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Hyphenation of multi-dimensional chromatography and mass spectrometry for the at-line-analysis of the integrity of recombinant protein drugs

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Abstract

A robust tool is proposed for the rapid at-line verification of the identity and integrity of (recombinant) proteins, namely the hyphenation of multidimensional chromatography and mass spectrometry (MS). A recombinant human antibody produced in Chinese hamster ovary cells is taken as pertinent example. The recombinant human antibody is first captured from the production environment by affinity chromatography (rProtein A, isolation/concentration of the target molecule) and automatically transferred to an enzyme reactor (immobilized trypsin column) for digestion, thereby yielding different peptides corresponding to the protein sequence. The peptides are then separated on a reversed-phase column before being analyzed and identified by MS. This step does not require a fine resolution since the mass spectrometer can identify a variety of substances at the same time. The results are then analyzed in silico with suitable bio-informatic tools. When the gene sequence of the protein product is known, proteolytic cleavages can be predicted and the exact mass and hence the amino acid sequence of each peptide can thereby be deduced. Fitting experimental data and reference peptide sequences then provides important information about the integrity of the protein and more particularly about its sequence. In our case, the integrity of 45% of the light and 75% of the heavy chain sequences of the antibody could be verified within minutes.

Keywords: Recombinant protein drugs

1. Introduction

The number of recombinant human proteins produced as therapeutic drugs is increasing rapidly. The financial returns are usually attractive, but development of suitable industrial-scale production processes is known to be labor-intensive, time-consuming, and costly [1]. Depending on the chosen production mode, a given product molecule may remain in this potentially degrading environment (proteases) for quite some time. After bioproduction, the product is purified [2], typically by a series of chromatographic separations, and hence again exposed to potentially degrading conditions, e.g., low-pH elution from the affinity column. Verifying the integrity and the purity of the final product is an important and strictly regulated task typically achieved by a number of specific off line assays [3]. While these assays

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provide sufficient data on the nature and quality of the final product, they allow no insight into the ''history'' of the compound. Process development and process monitoring/control would benefit significantly from a robust tool for the at-line verification of the product identity and integrity. The efficiency of the development of conventional batch bioprocesses, but also the ''trouble-shooting''-strategies would benefit from the ability to ''see'' the product and its quality as it is being produced and purified. Concomitantly, the number of acceptable production modes could be increased, including also a larger variety of continuous modes, where the characterization of the ''final batch'' is less straightforward. Such a monitoring tool must be quick, easy to handle and adaptable to different recombinant proteins. Finally, the data thus generated must enable rapid decision-making.

We have recently published a paper on the use of mass spectrometry to monitor individually chromatographic columns (affinity, ion-exchange, reversed-phase) during protein separation, taking the mass of the eluting protein as parameter [4]. In the present paper, we would like to extend the type of at-line data thus provided to the peptide map, i.e., a first indication of the identity and integrity of the recombinant product. For this purpose we have developed a tool coupling a multi-dimensional chromatographic separator/reactor with electrospray ionization mass spectrometry (ESI-MS) as a sensitive and informative detector. To reduce the possibility of mistakes and/or contamination of the protein product during analysis, the combination is fully automated and therefore does not require any human intervention else than sample injection.

2. Experimental

2.1. Reagents

All the chemicals and solvents were purchased from Fluka (Buchs, Switzerland). The highest available quality was used. Ultra-pure water, produced by a Millipore purification system, was used for buffer and mobile phase preparation. The recombinant human antibody used in the demonstration was produced in the laboratory by Chinese hamster ovary (CHO) cells and in serum-free culture medium. This antibody is composed by 669 amino acid residues, divided in four chains and reticulated by eight disulfide bridges [5,6].

