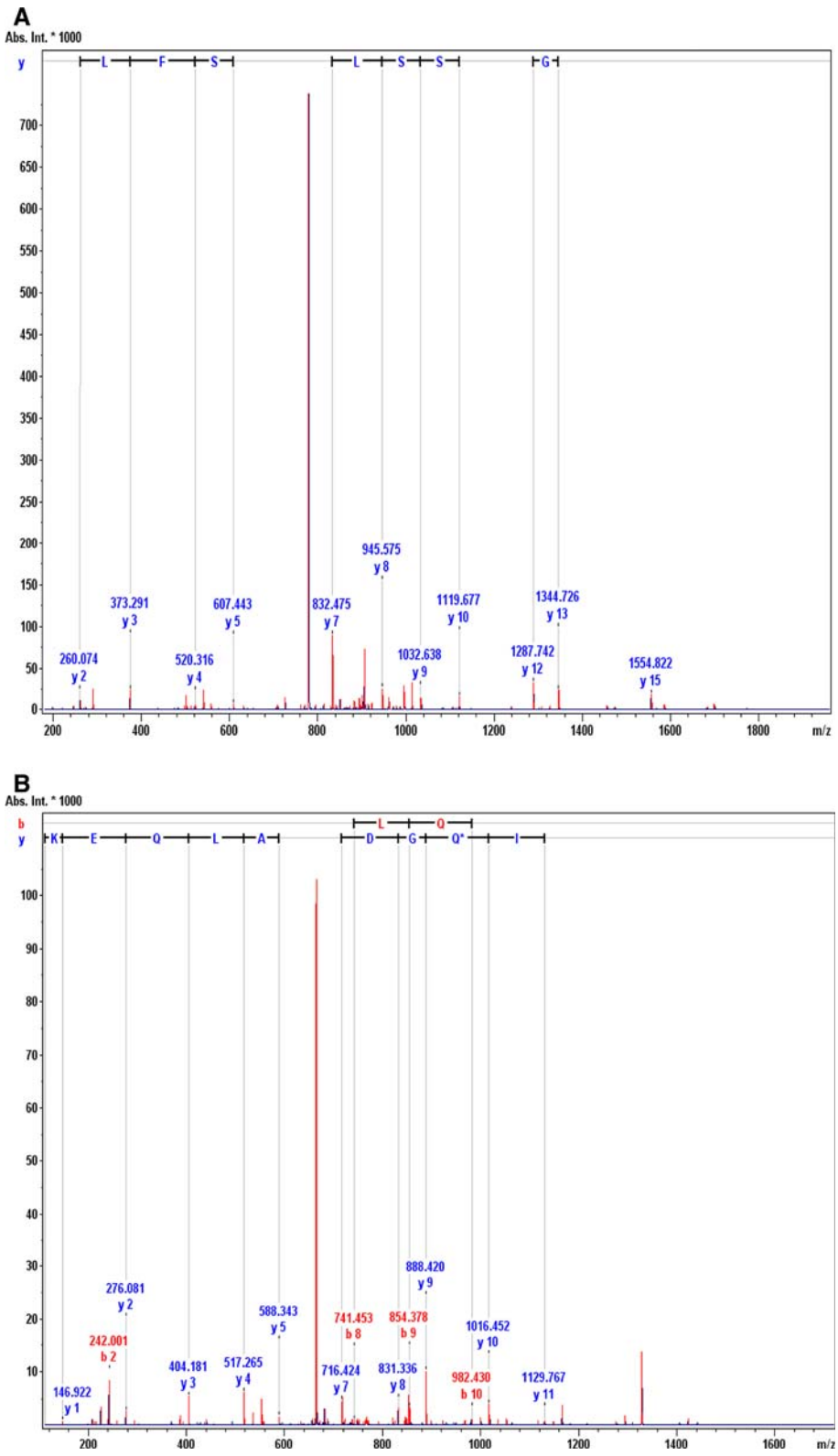

Fig. 2 **a** MS/MS spectrum of the *m/z* 1,842.92 (922.46, 2+) assignment of the identified sequence 1–17, MTPLGPASSLPQSFLLK, which contains N-terminally modification by both, N-terminal amidination and oxidation.of the methionine residue. **b** MS/MS spectrum of the *m/z* 1,567.80 (784.91, 2+) assignment of the identified sequence 24-35, KIQGDGAALQEK, which contains modification by PentylamineBiotin at amino acid Q-26

shield was set to 4,000 V. Drying nitrogen gas was heated to 300°C and the flow rate was 10 L/min. The MS spectrum was deconvoluted in Bruker Data Analysis 4.0 software, and shifted spectra were also generated.

Results and discussion

The non-sophisticated gel-based methodology (Fig. 1) used allowed complete sequencing just by in-gel digestion with

