Analytical techniques and formulation strategies for the therapeutic protein alkaline phosphatase

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Chapter 4

Feasibility of non-volatile buffers in capillary electrophoresis-electrospray ionization mass spectrometry of proteins

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Summary

The combination of capillary electrophoresis (CE) and electrospray ionization mass spectrometry (ESI-MS) *via* a triaxial interface was studied as a potential means for the characterization of intact proteins. To evaluate the possibility to use a nonvolatile electrolyte for CE, the effect of sodium phosphate and ammonium borate on the MS signal of the proteins insulin, myoglobin and bovine serum albumin (BSA) was investigated by employing infusion experiments, and compared to the effect of ammonium formate and formic acid. The study shows that with formic acid (50 mM, pH 2.4) the most intense protein signals were obtained, while the use of sodium phosphate buffer (5 and 10 mM, pH 7.5) almost completely diminished the MS response. Ammonium formate and ammonium borate (up to 100 mM, pH 8.5) also caused protein ion suppression, but especially with the borate buffer significant MS intensity remained. MS analysis of myoglobin revealed the loss of the heme group when an acidic CE electrolyte was used. Using a background electrolyte containing 25 mM ammonium borate (pH 8.5), it is demonstrated that a CE separation of a protein test mixture can be monitored with ESI-MS without degrading the MS performance allowing molecular weight determinations of the separated compounds. In presence of borate, detection limits were estimated to be 5-10 μM (ca. 100 fmol injected). The usefulness of the CE-MS system employing a borate buffer is indicated by the analysis of a stored sample of BSA revealing several degradation products. A sample of placental alkaline phosphatase (PLAP), a potential therapeutic agent, was also analyzed by CE-MS indicating the presence of a protein impurity. Probably due to insufficient ionization of the PLAP (a complex glycoprotein), no MS signals of the intact protein were observed.

4.1 Introduction

Quite a number of separation techniques have been used to monitor purity and stability of proteins. For example, size-exclusion chromatography (SEC), ion-exchange liquid chromatography (IEC), slab-gel electrophoresis, and reversed-phase liquid chromatography (RP-LC) are routinely applied to monitor changes in size, charge and hydrophobicity of proteins [1-8]. Over the last years, capillary electrophoresis (CE) has enriched the arsenal of protein analysis [9] by providing an efficient alternative to traditional and laborious slab-gel techniques. In addition, CE provides a charge-based mode of separation that often is complementary to the chromatographic techniques heavily used in analytical biotechnology. Presently, CE has become an accepted technique for the analysis of proteins [10-12] such as recombinant insulin [13, 14], erythropoietin [15, 16], and immunoglobulins [17]. CE can be highly useful for efficiently analyzing the differences and charge heterogeneity of intact proteins that might be difficult to assess with LC techniques.

Mass spectrometry (MS) nowadays is an important and powerful tool for the analysis and characterization of proteins [18, 19]. The multiple charging of proteins observed in electrospray ionization (ESI) allows their analysis on MS instruments with mass ranges far below the molecular masses of the proteins. MS detection can considerably enhance the utility of CE by providing information about the identity of the separated compounds. In purity and stability studies the availability of MS data is highly desirable. Characterization of peaks by molecular mass strongly adds to the reliability and is very helpful when conditions are changed and cross correlations have to be made. Therefore, for quality control, e.g., when changes in proteins during storage or exposure are monitored, CE-MS would be an attractive approach. CE-ESI-MS analysis of (test) mixtures of intact proteins such as insulin, cytochrome c, lysozyme and lactoglobulins has been reported [20-23]. In most of these cases, for CE volatile electrolyte systems at relatively low concentrations were used. However, optimum CE performance often requires nonvolatile electrolytes at considerable concentrations.