2.2. Instrumentation

For Protein A affinity chromatography, a fast protein liquid chromatography (FPLC) system from Amersham Pharmacia Biotech (Uppsala, Sweden) was used. The system was composed of three P-500 pumps and a liquid chromatography controller LCC-500-Plus. The samples were injected with a motor valve MV-7 from the same supplier. Detection was by a UV detector from Soma Optics (Japan) and at 280 nm. On-column digestion was performed in a thermostated water-bath (Haake, Karlsruhe, Germany) with the temperature set at 37 °C. An Agilent 1100 high-performance liquid chromatography (HPLC) system from Agilent Technologies (Folsom, CA, USA) was used for the reversed-phase experiments. The HPLC system consisted of a high-pressure binary pump, a degasser and a UV detector (detection wavelength set at 214 nm). Two six-way valves were used in this set-up; an EQ 60 motorized model from Valco Instruments (Houston, TX, USA) and a manual one from Knauer (Berlin, Germany). An additional stainless steel 1 ml loop was integrated in the system for the deliberate sample denaturation step (see below). All tubings, couplings, and fittings were from Upchurch Scientific (Oak Harbor, WA, USA). The mass spectra were acquired on a LCT mass spectrometer from Micromass (Manchester, UK). This instrument combines an ESI source with a time-of-flight (TOF) mass analyzer.

2.3. Chromatographic methods

Protein A affinity chromatography was carried out on a C10/10 (100×10 mm) column from Amersham Pharmacia Biotech packed with rProtein A Sepharose Fast Flow (Pharmacia). The column was equilibrated with a 50 m*M* sodium phosphate buffer (pH 7.0) containing in addition 150 m*M* sodium chloride. Elution was performed by injecting into the column via the MV-7 valve 500 μl of a solution containing

20 m*M* glycine, 3 *M* guanidine, and 10 m*M* dithiothreitol (DTT) (pH 3.0) at a flow-rate of 1 ml/min (''flash elution''). The antibody-containing fraction was transferred to the 1 ml loop and submitted for 5 min to a temperature of 65 °C (thermostated water-bath). The loop was out of flow during this time. During the injection into the loop the sample was diluted (1:1) with a suitable digestion buffer (100 m*M* ammonium hydrogencarbonate, pH 8.5) containing in addition 20 m*M* cysteine. Afterwards the contents of the loop were injected into the digestion cartridge (Poroszyme Immobilized Trypsin Cartridge; 30×2.1 mm) from Perseptive Biosystems (Framingham, MA, USA). Immobilization of trypsin on POROS medium minimizes enzyme autodigestion and preserved the enzyme activity for over one hundred injections. During injection the sample was once more on-line neutralized with the digestion buffer to reach the optimum pH value for the digestion. The digestion of the protein sample was achieved at a flow-rate of 50 μl/min and a temperature of 37 °C. The outlet of the digestion column was connected to the reversed-phase column via the Vici motorized valve. Reversed-phase chromatography was performed on a 250×2.1 mm type 218TP52 column from Grace-Vydac (Hesperia, CA, USA). The mobile phase was a water–acetonitrile mixture of varied composition. Formic acid at a concentration of 0.1% was used as ion-pairing reagent. The flow-rate was 200 μl/min and experiments were made at room temperature. The eluting peptides were both recorded by UV detection (214 nm) and by mass spectrometry.

2.4. ESI-MS conditions

The LCT mass spectrometer was used in the positive ionization mode for this experiment. The ES+ source parameters were adjusted as a function of the mass (MW) of the expected peptides. Taking into account all trypsin-cleavage sites of the antibody (parameters computerized using software from the Expasy web site, see below), these parameters were: capillary voltage 3.4 kV, sample cone voltage 35 V, extraction cone voltage 2 V, radio frequency (RF) lens voltage 300 V, desolvation temperature 250 °C, source temperature 120 °C, and desolvation flow-rate 500 l/h. Recording was performed on a 100–2000 m/z scale at a 0.5 s scan time.

3. Results and discussion

The set-up used to couple the multidimensional chromatographic system with the electrospray injection MS is shown in Fig. 1.

3.1. Capture of the target protein

The recombinant antibody discussed in this paper is excreted by the producing cells into the culture medium. During harvesting, a cell free culture supernatant containing the target antibody is obtained by centrifugation [1,7]. Afterwards the antibody is captured from the supernatant by Protein A affinity chromatography. In our experience, Protein A affinity chromatography is perfectly adapted for diluted sample streams, since a very large volume of sample solution can be passed through the column without biasing the specific retention of the desired product. Recovery of active antibody is usually between 70 and 80% [8]. Elution in Protein A affinity chromatography is usually performed by a shift to low pH (3.0). The eluent chosen here contains a comparatively low concentration of the acidic agent (20 m*M* glycine) and in addition both a denaturing and a reducing agent (3 *M* guanidine and 10 m*M* DTT, respectively). The eluent composition was optimized with regard to the subsequent digestion step, where a low pH is unsuitable to the digestion enzyme (trypsin), while a highly denatured and reduced (no disulfide bridges) substrate molecule is more easily digested. A 500 μl flash elution was used to preserve the protease column.

