

Mass spectrometry analysis of liquid chromatography fractions using Ettan LC-MS system

Key words:

Protein purification • automated sample desalting • protein fraction analysis • deconvolution of intact protein masses

Summary

Mass spectrometry provides a faster and more accurate tool for analyzing liquid chromatography fractions during protein purification than traditionally used techniques (for example electrophoresis). Liquid chromatography-mass spectrometry (LC-MS) using Ettan™ LC-MS offers an excellent means of monitoring protein purification by determining molecular weight, identity, and purity of fractions. The procedure described involves initial protein purification on ÄKTA™purifier 10, automated and rapid desalting of fractions on Ettan microLC, and MS analysis by Electrospray Ionization Time-of-Flight mass spectrometry (ESI-ToF MS) using Ettan ESI-ToF. Ettan LC-MS provides high quality mass spectra of a wide range of high molecular weight proteins including bovine serum albumin, cytochrome C, transferrin, ovalbumin, myoglobin, a monoclonal antibody, and two β -lactoglobulin variants.

Introduction

Mass spectrometry offers an excellent means of monitoring protein purification and performing confirmation analysis of protein fractions collected from chromatographic purification. However, chromatography techniques normally used rely on the ion exchange, size exclusion, or hydrophobic interaction properties of the proteins. With these techniques the mobile phase is ionic and at about neutral pH, i.e. conditions that are poorly or not at all compatible with electrospray ionization mass spectrometry (ESI MS, Fig 1). We show results of an efficient technique for automated desalting in combination with mass analysis using Ettan ESI-ToF. The mass spectrometer is suited to mass analysis over a wide mass range. It is equipped with dual ionization probes, enabling internal calibration by simultaneous spraying of sample and calibrants, which facilitates high mass accuracy.

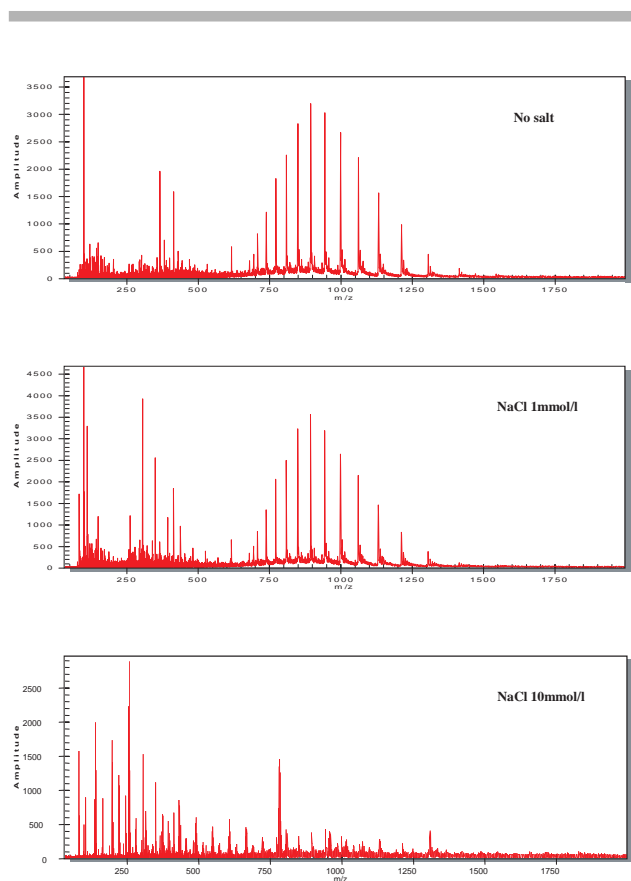


Fig 1. Effect of 0, 1, and 10 mmol/l NaCl on the mass spectrum of myoglobin (50 μ g/ml; 3 μ mol/l). The Figure demonstrates the importance of desalting liquid chromatography fractions to obtain high quality mass spectra.

