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Potential of capillary electrophoresis for the monitoring of the stability of placental alkaline phosphatase

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Abstract

Alkaline phosphatase (AP) is a potential therapeutic agent in the treatment of sepsis. In this paper the potential of capillary zone electrophoresis (CZE) for the monitoring of the degradation of placental alkaline phosphatase (PLAP) was investigated. To induce degradation PLAP samples were exposed to high temperatures, low and high pH and freeze-drying. The samples were then analyzed by CZE and enzymatic activity assay. Upon exposure to temperatures above 65 °C, PLAP lost its activity exponentially over time, while CZE revealed both a linear decrease of the area of the main peak and a rise of degradation products. At acidic pH the enzyme appeared to lose its activity. CZE revealed a decrease of the area of the main peak, but no degradation products could be detected. At pH 12 the enzymatic activity and the area of the main peak both decreased linearly over time and, in addition, formation of degradation products could be detected by CZE. Activity and CZE profile of PLAP remained unchanged upon freeze-drying in the presence of inulin. Prolonged storage of freeze-dried samples at room temperature caused a slight decrease of enzymatic activity, while the potential formation of oligomers was revealed by CZE analysis. The examples in this study show that, in combination with activity assays, CZE can provide useful complementary information, especially on the status of the protein and the presence of degradation products.

Keywords: Alkaline phosphatase; Therapeutic protein; Protein stability; Capillary electrophoresis; Enzymatic activity

1. Introduction

Pharmaceutically active proteins have been applied for decades. However, their number has been small until the 80s, but since that time rapid developments in molecular biology resulted in a fast increase in their number. Currently, the FDA has approved over 30 different recombinant DNA-

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derived proteins, e.g. erythropoietin, interferon alpha-2a/b, somatropin, and follitropin beta and many more are already in a far stage of development. This fast growth asks for improved analytical methods, which can be used for quality control and stability studies.

Proteins are complex molecules containing many functional groups, which can undergo a variety of reactions, such as deamidation, hydrolysis, oxidation, and racemisation of the amino acids. The type and rate of these reactions depend on the amino acids involved and the conditions applied. These reactions can alter the conformation, size, charge and hydrophobicity of the protein, or may totally degrade it, thereby affecting the activity of the protein or even leading to formation of toxic compounds. Clearly, from the viewpoint of quality control and safety, monitoring of the stability and degradation of pharmaceutical proteins is of utmost importance, as is recognised by the ICH by formulating guidelines for stability testing of biological products [1]. Size-exclusion liquid chromatography (SEC), ion-exchange liquid chromatography (IEC), slab-gel electrophoresis and reversed-phase liquid chromatography (RP-LC) have been used to monitor changes in size, charge and hydrophobicity of proteins [2–9]. Capillary zone electrophoresis (CZE) has a high resolving power and the electrophoretic migration of compounds in free solution basically depends on their molecular charge and size. Therefore, CZE may be a powerful tool to monitor the degradation of proteins.

Recently, it was discovered that the endogenous protein alkaline phosphatase (AP, E.C. 3.1.3.1) can dephosphorylate (and thus detoxify) endotoxins, and that it might be used as a pharmaceutical in the treatment of sepsis [10]. AP is a dimeric enzyme, where each subunit has a molecular weight of 58 kDa [11]. The pI of AP has been reported to be 4.3–4.5 [12] and it exhibits optimum activity at approximately pH 9.8 [10]. Obviously, successful therapeutic use of AP requires availability of material with adequate purity and the development of appropriate dosage forms and formulations. Also, analytical methods for the determination of the purity and stability of (treated) AP are needed. In a previous paper, we demonstrated the usefulness of CZE to study the composition of AP samples during several stages of isolation/purification from human placenta [13]. In these experiments a CZE run buffer containing putrescine (1,4-diaminobutane) was used to prevent protein adsorption to the capillary [14], and to improve the peak shape and resolution. In this paper the possibility to employ CZE as a tool to monitor AP degradation is investigated and compared to enzymatic activity assay.