The corresponding chromatogram at 280 nm shows a wide peak corresponding to the elution of the antibody with a tailing due to the relative absorbance of one of the eluent components (guanidine), Fig. 2. To determine whether the antibody fraction was fully eluted, a 100 m*M* glycine buffer at pH 3.0 was applied to the column after the standard flash elution had taken place. A small amount of antibody was eluted by this step, however this amount was less than 5% of the total amount

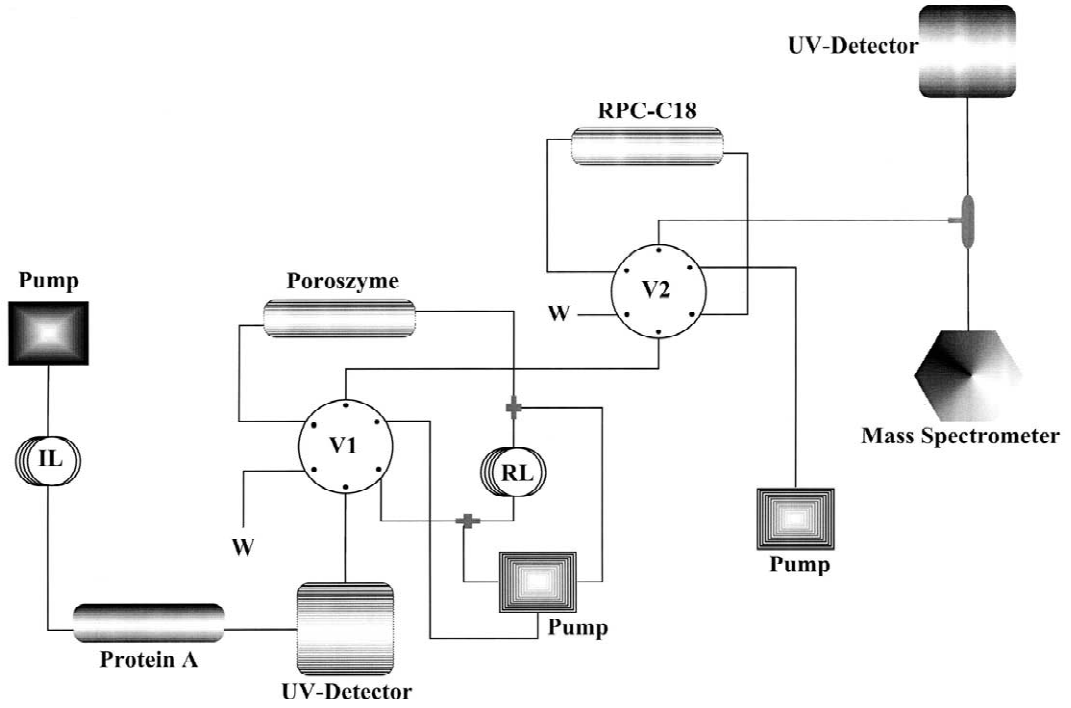

Fig. 1. Schema illustrating the direct coupling of the chromatographic set-up to electrospray ionization mass spectrometry. IL: Injection loop, RL: reaction loop, W: waste, V1 and V2: six-way valves.

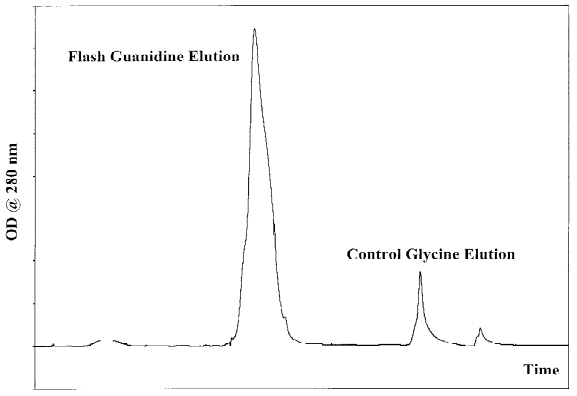

Fig. 2. Antibody capturing by the Protein A column. Column: C10/10, stationary phase: rProtein A Sepharose Fast Flow, mobile phase for column equilibration: 50 m*M* sodium phosphate buffer (pH 7.0) containing in addition 150 m*M* sodium chloride, elution (''flash''): 500 μl of a solution containing 20 m*M* glycine, 3 *M* guanidine, and 10 m*M* dithiothreitol (DTT) (pH 3.0) at a flow-rate of 1 ml/min.

applied to the column, indicating that the flash elution was very effective (data not shown).