Ettan LC-MS is a totally integrated system comprising Ettan ESI-ToF mass spectrometer and either Ettan LC or Ettan microLC. In this study, an LC-MS system including Ettan microLC was used for automated sample desalting mass analysis.

Products used

Amersham Pharmacia Biotech products used in this study:

Ettan LC-MS system **Inquire**

ÄKTApurifier 10 **Inquire**

Mini Q PC 3.2/3 **17-0686-01**

Mini S PC 3.2/3 **17-0687-01**

Materials and methods

Protein Purification

A protein mixture consisting of bovine serum albumin (BSA), cytochrome C, transferrin, ovalbumin, and myoglobin was separated by cation exchange chromatography on a Mini S™ PC 3.2/3 column connected to ÄKTApurifier 10. A second protein mixture containing a monoclonal antibody and two β -lactoglobulin variants was separated by anion exchange chromatography on a Mini Q™ PC 3.2/3 column. Sample concentrations ranged between 0.25 and 0.90 mg/ml (10 or 20 μ l injected.) The elution buffer was 50 mmol/l Tris-HCl (in buffer B containing 1 mol/l NaCl), pH 7.5 for both column types. Flow rate was 0.2 ml/min and the gradient was 0–100% B over 20 column volumes (CV) with a column wash at 100% B of 3 CV and a re-equilibration phase of 7 CV. The fraction size was 0.1 ml and samples were collected in microplates using Fraction Collector Frac-950.

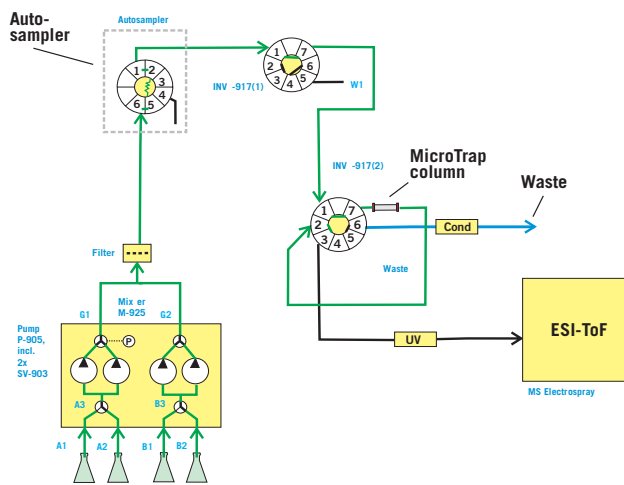
Desalting on Ettan microLC

After purification on ÄKTApurifier 10, the microplates with collected samples were transferred to the autosampler of Ettan microLC for desalting and sample injection into the Ettan ESI-ToF mass spectrometer. Ettan microLC was configured with Autosampler A-905, two switch valves (INV 917) and with a MicroTrap™ column connected to the second valve (Fig 2). Prior to each sample injection the column was equilibrated with a minimum of 150 μ l of eluent A (5% acetonitrile containing 0.1% TFA). The equilibration continued during the first part of the autosampler injection. The flow rate at sample application was generally 400 μ l/min. Samples (<10 μ l, <20 μ g) were applied to the column and subsequently washed with 0.2 ml of eluent A to remove salt. At sample elution, the flow rate was lowered to 100 μ l/min and the concentration of eluent B (70% acetonitrile with 0.5% acetic acid) was raised in one step to 100% B.

The conductivity monitor was positioned in the line going to Waste for monitoring of the salt peak during the sample load and desalting phase of the operation (Fig 2).