In the present study, the possibility of directly introducing non-volatile buffer into the mass spectrometer is studied using a triaxial ESI interface. Using three proteins of different molecular weight as test proteins, *viz.* insulin, myoglobin and bovine serum albumin (BSA), the influence of

several background electrolytes on the intensity and character of the resulting mass spectra, and the CE-ESI-MS performance is studied. The analysis of degraded BSA with CE-ESI-MS using a non-volatile background electrolyte is investigated. Finally, attempts are made to analyze samples of placental alkaline phosphatase, a potential therapeutic [24], with the CE-ESI-MS system.

4.2 Materials and methods

Materials

Sodium hydroxide, phosphoric acid, methanol, sodium dihydrogen phosphate, disodium hydrogen phosphate, ammonium hydroxide, formic acid, and acetic acid were from Merck (Darmstadt, Germany), and boric acid, and ammonium formate were from Fluka (Zwijndrecht, The Netherlands). Myoglobin from horse heart, bovine serum albumin and human placental alkaline phosphatase were from Sigma (St. Louis, MO, USA). Insulin (Actrapid®) was from Novo Nordisk A/S (Bagsvaerd, Denmark). Deionized water was filtered and degassed before use.

The electrolytes studied were 50 mM formic acid (pH 2.4), 5 and 10 mM sodium phosphate (pH 7.5), 10-100 mM ammonium borate (pH 8.5), and 10-100 mM ammonium formate (pH 8.5). The ammonium formate and borate buffers were brought to pH by adding ammonium hydroxide (25%), while the pH of the phosphate buffer was adjusted with either 1M NaOH or 1M phosphoric acid. For the infusion experiments, 100 µM solutions of insulin, myoglobin and BSA, respectively, were prepared in each electrolyte and water. A test mixture of insulin, myoglobin and BSA (~50 µM each), and a solution of placental alkaline phosphatase (~50 µM) were prepared in water.

CE system

For CE-ESI-MS a PrinCE CE system (Prince Technologies, Emmen, The Netherlands) equipped with a 75 µm I.D. fused-silica capillary of 75 cm was used. The capillaries were from Composite Metal Services (The Chase, Hallow, UK) and flushed with 0.1 M sodium hydroxide and water (each 30 min at 1000 mbar) before use. Prior to every CE analysis the capillary was flushed with fresh background electrolyte for 1 min at 1000 mbar. Hydrodynamic injection of sample was performed at 35 mbar for

6 seconds, and CE was performed at a potential of 30 kV while applying an underpressure of typically 35 mbar to the capillary inlet (for reason, see Results and Discussion). During infusion experiments, the sample solution under study was led continuously from the inlet vial through the capillary to the ESI interface at a flow rate of approximately 400 nl/min.

MS system

MS experiments were carried out on an Agilent 1100 Series LC/MSD-SL ion-trap mass spectrometer (Agilent Technologies, Waldbronn, Germany) operated in the positive ion mode and equipped with an ESI source. CE-ESI-MS was performed using a triaxial interface from Agilent in which capillary effluent is mixed with sheath liquid (5 µl/min) and nebulized by nitrogen gas (pressure, 15 p.s.i.). The sheath liquid was water-acetonitrile-formic acid (50:50:1, v/v/v) or water-methanol-acetic acid (50:50:1, v/v/v), and was supplied by a syringe pump. Drying gas temperature and flow rate was set at 150 °C and 4 L/min, respectively. The electrospray voltage was 5.0 kV and the mass spectrometer was operated in full scan mode (range, 800-2200 m/z) and three scans were averaged for one spectrum. The ion accumulation time was automatically adjusted using the Ion-Charge-Control option of the instrument. The MS settings, such as capillary exit, skimmer and lens voltages, were optimized and tuned by instrument and data acquisition software during infusion of a 50 µM solution of insulin.

4.3 Results and discussion

Infusion experiments

The influence of various electrolytes on the MS signal of the test proteins was determined by infusion of sample solutions into the ion trap mass spectrometer. In order to mimic CE-ESI-MS conditions as much as possible, the protein solutions in each respective electrolyte were led through the CE capillary to the ESI interface in which the capillary effluent is merged with sheath liquid prior to nebulization. The underpressure caused by the nebulizing gas at the capillary outlet causes a flow through the capillary similar in rate to a common electroosmotic flow (EOF). No separation voltage was applied in this instance because it would have led to different

flow rate and mobility of the proteins amongst the various electrolytes, thereby hindering a proper comparison.