3.2. Digestion of the target protein

The protease activity is strongly pH dependent and can be modulated by the composition of the digestion buffer. The optimum pH for protein digestion by trypsin is around 8.0. Denaturation conditions must be avoided so that the structure of the enzyme and therefore its activity are retained. To render the eluted antibody suitable for the subsequent digestion, a first neutralization was performed just after the elution from the Protein A column. To this end, the sample was mixed (1:1) with the digestion buffer (100 m*M* ammonium hydrogencarbonate, pH 8.5 containing 20 m*M* cysteine). The cysteine was added to quench the excess of DTT used for eluting the antibody from the protein A column. The sample

was stored ''out of flow'' in a loop and heated for 5 min to 65 °C to increase the denaturation of the protein and to some extent still the reduction of the disulfide bridges of the antibody molecule (for details of the experimental set-up see Fig. 3). The flow was stopped during this period of time. Finally, the sample was injected into the digestion column. Concomitantly a second dilution in the digestion buffer was carried out in order to fully quench the DTT. The digestion of the denatured sample was done under flow conditions (flow-rate 50 μl/min). The digestion column was thermostated at 37 °C in a water-bath during this time.

Tryptic digestion can result in different types of products. The most abundant molecules should be the peptides generated by the complete digestion of the target molecule. However, some of them may be the result of only a partial protein digestion, especially if the time allowed for digestion is rather short. Here, it was found that the cleavage efficiency achieved within minutes by the outlined protocol is comparable that of the conventional overnight method of digestion. A second population of peptides may be produced by auto-proteolysis of the digestive enzyme (protease). Here auto-proteolysis was kept to a minimum by using an immobilized enzyme.

3.3. Peptides separation and detection

Reversed-phase chromatography (RPC) remains the method of choice for peptide separation especially in the case of detection/analysis by mass spectrometry [9–12], since RPC uses hydro–organic mobile phases that are fully compatible with the electrospray ionization technique. In our case, the analytical reversed-phase column was connected directly to the digestion column. After the peptide mixture had been transferred to the C_{18} column (''sample loading''-position), the motor valve was switched to the ''RPC-operating''-position. First a desalting step was performed, i.e., a 15 min wash under isocratic condition at low organic solvent content (10% acetonitrile) to remove all components of the sample buffer (mainly salts) not compatible with mass spectrometry. This step is necessary to optimize the signal-to-noise ratio and to reduce adduct formation, which would lead to more complex and hence more difficult to interpret MS spectra. The desalting progress was monitored by UV absorption at 214 nm. After desalting, the peptide elution and separation was achieved by a 30 min gradient from 10 to 70% acetonitrile. The column effluent was monitored both by UV and mass