(a). Sample loading

Equilibrate: 5% CH₃CN with 0.1% TFA
Load: Maximum 20 μ g
Wash: 5% CH₃CN with 0.1% TFA



(b). Sample elution (injection)

Elute: 70% CH₃CN with 0.5% acetic

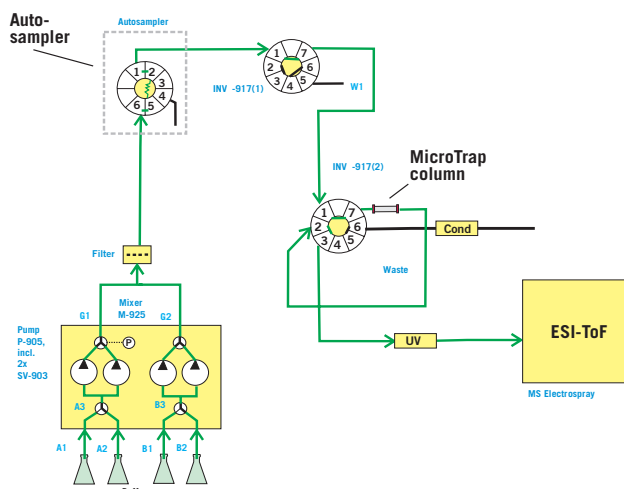


Fig 2. Configuration of Ettan microLC system showing flow path during (a) sample load and (b) elution (injection) operations. Microplates containing fractions collected at the ion exchange chromatography separation of the proteins were placed in Autosampler A-905. After equilibration, 10 μ l aliquots of each fraction were loaded onto the MicroTrap column according to (a) and subsequently eluted to the ESI-ToF (see [b]).

Mass spectrometry analysis

Ettan ESI-ToF was operated at pulse mode with a capillary exit voltage setting between 150 and 250 V depending on protein mass. Generally a larger protein demands the higher capillary exit voltage setting. Spectra were acquired at

1 spectrum/sec with a detector voltage setting of 1850 V.

Results

Desalting and Mass Spectrometry Analysis

The MicroTrap column positioned in Ettan microLC, in combination with a valve system and automatic injection of samples proved to be a very efficient way of performing simultaneous desalting and on-line mass spectrometry analysis. The cycle time was about 5 min. Consequently, fractions collected after ion exchange chromatography were analyzed in less than 30 min.

The results of the protein separation and the mass spectrometry analysis are shown in Figure 3. Each mass

spectrometry analysis is presented with the crude spectrum in the lower panel and the deconvoluted spectrum in the upper panel. Protein fractions proved to be very pure although sodium adducts were detected in the RNase A-containing fraction. Transferrin was present in two forms differing 284 Da in mass, presumably representing forms with different glycosylation patterns.

Monoclonal antibody

The monoclonal antibody sample was previously analyzed by SDS-PAGE. However, when the sample was separated by liquid chromatography on the Mini Q column, two peaks were detected. The two peaks corresponded to two different variants of the monoclonal antibody with slightly different masses—150073 Da and 150132 Da respectively (Fig 4). These peaks might represent different glycosylation patterns of the antibody.