2. Experimental

2.1. Chemicals

Magnesium chloride, ammediol (2-amino-2-methyl-1,3-propanediol), sodium chloride, benzyltrimethylammonium chloride and 4-nitrophenyl phosphate (*p*NPP) were from Fluka (Buch, Switzerland). Boric acid, hydrochloric acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Putrescine (1,4-diaminobutane) was from Aldrich (Gillingham-Dorset, UK) and placental alkaline phosphatase (PLAP, E.C. 3.1.3.1) (14 U/mg) was from Sigma (St. Louis, MO, USA). Water used in the experiments was from an Elga Maxima Ultra Pure Water apparatus (Salm & Kipp, Breukelen, The Netherlands). Inulin with a number/weight average degree of polymerisation (DP_n/DP_w) of 23/26 was a gift from Sensus (Rosendaal, The Netherlands).

2.2. Analytical techniques

2.2.1. Enzymatic activity assay

Activity of the PLAP was determined using the enzymatic conversion of *p*NPP and measuring the absorbance of the yellow product (*p*-nitrophenol) at 405 nm. For the assay 475 μl of a mixture of 97.9% v/v of 0.05 M ammediol (pH 9.8) with 2.1% v/v of 100 mM $MgCl_2$ and 25 μl of 10 mg/ml *p*NPP were mixed with 12.5 μl of sample. After incubation for 30 min at 37 °C, 2.50 ml of 0.1 M NaOH was added to stop the conversion and the absorbance of the samples was measured on a Hitachi U-2001 Spectrophotometer (Tokyo, Japan).

2.2.2. Capillary zone electrophoresis

CZE experiments were performed on a Beckman P/ACE System 5500 equipped with a diode array detector (Fullerton, CA, USA), using an uncoated fused-silica capillary (Supelco, Bellafonte, PA, USA) with 75 μm I.D. and a length of 37 cm (effective length, 30 cm). Before use the capillary was flushed for 30 min with 0.1 M NaOH, water and run buffer, respectively. The run buffer was 50 mM boric acid, containing 2 mM putrescine, adjusted to pH 8.5 using 1 M sodium hydroxide. Before addition of putrescine, the run buffer was filtered through a 0.45-μm membrane filter from Schleicher and Schuell (Dassel, Germany). Between injections the capillary was rinsed with run buffer (2 min). The separation voltage was 15 kV and the temperature was set to 25 °C. Commonly, samples were hydrodynamically injected at 0.5 p.s.i. for 3.5 s, and detection was carried out at 200 nm. Each sample was analyzed in duplicate. For quantitative analysis, migration time-corrected peak areas were used.

2.3. Stress conditions

2.3.1. Heat

For the exposure of PLAP to high temperatures a water bath set to the desired temperatures was used. PLAP was dissolved in 0.05 M ammediol buffer pH 9.8 at a concentration of 5 mg/ml. Typically, samples of 10 μl for enzymatic activity assay and 25 μl for CZE analysis were taken every hour for up to 5 h.

2.3.2. Low and high pH

Exposure of PLAP to extreme pH-values was accomplished by adding either HCl or NaOH to the sample solution. 0.05 M ammediol pH 9.8 was titrated with acid or base to the desired pH and then PLAP was added to yield a final concentration of 5 mg/ml. Samples for enzymatic activity assay and analysis by CZE were taken as described above.

2.3.3. Freeze-drying

Ammediol solutions of inulin and PLAP (inulin/PLAP, 9/1 w/w) were freeze-dried by using a CHRIST freeze-dryer equipped with temperature and pressure control. As sample containers 20-ml glass bottles were used, and before the drying process the samples were rapidly pre-frozen in liquid nitrogen. The lyophilization was then performed using a shelf temperature of −30 °C, a condenser temperature of −53 °C and a pressure of 0.220 mbar for 18 h. Then, the shelf temperature and pressure were gradually raised to 20 °C and 0.520 mbar, respectively, during 6 h. Finally, the drying process was continued for another 20 h under these conditions.