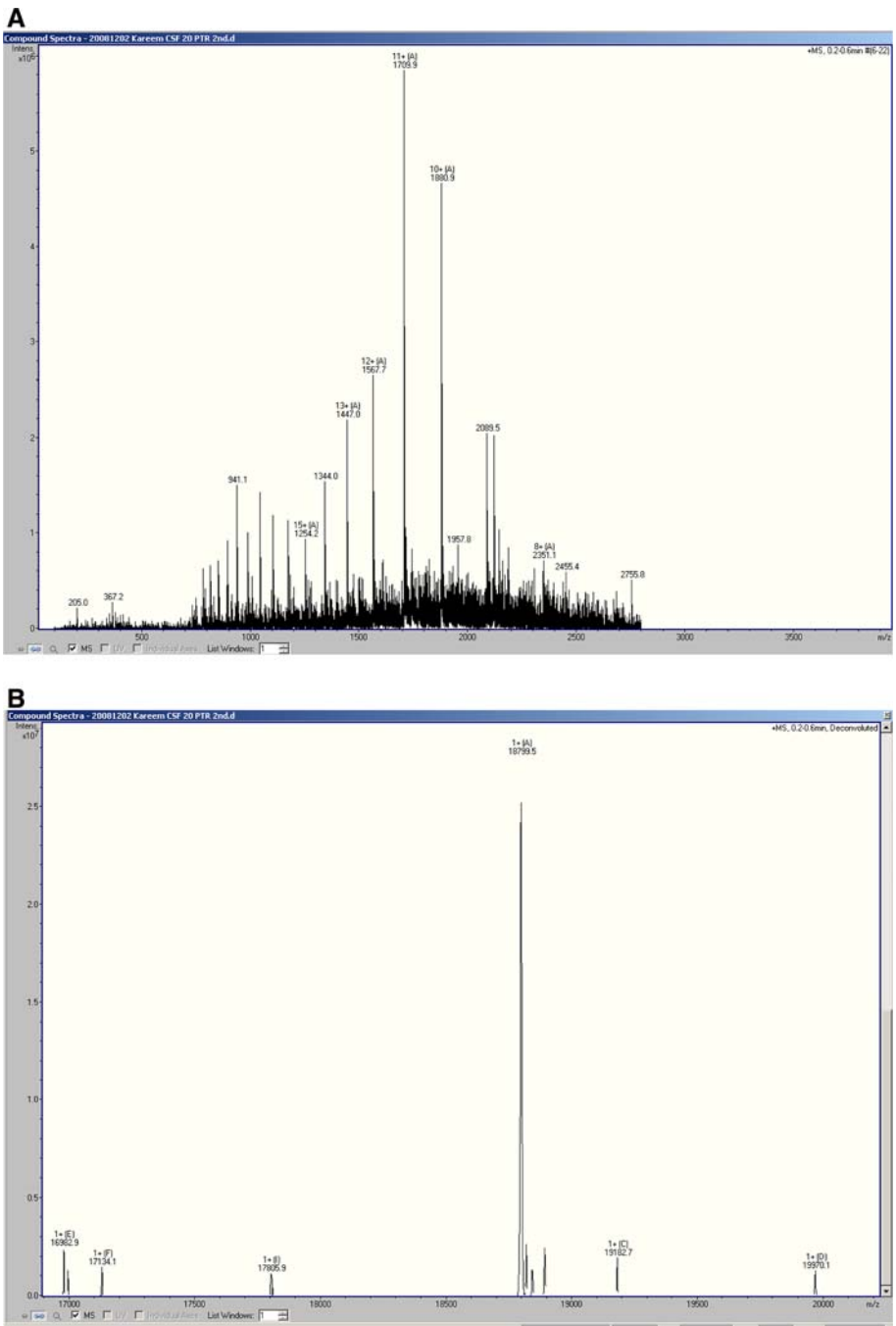

Fig. 3 **a** The raw MS spectrum of G-CSF. 8, 10, 11, 12, 13, 15 charged G-CSF peaks are labeled. **b** Deconvoluted MS spectrum of G-CSF. The *m/z* of single charged G-CSF was 18,799.5

only two enzymes, trypsin and chymotrypsin and the concomitant application of the Mascot and the Modiro™ software. Bioinformatic handling with Mascot alone revealed 96% sequence coverage (Table 1), Modiro™ alone revealed 94% sequence coverage of rhG-CSF (Table 2). This is of particular interest because in previous work, multi-enzyme digestion with several proteases has to be applied in the majority of mass spectrometry techniques to obtain this high sequence coverage (John et al. 2007). The published sequence derived from database UniProtKB (P09919) and NCBI (1CD9_A) was confirmed.

Apart from N-terminal oxidation of methionine along with amidination (Unimod modification #141, Fig. 2a), N-terminal methionine loss was observed in another expression form of the rhG-CSF. A modification compatible with glutamine biotinylation, PentylamineBiotin (Unimod accession number #800) was observed (Fig. 2b) (Cernuda-Morollon et al. 2001). This chemical modification of glutamine 26 may have been generated by the manufacturer by biotinylation with 5-(biotinamido) pentylamine. This finding was observed using both bioinformatic tools, Mascot and Modiro™. It is not known whether

this modification leads to functional impairment as, e.g., modified receptor-binding, changes of antigenicity or even may be responsible for side effects following administration of this protein drug.

No oxidative modifications as published before, except the abovementioned N-terminal methionine oxidation were observed and therefore no oxidative conformational or antigenic changes of the molecule are not to be expected that would interfere with bioactivity (Lu et al. 1992). It also remains open whether N-terminal methionine loss may lead to functional changes.

The observed amidination may have potential functional consequences: amidinations are known to interfere with function in the case of lysine amidination in haemoglobin reducing oxygen affinity (Zolock and Niehaus 1975). Moreover, amidination may well lead to conformational changes that in turn may affect rhCSF binding or activity (Janecki et al. 2005).

Determination of the molecular mass of the entire protein revealed 18,799 Da (Fig. 3), the calculated molecular weight was 18,800 Da that would show integrity of rhG-CSF and probably indicate that there is only modifications at low percentage.

Conclusion

We learn from this work that two proteolytic enzymes and the use of two bioinformatics tools led to complete sequencing of a protein, thus, representing a non-sophisticated approach. Complete sequencing of a protein can be very time consuming and costly and herein a non-expensive and non-time consuming analytical tool is proposed.

Acknowledgments We are highly indebted to the Verein zur Durchführung der wissenschaftlichen Forschung auf dem Gebiet der Neonatologie und Kinderintensivmedizin "Unser Kind".

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