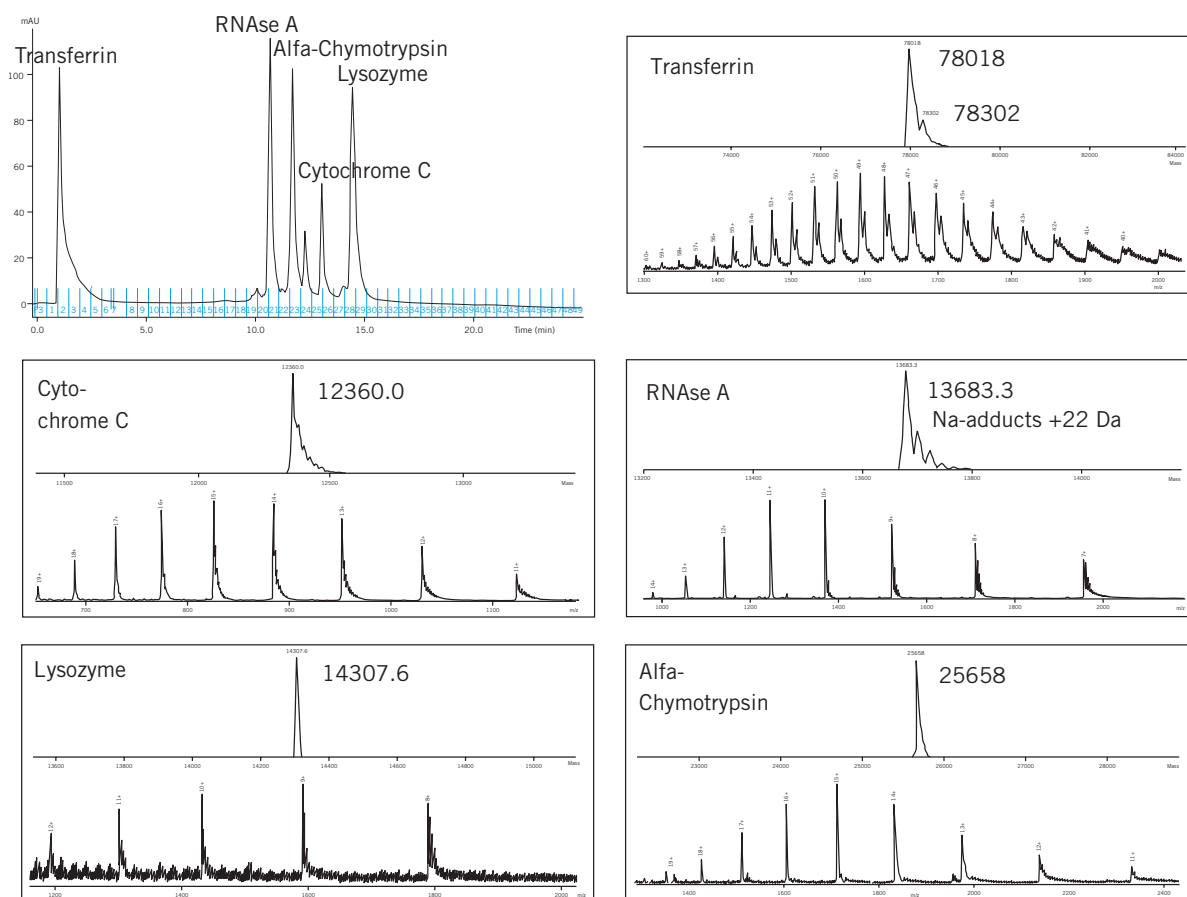


Fig 3. Separation of a protein mixture by cation exchange chromatography on Mini S PC 3.2/3 and results from the mass spectrometry analysis of the fractions corresponding to the main peaks in the chromatogram. Crude spectra are shown in the lower panels while the upper panels show deconvoluted spectra.

Beta-lactoglobulin

The two variants of β -lactoglobulin, A and B, were effectively separated on the Mini Q column. Masses from the two forms differed 0.06 Da from the theoretical values. Spectra taken from the analysis of the valley fraction between the peaks revealed exactly the same values as were analyzed for the separated entities (Fig 5).

Conclusions

Ettan LC-MS is suitable for analysis of high molecular weight proteins. Automated desalting was efficiently performed by connecting a MicroTrap column in Ettan microLC for automatic injection of samples and MS analysis. Cycle times of about 5 min are possible. Mass spectra of high quality were achieved revealing two different forms of a monoclonal antibody. The two forms of β -lactoglobulin were successfully separated and analyzed.