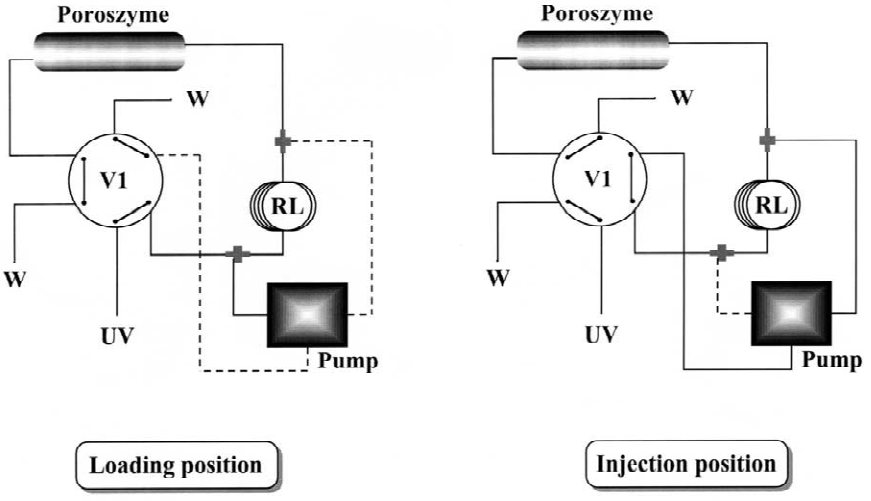

Fig. 3. Set up for protein digestion. Loading position: transfer and partial neutralization of the antibody coming from the Protein A column, injection position: final neutralization of the sample and transfer to the digestion column. Solid and dotted lines indicate off-line and in-flow conditions, respectively.

spectrometry, see Fig. 4 for an example. UV detection served mainly to verify that all eluted peptides were well ionized.

3.4. Interpretation of the mass spectra

Even if all possible precautions are taken, the digestion of a large protein generates a complex peptide mixture. While the mass determination of these peptides by MS a priori presents no problem, the correct interpretation of the high information content generated by MS may very well do so [13,14]. In addition to the inherent complexity of the peptide mixture, the peptides themselves may bear one or several charges (usually between 1 and 5), which in addition may stem from different types of ionization (mostly protonated and/or sodium adducts). In spite of the use of reversed-phase chromatography for contaminant removal, some adducts can usually also be still observed.

Starting from the experimentally obtained mass spectra, peptide mass collection was done by taking in account the type of adducts and the ionization states of the peptide molecules observed using isotopic resolution, see Fig. 5 for an example. Singly to fivefold charged molecules could usually be detected without major problems confirming thus the presence of the corresponding peptide. For the larger molecules, where missed cleavages (partial digestion) can frequently be observed, the peptide collection is best performed manually. For smaller molecules, where cleavage is usually more effective, the peptide collection can be realized in the single ion recording (SIR) mode, but only with protonated ions. Peptides with masses from 400 Da to about 5 kDa could be determined from the whole set of detected peaks on a single run. By integrating the sum of all