3. Results and discussion

3.1. Heat exposure

In order to examine the heat stability of PLAP, several PLAP solutions were exposed to temperatures between 25 and 75 °C. These tests were performed with PLAP dissolved in 0.05 M ammediol (pH 9.8), which also was used for the enzymatic activity assay. Up to 65 °C no loss of activity of the exposed PLAP was observed within 5 h, and also no change was observed in the electropherograms. This is in agreement with the reported heat stability of the placental form of AP (in contrast to the other isoforms) [15,16]. Upon further increase of the temperature a dramatic change in activity was observed in a relatively small temperature interval (Fig. 1A). At 67.5 °C still a rather slow and gradual loss of activity is observed, but at 70 and 72.5 °C the activity is clearly decreasing at a much higher rate. Between 67.5 and 72.5 °C the enzymatic activity changes in an exponential manner, showing the most rapid loss of activity in the first hour. At 75 °C, 90% of the activity diminished within 1 h. At this temperature the sample showed some turbidity, indicating partial protein precipitation. To prevent clogging of the capillary, this sample was not analyzed by CZE.

Distinct changes in the electropherograms of the taken samples were observed when the PLAP was exposed to temperatures above 65 °C. The degree of change was larger when the exposure time and/or temperature were increased. As an example,

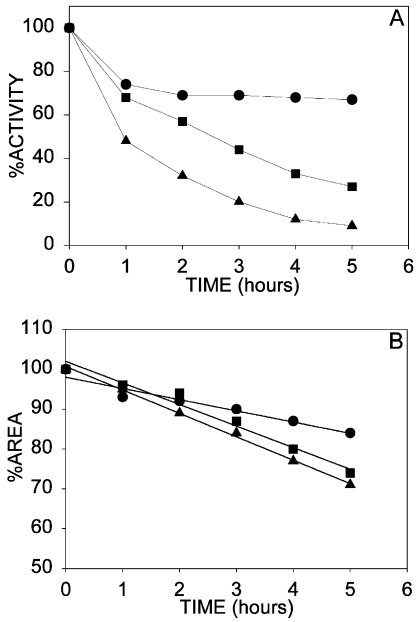

Fig. 1. Analysis of PLAP (5 mg/ml) in 0.05 M ammediol (pH 9.8) after exposure to 67.5 (●), 70 (■), and 72.5 °C (▲), respectively. (A) Relative activity; and (B) relative peak area of main peak after CZE. The activity and peak area of PLAP before exposure is set to 100%. Further conditions, see Section 2.

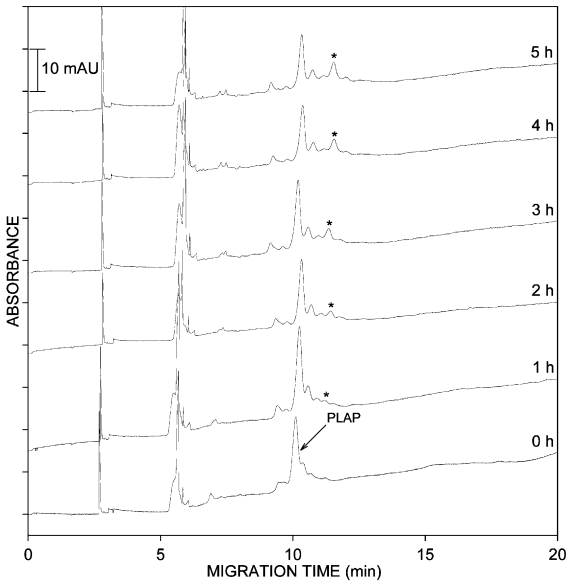

Fig. 2. CZE of PLAP (5 mg/ml) in 0.05 M ammediol (pH 9.8) exposed to 72.5 °C for up to 5 h. Further conditions, see Section 2.