The effect of four electrolytes (formic acid, ammonium formate, sodium phosphate and ammonium borate) on the MS response of insulin was investigated. For all evaluated electrolytes the most abundant signals produced by insulin in the mass spectra were detected at m/z 1452.9 and 1162.7, which correspond to the 4+ and 5+ ions. The shape and position of the charge envelopes observed for insulin at the respective electrolytes were very similar yielding a reconstructed molecular mass of 5808.1 (± 0.6). However, the signal intensities differed markedly (Fig. 1).

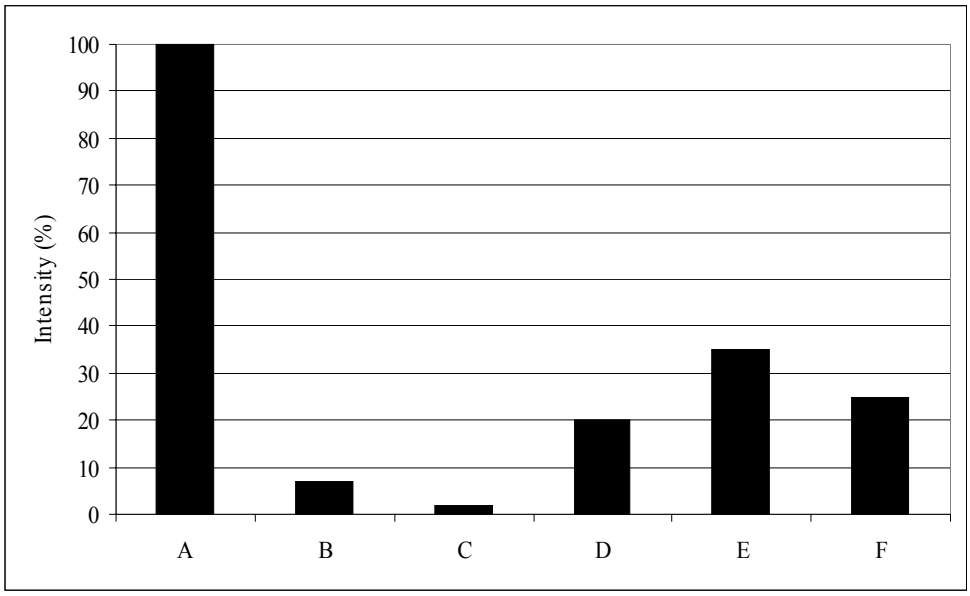

Fig. 1 *Relative MS signal of insulin (100 μM) in various background electrolytes measured by infusion: (A) formic acid (50 mM), (B) sodium phosphate (5 mM, pH 7.5), (C) sodium phosphate (10 mM, pH 7.5), (D) ammonium formate (25 mM, pH 8.5), (E) ammonium borate (10 mM, pH 8.5), and (F) ammonium borate (25 mM, pH 8.5). The signal obtained for insulin in 50 mM formic acid was set to 100%, and the insulin intensity is expressed as the sum of the signals of the two most abundant ions at m/z 1162.7 and 1452.9. Values are averages of three replicate measurements; relative standard deviations (RSDs) were always less than 10%. Sheath liquid, water-acetonitrile-formic acid (50:50:1, v/v/v); further conditions, see Experimental section.*