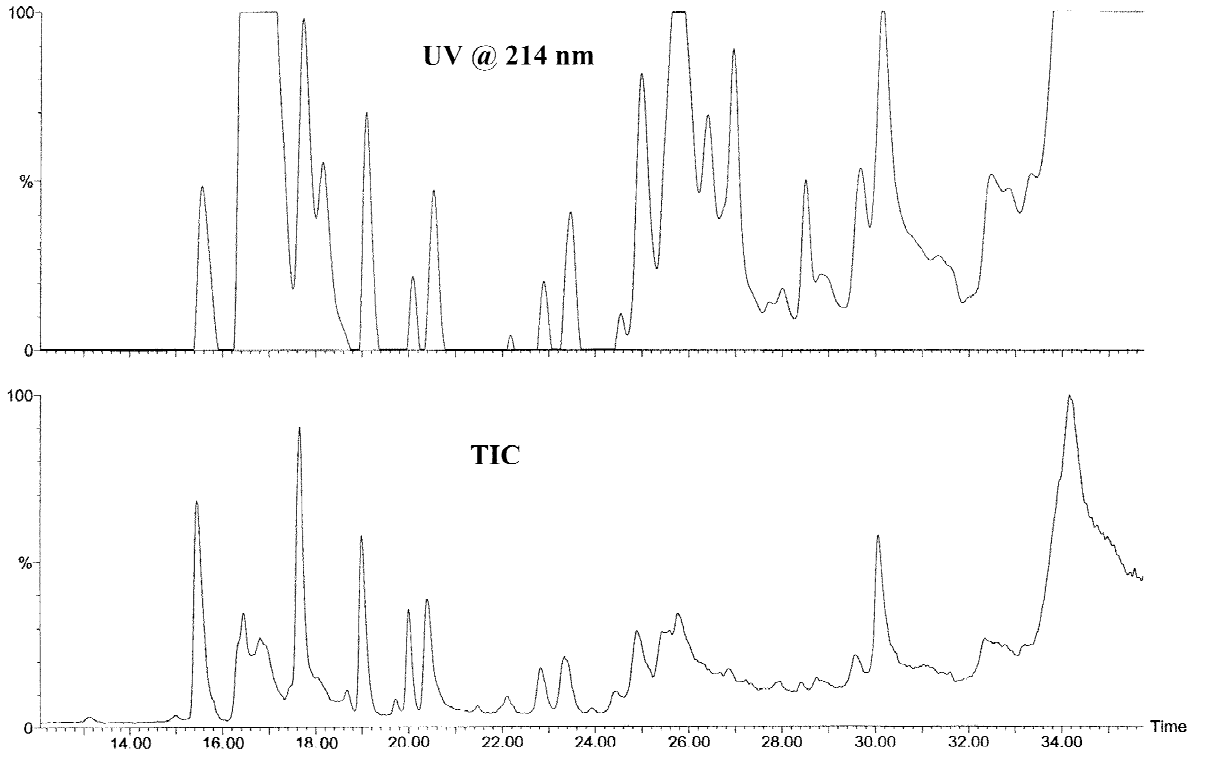

Fig. 4. Tryptic digest of the sample antibody. The peptide mixture is resolved by RPC on a C_{18} column. Column: 218TP52 C_{18} column (250×2.1 mm), ion pairing agent: 0.1% (v/v) formic acid, gradient: from 10 to 70% of acetonitrile in water in 30 min, flow-rate: 200 μl/min, detection: UV at 214 nm and mass spectrometry. Top: chromatogram obtained by UV-absorption. Bottom: chromatogram obtained by measuring the total ion current (TIC) in the positive mode.

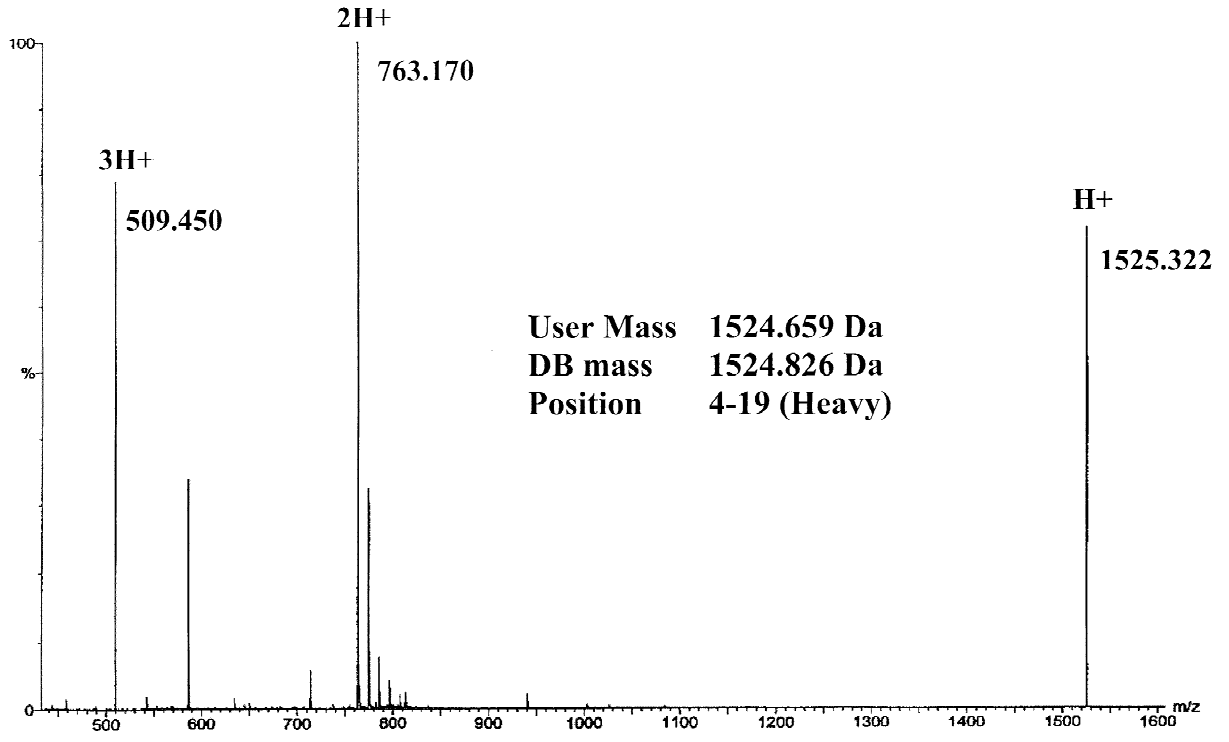

Fig. 5. Example of an ESI-MS spectrum of a peptide. Three charge states are shown and the result of the computerized data processing is summarized. The corresponding sequence on the heavy chain and the cleavage sites are also indicated. User mass: experimental resulting mass determined from the three peaks, DB mass: database mass from a theoretical digestion of the (known) antibody sequence, position: location of the peptide in the full sequence of the antibody.