Fig. 2 shows the CZE results obtained for a PLAP sample which was exposed to 72.5 °C for 5 h. CZE analysis of unexposed PLAP using a background electrolyte of borate buffer (pH 8.5) with 2 mM putrescine reveals one major peak with some minor shoulders. The peaks at $t_{mig} = 3$ and 6 min stem from benzyltrimethylammonium chloride, which was used as a marker, and the ammediol present in the sample, respectively. As described previously [13], the shoulders of the main peak ($t_{mig} = 10$ min) probably comprise some glycoforms of PLAP, but this should still be confirmed by mass spectrometric detection. In contrast to our previous study [13], no late migrating, broad band was detected. Probably, this is related to the fact that two different batches of AP were used in the two studies. Clearly, upon heat exposure the main peak decreases while a degradation product (marked with an asterisk) emerges. In contrast to the activity, the decrease of the area of the main peak was found to be linear over time during exposure to 67.5–72.5 °C (Fig. 1B). After 5 h at 67.5 °C the peak area was 85% of the original value, while at 72.5 °C the area had diminished to 69%. An explanation of the difference in decay of enzymatic activity and peak area of the main peak (exponential vs. linear), might be that the increased temperature caused both chemical degradation of PLAP and small conformational changes. It is possible that both effects are reflected in the enzymatic activity, while CZE reveals only the effects of chemical modifications of the protein.

3.2. Exposure to acidic and basic conditions

In order to study the stability of PLAP under acidic conditions, PLAP (5 mg/ml) was dissolved in 0.05 M ammediol that had been adjusted to pH 5, 4, 3 and 2 with 1 M HCl. These samples were then monitored over time by enzymatic activity and CZE analysis. Already at pH 4, a considerable loss of activity of PLAP was observed down to 13% within 4 h. The decay was exponential, showing a 60% loss in the first hour of exposure. CZE analysis of the sample exposed to pH 4

showed an exponential decay of the main peak over time, and after 4 h the area of the main peak had decreased to 25% of its original value. However, no new peaks emerged in the electropherogram, i.e. no degradation products were observed. Probably, the PLAP (pI ≈ 4.3–4.5 [12]), simply precipitated at pH 4, although the loss of enzymatic activity could also be caused by an acid-induced loss of Zn^{2+} [17,18], which is vital for the enzyme [19]. At pH 3 these effects were much stronger and PLAP instantaneously lost all of its activity and no peaks were observed when this sample was analyzed by CZE.

When PLAP solutions were brought to pH 8–10 for a period of 5 h no changes in the activity or the CZE profiles were observed. Naturally, this can be expected for a protein that shows optimum activity in an alkaline environment. Even at pH 11, PLAP degradation appeared to be negligible. When PLAP was exposed to pH 12 for 5 h, the activity decreased linearly to 74% of its original value (Fig. 3), and CZE analysis of the same samples (Fig. 4) revealed that the composition of the exposed sample changed with a similar rate. The area of the main peak decreased in 5 h to about 65% with respect to the non-exposed sample (Fig. 3). Simultaneously, around $t_{mig} = 19$ min a new peak (marked by an asterisk) appears. In front of the PLAP-cluster a broad shoulder (marked by an arrow) emerges, which we suggest is an effect of oligomerisation of PLAP, yielding products with similar mass to charge ratios.

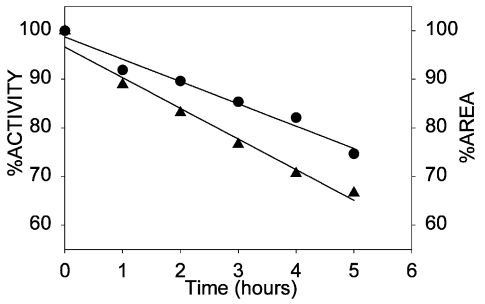

Fig. 3. The analysis of PLAP (5 mg/ml) in 0.05 M ammediol (pH 12) after exposure for up to 5 h, (●) relative activity and (▲) relative peak area of main peak after CZE. The activity and peak area of PLAP immediately after pH adjustment is set to 100%. Further conditions, see Section 2.