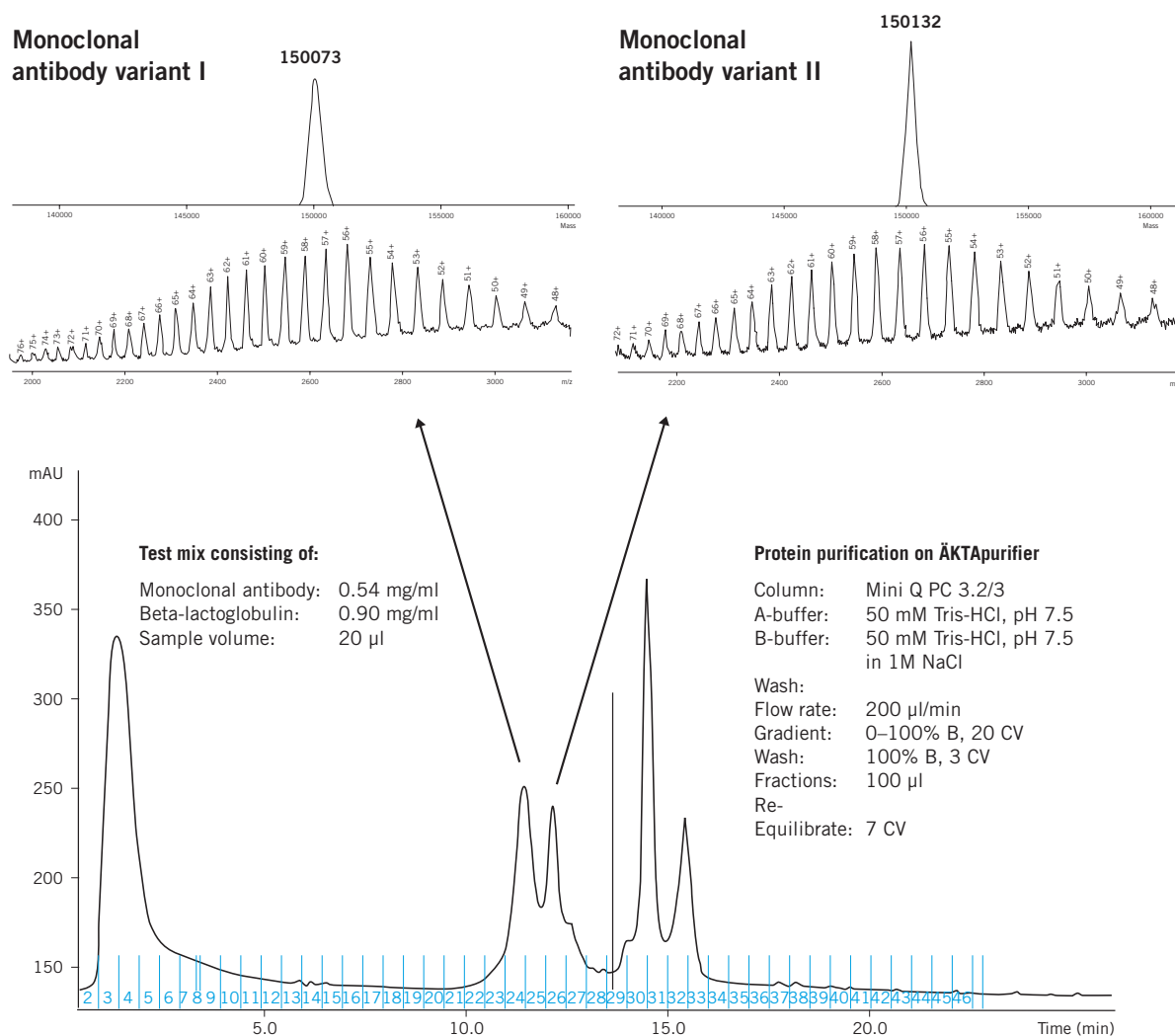


Fig 4. Separation of a mixture of a monoclonal antibody and β -lactoglobulin by anion exchange chromatography on Mini Q PC 3.2/3. The results from mass spectrometry of the fractions corresponding to the two first peaks in the separation are shown above the chromatogram. Crude spectra are shown in the lower panels while the upper panels show deconvoluted spectra.