ions recorded by MS, it was possible in the case of the recombinant antibody to recover more than 150 different ions of interest corresponding to 70 different peptides. Most of the peptides were hence represented by two states of charge.

While manual peptide mass collection can be handled even with little experience in the field, one should not try to recover in this manner the sequence of a large protein such as the antibody considered here. The use of an adapted bioinformatics software (see, e.g., www.expasy.ch) is recommended instead, due to the large number of peptides generated and the total size of the antibody molecule, which after all contains more than 600 amino acid residues. Once the target protein sequence, the protease, and the experimental peptide mass have been entered into the database, it is possible by using the ''FindPept''-tool to immediately know if a given peptide stems from the target protein. Several peptides masses can be processed at the same time and the software is also able to take into account posttranslational modifications and up to five consecutive missed cleavage sites. Peptides corresponding to the protease itself can also be identified.

Starting from the whole set of peptide masses and using this computer tool, 12 peptides fitting to sequences of the light chain and 42 peptides for the heavy chain were identified, see Fig. 6. This corresponds to 45 and 75% of the entire sequences of the light and heavy chains, respectively. These results were obtained in a single run using less than 200 μg (i.e., approximately 1 n*M*) of antibody. This amount (mass) represents an optimum. If less is used, the percentage of identified peptide sequences is reduced, while no further improvement in this regard could be achieved, even when a larger amount of antibody was subjected to the analysis. The fact that portions of the protein sequence were not identified,

Light Chain

DIVMTQSPSSLSASVGDRVTITCRASQSIIRYLNWYQHKPGKAPKLLIHTASSLQSGVPSRFSGSVS
GTDFTLTISSLQPEDFATYYCQQSYTTPYTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVC
LLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL
SSPVTKSFNRGEC

Heavy Chain

QVKLLESGGGVVQPGGSLRVACVASGFTFRNFGMHWVRQAPGKGLEWVAFIWFDASNKGYGDSVKGR
FTVSRDNSKNTLYLQMNGLRAEDTAVYYCAREKAVRGISRYNYYMDVWGKGTTVTVSSASTKGPSVF
PLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
GTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT
CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKA
LPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

Fig. 6. Recovered sequences of the antibody molecule. The underlined parts represent matching amino acid residues.

may be due to the fact that the reduction process was not completed and some peptides remained linked by disulfide bridges. The complete processing of such disulfide bounded peptides is in principle also possible, but time-consuming. Some peptides may also have been lost, e.g., by adsorption on the digestion column or by a lack of retention on the reversed-phase column. Finally, for the heavy chain, previous experiments have shown a glycosylation site and the corresponding peptides are very difficult to identify with MS due to the heterogeneity of the modification [15–17].

It should be decided on a case by case basis whether the routine at line monitoring of only a part of the sequence of a recombinant product in combination with the overall peptide pattern is sufficient for process development or whether a more involved sample preparation is called for. The latter may render the method too complex for fully automated at line analysis and and should be carried out off line, for example when changes in the peptide pattern are observed and a closer examination of the product becomes necessary.

4. Conclusions

In this paper, we describe an easy-to-handle, informative tool for the at-line characterization of recombinant proteins, e.g., prior a downstream processing or during bioprocess development. The analysis was developed for antibody monitoring but could easily be adapted to other proteins by choosing the appropriate capture and digestion column and conditions. The reversed-phase column for desalting and peptide separation, on the other hand, is prerequisite in order to allow for the coupling of MS. However, this type of chromatography should be applicable to most if not all peptide mixtures. For each target a data bank can be generated and used as

reference for subsequent monitoring purposes. This set-up may therefore become a quick and very efficient decision making tool in the production of recombinant proteins as novel therapeutic drugs.

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