As expected, the highest intensities for insulin were obtained in the presence of the volatile formic acid. Phosphate is a well-known ion suppressor in ESI, and indeed the phosphate buffer caused a dramatic decrease of the protein signal; using 10 mM phosphate (pH 7.5) less than 3% of the signal remained with respect to formic acid. This reduction can

be fully attributed to ion suppression effects, and not to source fouling as the intensity was restored when the insulin solution in formic acid was measured again. When applying an insulin solution in 25 mM ammonium formate (pH 8.5), the observed signal was considerably higher than with phosphate, although still about five times lower than for formic acid. Ammonium borate buffer (25 mM, pH 8.5) also led to a decrease of the insulin signal but it should be noted the MS signal was still significant and could be measured reliably. Somewhat surprisingly, the intensity for insulin in the presence of the nonvolatile borate was even stronger than the signal found for the same concentration in ammonium formate (Fig. 1). For all electrolyte systems studied no increased background signals or noise were observed. The buffer ions and their clusters typically exhibit m/z values below 600 and are therefore not detected in the applied scan range (m/z 800-2200).

The influence of the various electrolytes on the MS response of myoglobin (100 µM) was also studied by infusion of the respective solutions into the mass spectrometer *via* the triaxial interface. In general the effect of the electrolytes on the signal intensity of myoglobin was quite the same as observed for insulin. Phosphate buffer (5 and 10 mM) almost fully diminished the myoglobin signal and no clear charge envelope could be observed. Much better results were obtained for myoglobin in ammonium formate and ammonium borate showing a quite symmetric charge distribution with the most abundant signal at m/z 1598.1. Again the signal suppressing effect of the buffer was more pronounced for ammonium formate than for ammonium borate. For example, addition of 25 mM ammonium formate yielded a decrease of 73% of the total signal intensity of myoglobin (accumulation of intensities of ten most abundant ions), whereas for 25 mM ammonium borate only 20% reduction of the signal was observed. Over the range of 0-100 mM ammonium borate, the protein signal decreased from 100 to 37% in a linear fashion, so even in the presence of 100 mM of this buffer significant protein-ion intensities could still be measured. As could be expected, highly intense spectra were obtained for myoglobin dissolved in 50 mM formic acid (pH 2.4), but quite remarkably, a clearly different charge envelope was found when compared to myoglobin in ammonium borate (Fig. 2).