Beta-lactoglobulin A and B Variants

Theoretical: MW difference A and B variants = 86.04 Da

Experimental: MW difference A and B variants = 86.10 Da

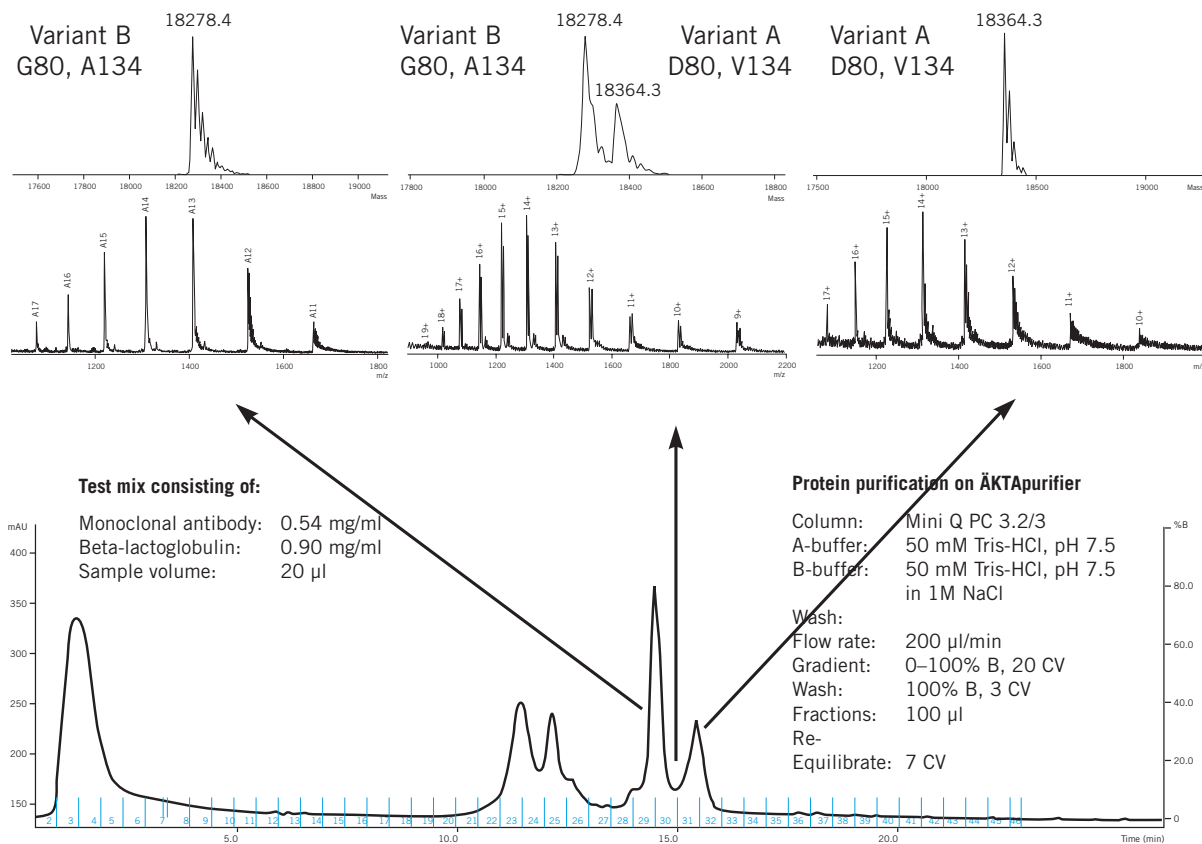


Fig 5. Separation of a mixture of a monoclonal antibody and β -lactoglobulin by anion exchange chromatography on Mini Q PC 3.2/3. The results from the mass spectrometry analysis of the fractions corresponding to the two last peaks in the separation are shown above the chromatogram. Crude spectra are shown in the lower panels while the upper panels show deconvoluted spectra.

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