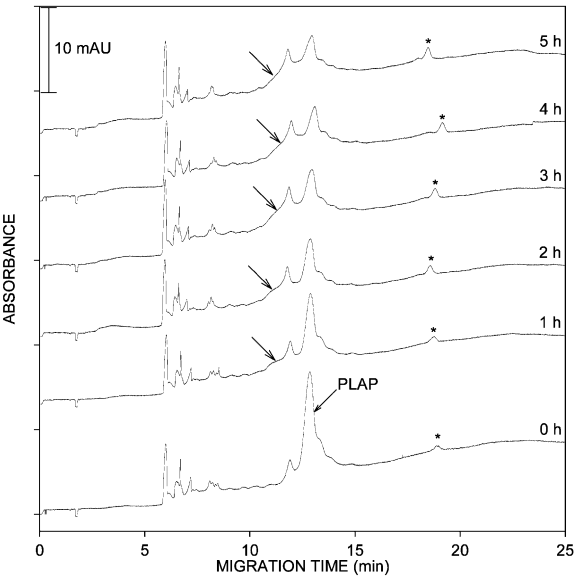

Fig. 4. CZE of PLAP (5 mg/ml) in 0.05 M ammediol adjusted to pH 12 during 5 h after pH adjustment. Sample at 0 h is taken just after pH adjustment. Further conditions, see Section 2.

3.3. Freeze-drying

The effect of freeze-drying of PLAP in the presence of inulin (a protective sugar, [20]) was investigated by analysing the PLAP before and immediately after the process. The enzymatic activity of PLAP after freeze-drying appeared to be unaffected, and also with CZE a similar peak pattern was obtained (Fig. 5). The inulin had no adverse effect on the CZE performance; the large peak at $t_{mig} = 6$ min is caused by the ammediol in the freeze-dried product. The difference in the height of the peaks (Fig. 5a and b) is due to a difference in injection times. After storage of the freeze-dried PLAP for 22 days at room temperature, the enzymactic activity decreased to 83% of the original value. CZE analysis showed a different peak pattern when compared to the pattern obtained at 0 h (Fig. 6). In our previous study [13] the analysis of pure PLAP also resulted in a cluster of peaks at ca. 10 min in the electropherogram, which was attributed to the microheterogeneity of the protein. Possibly, storage of the

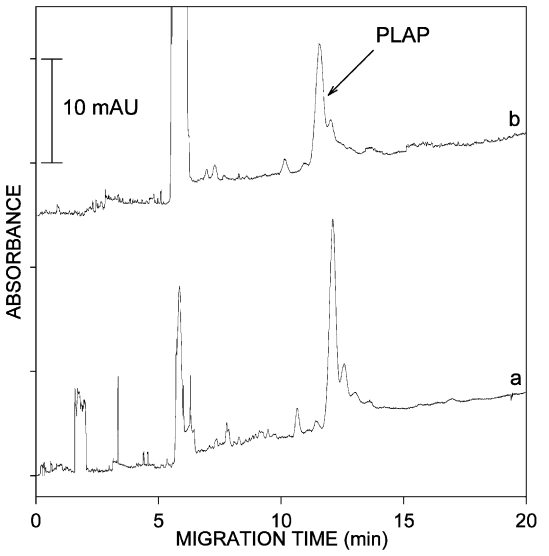

Fig. 5. CZE of PLAP (5 mg/ml) in 0.05 M ammediol (pH 9.8) before (a) and after (b) freeze-drying. Further conditions, see Section 2.

freeze-dried material led to oligomerisation/aggregation of PLAP, a phenomenon that also has been reported for lyophilised bovine serum albumin [21].

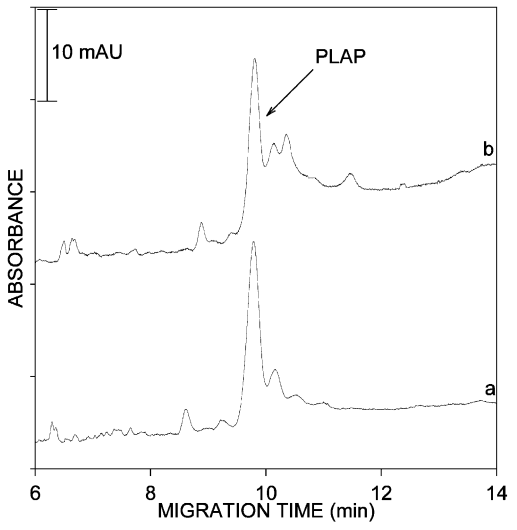

Fig. 6. CZE of PLAP freeze-dried in presence of inulin (a) and then stored for 22 days in a vacuum dessicator at room temperature (b). Further conditions, see Section 2.