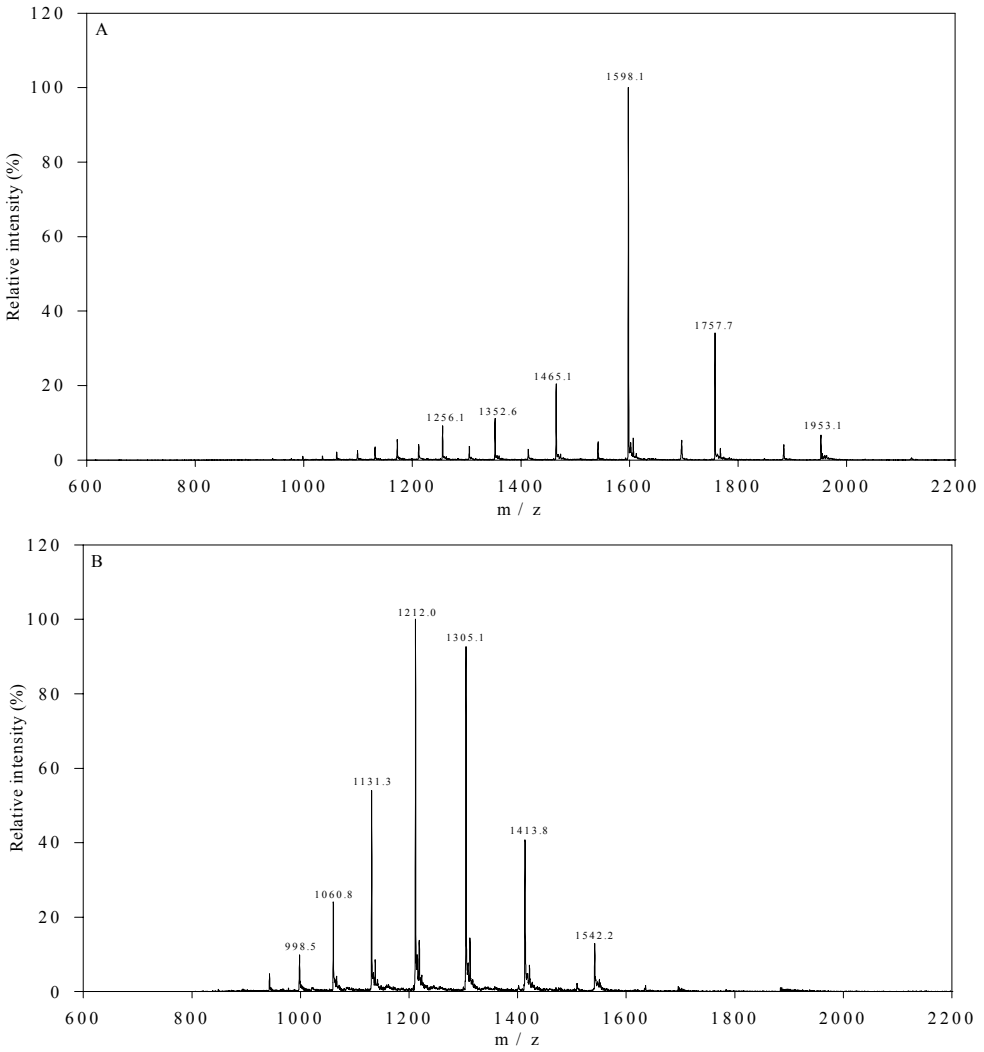

Fig. 2 *Mass spectra of myoglobin (100 μM) in (A) 25 mM ammonium borate (pH 8.5) and (B) 50 mM formic acid (pH 2.4) acquired during infusion. Sheath liquid, water-methanol-acetic acid (50:50:1, v/v/v); further conditions, see Experimental section.*

In formic acid the centre of the charge distribution (most abundant ion now at m/z 1212.0) as well as the m/z positions of all protein ions were shifted. The shift of observed m/z values towards lower values indicates more charging of the protein. This could be an effect of unfolding of the protein, which would expose additional ionizable groups and thus lead to higher ionization. Based on the obtained spectra, the molecular mass of the

analyzed protein in formic acid was calculated to be 16 955.0 (±1.7), whereas a molecular mass of 17 569.2 (±1.6) was found for myoglobin in ammonium borate (using the positions of the most abundant ions), a difference of 616 Da. We presume that this discrepancy originates from the heme group of myoglobin, which is not covalently bound to the protein and may be lost under acidic conditions [25-28]. Closer inspection of the mass spectrum of myoglobin obtained in ammonium borate at pH 8.5 (Fig. 2A) shows that also signals of myoglobin without the heme moiety are found, but at relatively low intensity. Possibly, the acidic sheath liquid may cause some detachment of the heme group, but apparently the contact time is too short to result in a full loss of the heme as occurs when myoglobin is dissolved in formic acid prior to MS analysis (Fig. 2B).

The influence of various electrolytes on the ESI-MS performance of BSA (100 µM) was essentially in line with results obtained for the other proteins. With formic acid (50 or 100 mM, pH 2.4) a good-quality charge envelope of BSA was obtained. During infusion of BSA (100 µM) in phosphate buffer (5 and 10 mM, pH 7.5), no clearly resolved spectra could be measured. Infusion of BSA dissolved in clear ammonium borate buffer (25 and 50 mM, pH 8.5) still yielded spectra of good quality allowing the molecular mass to be measured: 66 568 (± 23). Yet, the protein signals obtained with borate were considerably lower in intensity than in the presence of formic acid (50 or 100 mM, pH 2.4).

CE-MS of test proteins

The infusion experiments described above indicate that it should be possible to record significant MS signals from proteins under common CE conditions, i.e. using borate as background electrolyte. In order to evaluate this, a protein test mixture consisting of insulin, myoglobin and BSA (50 µM each) was analyzed by CE-ESI-MS applying a 25 mM ammonium borate buffer (pH 8.5). As mentioned above, the nebulizing gas in the ESI interface caused an underpressure at the capillary outlet and consequently an additional flow through the capillary, which obviously is undesirable when performing CE. In an attempt to avoid this effect, an underpressure of 35 mbar was applied to the capillary inlet during CE-ESI-MS. Fig. 3A shows a typical CE-ESI-MS result of the test mixture with the proteins eluting in the order myoglobin, insulin, BSA. The proteins are well separated and can easily be detected in the total-ion-current (TIC) trace with a satisfactory signal-to-noise ratio. In full-scan mode the detection

limits (S/N of 3) for these proteins are estimated to be in the low µM region (corresponding to ca. 100 fmol injected), which is quite favourable for a CE-ESI-MS method using a nonvolatile background electrolyte. The protein peaks (and especially BSA) are somewhat broadened, indicating that the CE performance was not optimal. It should be noted, however, that in this study no attempts were made to prevent the proteins from adsorbing to the capillary wall, a notorious source of band broadening in protein CE [9, 29-31]. Moreover, the mixing of the capillary effluent with sheath liquid and the potentially unsuccessful circumvention of pressure differences across the capillary inherently may lead to increased peak widths.

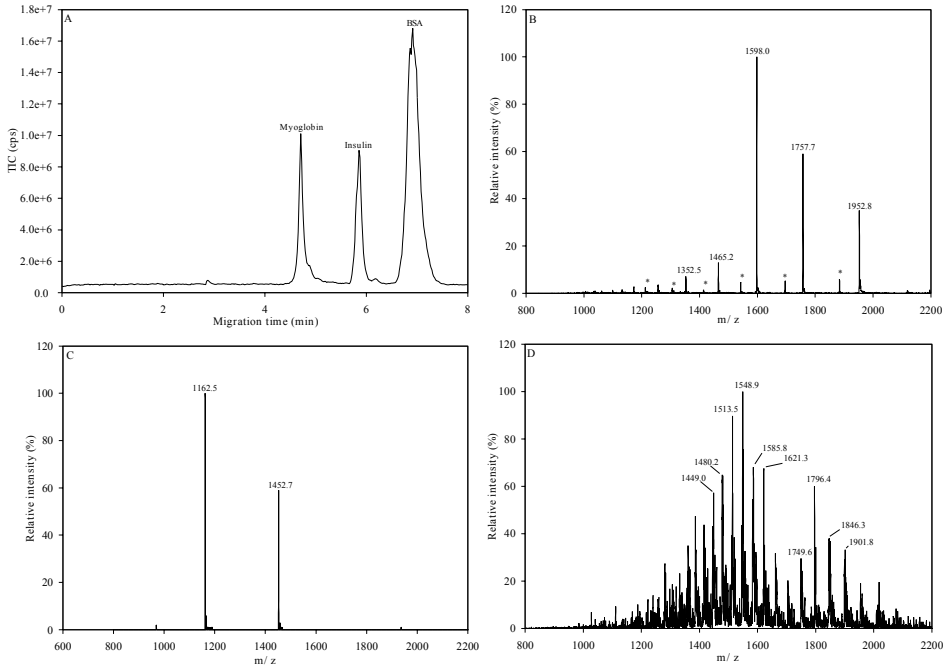

Fig. 3 *CE-MS of a mixture of myoglobin, insulin, and BSA (50 µM each). (A) TIC trace, and (B-D) mass spectra recorded at the apices of the peaks of myoglobin, insulin and BSA, respectively. In (B) ions originating from myoglobin without heme group are indicated with an asterisk. Run buffer, 25 mM ammonium borate (pH 8.5); sheath liquid, water-methanol-acetic acid (50:50:1, v/v/v); further conditions, see Experimental section.*

CE-ESI-MS analysis yielded good-quality mass spectra for the separated proteins (Figs. 3B-D) permitting the determination of the respective molecular masses: 17 570.9 (±4.4), 5807.2 (±0.5), and 66 510 (±69) for myoglobin, insulin and BSA, respectively. The spectrum obtained for myoglobin (Fig. 3B) is mainly dominated by ions of the intact protein,

although some weak signals of myoglobin without the heme group can be discerned in the spectrum (*cf.* Fig. 2A). CE-ESI-MS analysis of BSA (about 50 μM in water) that had been stored for two weeks at ambient conditions revealed several new peaks next to BSA, indicating degradation of the protein. In other words, the developed CE-MS method could be potentially useful in the stability monitoring of proteins.