4. Conclusions

The role of CZE to monitor the stability/degradation of a therapeutically interesting protein has been demonstrated. Determination of only the enzymatic activity would provide no information on the state of the protein and/or presence of degradation products. With CZE molecular changes in the protein can be monitored by comparing the electropherograms of the protein sample obtained before and after exposure to stress conditions. For instance, CZE analysis of PLAP shows that the reduction of activity caused by exposure to high temperature or high pH is accompanied by a decrease of the main-peak area as well as with the formation of degradation products. On the other hand, upon freeze-drying and subsequent storage of PLAP, only a minor change in activity of PLAP is observed while CZE reveals a clear change in peak profile, probably indicating aggregation of the protein. Admittedly, we are not yet able to reliably assign observed changes in the CZE profile to specific protein alterations or degradation products. Partly, this is due to the use of UV-detection so that hardly any information on the character of the separated compounds is obtained. Therefore, we are currently investigating the use of coupled CZE and mass spectrometry to characterize the observed protein peaks and to identify degradation products.

References

[1] International conference on harmonization of technical requirements for registration of pharmaceuticals for human use, in: Q5C, ICH Harmonised Tripartite Guideline, Quality of Biotechnological Products: Stability Testing Biotechnological/Biological Products. http://www.ich.org
[2] A. Arroyo-Reyna, A. Hernandez-Arana, Biochim. Biophys. Acta 1248 (1995) 123–128.
[3] S.R. Davio, K.M. Kienle, B.E. Collins, Pharm. Res. 12 (1995) 642–648.
[4] V.N. Uversky, Biochemistry 32 (1993) 13 288–13 298.
[5] T. Daiho, T. Kanazawa, J. Biol. Chem. 269 (1994) 11 060–11 064.
[6] A. Zapun, D. Missiakas, S. Raina, T.E. Creighton, Biochemistry 34 (1995) 5075–5089.

[7] J. Eichler, D.I. Kreimer, L. Varon, I. Silman, L. Weiner, J. Biol. Chem. 269 (1994) 30 093–30 096.
[8] M.C. Manning, K. Patel, R.T. Borchardt, Pharm. Res. 6 (1989) 903–918.
[9] M.F. Powell, ACS Symp. Ser. 567 (1994) 100–117.
[10] K. Poelstra, W.W. Bakker, P.A. Klok, M.J. Hardonk, D.K. Meijer, Lab. Invest. 76 (1997) 319–327.
[11] A.J. Gottlieb, H.H. Sussman, Biochim. Biophys. Acta 160 (1968) 167–171.
[12] P.J. Greene, H.H. Sussman, Proc. Natl. Acad. Sci. USA 70 (1973) 2936–2940.
[13] H.J.C. Eriksson, G.W. Somsen, W.L.J. Hinrichs, H.W. Frijlink, G.J. de Jong, J. Chromatogr. Biomed. Appl. 755 (2001) 311–319.
[14] B. Verzola, C. Gelfi, P.G. Righetti, J. Chromatogr. A 868 (2000) 85–99.
[15] E.R. Harkness, Arch. Biochem. Biophys. 126 (1968) 503–512.
[16] F.C. Neale, J.S. Clubb, D. Hotchkis, S. Posen, J. Clin. Path. 18 (1965) 359–363.
[17] K. Hiwada, E.D. Wachsmuth, Biochem. J. 141 (1974) 283–291.
[18] M.J. Schlesinger, K. Barrett, J. Biol. Chem. 240 (1965) 4284–4292.
[19] B.H.J. Hofstee, Arch. Biochem. Biophys. 59 (1955) 352–365.
[20] W.L. Hinrichs, M.G. Prinsen, H.W. Frijlink, Int. J. Pharm. 215 (2001) 163–174.
[21] G.M. Jordan, S. Yoshioka, T. Terao, J. Pharm. Pharmacol. 46 (1994) 182–185.