CE-ESI-MS of alkaline phosphatase

Alkaline phosphatase is a promising agent for the treatment of sepsis [24] and recently it has been demonstrated that CE with UV absorbance detection can be useful in the assessment of the purity and stability of this dimeric protein (monomer molecular mass, about 58 kDa) [32, 33]. Clearly, it would be advantageous to have additional mass information on the CE separated species as well. To check the feasibility of this, a commercially available sample of placental alkaline phosphatase (PLAP) was analyzed with the developed CE-ESI-MS system using ammonium borate as background electrolyte. The result shows an electropherogram with a broad hump followed by a rather sharp peak with a shoulder (Fig. 4A). In a previous study with CE-UV [32] a shouldered peak was also found, but the hump was not detected. Unfortunately, the mass spectra acquired in the 5-7 min region (i.e. under the broad band) appeared to be rather featureless and hard to interpret. In contrast, for the PLAP peak at 7.2 min a distinct ion pattern was observed in the acquired mass spectrum (Fig. 4B).

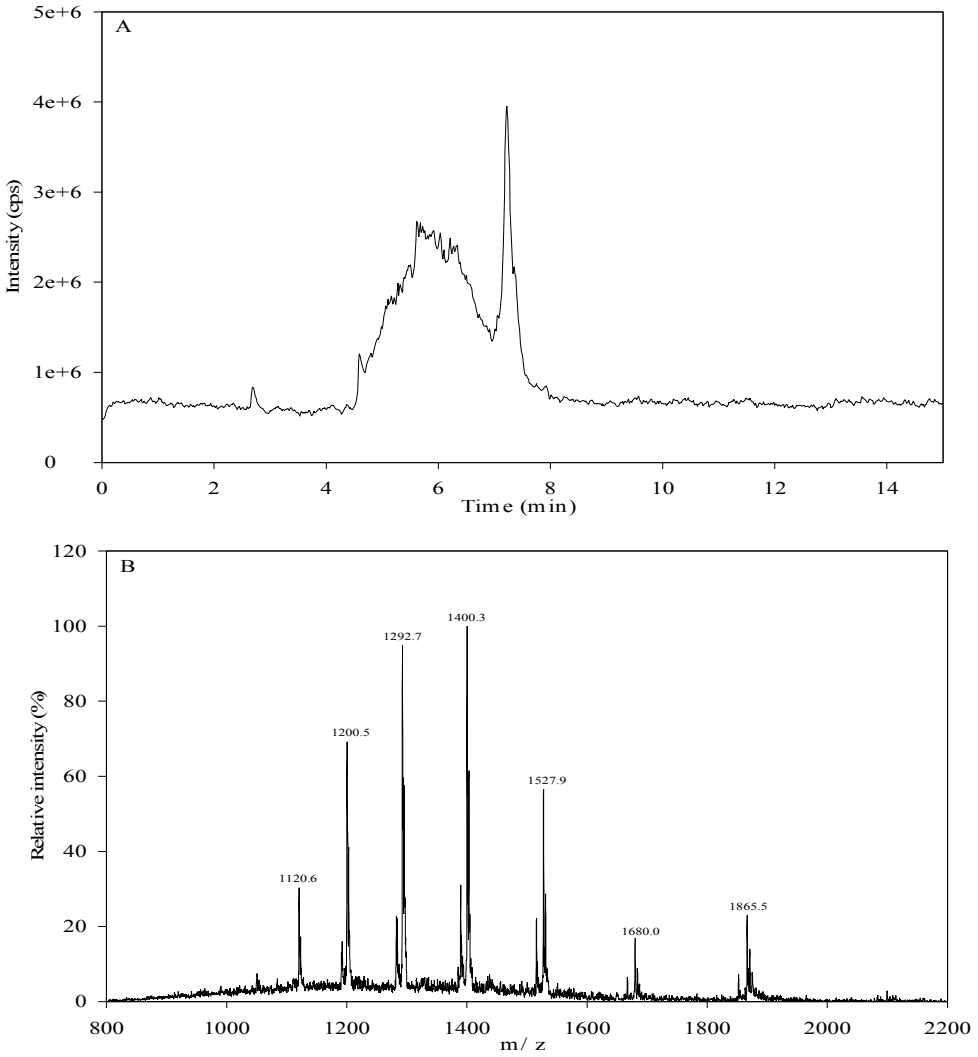

Fig. 4 *CE-MS of PLAP (50 μM). (A) TIC trace, and (B) mass spectrum recorded at the apex of peak at 7.2 min. Experimental conditions: see Fig. 3.*

Oddly, the molecular mass that was derived from the positions of the most intense ions in this spectrum was 16 792 Da, and not about 58 000 Da as should be expected for the PLAP monomer. Moreover, considering the injected amount of protein, the absolute intensity of the observed signals was relatively low indicating that the actual intact protein has not been detected. Direct ESI-MS analysis of the PLAP sample by infusion also yielded a spectrum quite similar to Fig. 4B without any signals of native

PLAP. An enzymatic activity assay on the sample, on the other hand, indicated that PLAP was actually present in the expected amounts. Moreover, gel electrophoresis previously carried out on the PLAP revealed a band at the appropriate molecular weight. Therefore, we presume that during CE-ESI-MS only an impurity present in the PLAP sample is detected. The determined mass of 16.8 kDa corresponds with the molecular weight of Interferon-γ which indeed can be present in human placenta [34]. The absence of a PLAP signal in the mass spectrum might be caused by the fact the ionization of PLAP, like for other glycoproteins [35], is troublesome and incomplete so that the resulting ions are outside the range of the mass spectrometer and consequently not detected. Earlier attempts to analyze this commercial PLAP by MALDI-TOF-MS were also not successful, although for a sample of in-house purified PLAP a good MALDI-TOF spectrum was obtained indicating a protein monomer mass of about 58 100 Da [35]. ESI-MS analysis of this purified PLAP, however, did not yield a proper spectrum. Further strategies to improve the susceptibility of PLAP for MS detection include deglycosylation prior to MS analysis and tryptic digestion followed by MS analysis of the produced peptides.

4.5 Concluding remarks

The influence of some nonvolatile electrolytes on the analysis of proteins by CE-ESI-MS was evaluated. It was found by infusion experiments that sodium phosphate strongly suppressed the MS signals of the proteins yielding no interpretable spectra. However, when ammonium borate buffer was used satisfactory MS intensities for the proteins could be achieved allowing reliable molecular weight determinations on the basis of the recorded charge envelopes. For a background electrolyte containing 25 mM ammonium borate, detection limits of about 100 fmol were found for the proteins. Analysis of a degraded BSA sample indicated that CE-ESI-MS using non-volatile background electrolyte can be employed for the study of the stability of (therapeutic) proteins, although the ionization of glycoproteins might be troublesome.

Generally, it can be concluded that the use of nonvolatile electrolytes as ammonium borate for CE-MS is possible. Further research includes the long-term stability of the ESI interface while employing nonvolatile buffer, and a more in-depth evaluation of the influence of these buffers on the

character of the charge envelope of different proteins. On the CE side, we are now evaluating the usefulness of some charged polymers for noncovalently coating the inner wall of the capillary in order to improve the CE performance of proteins, and we will also test the applicability of these coatings in combination with MS detection. We are also interested in investigating whether the application of nonvolatile buffers is feasible in CE-MS with sheathless interfacing.

4.6 References

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