FFE Weber GmbH

ProMetHEUS™ FFE

Separation of a protein mixture by free-flow electrophoresis with special focus on the day-to-day reproducibility

Introduction

Free-flow electrophoresis (FFE) is a versatile fractionation technology that is particularly well suited for the fractionation of protein samples by isoelectric focusing (IEF). It offers nearly unrestricted compatibility to post-fractionation workup by 2D-electrophoresis, MS, LC-MS, simple fraction- concentration, etc.Moreover, FFE provides high resolution in combination with great longterm reproducibility. To demonstrate the latter qualities, this application note describes the nearly identical results of an experiment, that was carried out 6 times by 3 different operators within a period of 45 days: the fast IEF-fractionation of a protein mixture consisting of 3 different compounds, i.e.

- trypsin inhibitor from soybean (plurea = 5.0)
- carbonic anhydrase B from bovine erythrocytes (plurea = 7.3)
- cytochrome C from horse heart (plurea = 9.8).

Methods

Sample preparation
Trypsin inhibitor from soybean (plurea = 5.0), carbonic anhydrase B from bovine erythrocytes (plurea = 7.3), and cytochrome C from horse heart (plurea = 9.8) were obtained from Serva. For each experiment, approximately 2 mg of each protein was combined and dissolved in 2 ml of separation media (see below). Subsequently, the samples were diluted with the same media to final concentrations of 0.01-0.1 mg/ml. Traces of the red, acidic dye 2-(4-Sulfophenylazo)-1,8-dihydroxy-3,6-naphthalenedisulfonic acid (SPADNS, Aldrich) were added to facilitate the optical control of the migration of the sample within the separation chamber.

Free-flow electrophoresis
7 FFE experiments were carried out by 3 different operators over a period of 45 days. All separations were conducted at 15°C using freshly prepared media:

Anodic stabilization medium (I1+2) 17.5% (w/w) glycerol; 42% (w/w) 8M urea; 0.03% (w/w) HPMC; 150 mM H_2SO_4
Separation medium (I3+4) 14.5% (w/w) glycerol; 42% (w/w) 8M urea; 0.03% (w/w) HPMC; 19% (w/w) Prolyte™ 2, 10 mM DTT, 1% (w/w) CHAPS
Cathodic stabilization medium (I5-7) 17.5% (w/w) glycerol; 42% (w/w) 8M urea; 0.03% (w/w) HPMC; 150 mM NaOH
Counterflow medium 14.5% (w/w) glycerol; 42% (w/w) 8M urea
Anodic circuit electrolyte 100 mM H_2SO_4
Cathodic circuit electrolyte 100 mM NaOH

The experiments were run in a horizontal separation chamber using a 0.4 mm spacer. The separation chamber was artificially narrowed from 10 cm to approximately 3 cm by the application of the stabilization media via inlets 11, 12, 15, 16, and 17 instead of merely inlets 11 and 17. Flow rates of ~ 80 g/h (Inlet 11-7) were used in combination with a voltage of 500 V which resulted in currents of ~ 20 mA. Samples were perfused into the separation chamber via the middle inlet at ~ 1 g/h. Residence times in the separation chamber were ~ 15 min. Fractions were collected in polypropylene microtiter as well as minititer plates, numbered 1 (anode) through 96 (cathode).

Data analysis

The pH-values of the individual microtiter plate fractions were measured manually. Subsequently, the protein fractions were analysed by SDS-PAGE using an XCell SureLock™ Mini-Cell (Novex) in combination with precast NuPAGE® Novex 4–12% Bis- Tris gels. Silver-staining of the proteins was carried out using the SilverQuest™ kit (Novex). Based

on these gels, the distribution and relative quantity of each protein was determined using the software Quantity One® (Bio-Rad). Together with the pHvalues, the protein patterns were transferred to appropriate diagrams and superimposed according to the fraction numbers.

Results

Typically, the fractionation of protein samples using the 2 outer inlets for stabilization media leads to a “net width” of the FFE separation chamber of approximately 7 cm corresponding to 70 fractions.We decided to reduce this width to approximately 3 cm corresponding to 30 fractions by using 5 out of 7 inlets for stabilization media. This was done for the following purposes: FFE being understood as a pre-fractionation technology prior to 2D electrophoresis or LC-MS should provide a reduced number of fractions. In addition, the transit time of the sample should be decreased. The silver-stained SDS-gel in Figure 1 exemplifies the excellent fractionation of the proteins trypsin inhibitor, carbonic anhydrase B, and cytochrome C by isoelectric focusing FFE using Prolytes™-based separation media. Furthermore it shows the perfect resolution of the proteins: based on the nature of the fractionation- technique even an infinite resolution could at best guarantee a distribution of a protein to 2 fractions. The distribution of carbonic anhydrase B to three fractions was due to the heterogeneity of the protein according to the analysis of the fractions by isoelectric focusing (data not shown). To show the perfect long-term reproducibility of the results – independent of the operator – the experiment was carried out 6 times within 45 days (Figure 2). The superposition of the protein distributions of all experiments impressively shows the nearly identical results. The number of the fraction containing the main share of each protein maximally differs by one – if at all – which is more or less the best result to expect based on the nature of the fractionation technique.

We further observed that even if the functionality of the instrument was affected by problems like inaccurate assembly of the separation chamber, partially plugged tubing, questionable state of spare parts like tubings, gaskets and membranes, or inaccurate preparation of the separation media there was still a rather good chance to get a (partially) significant result – not reproducible of course.

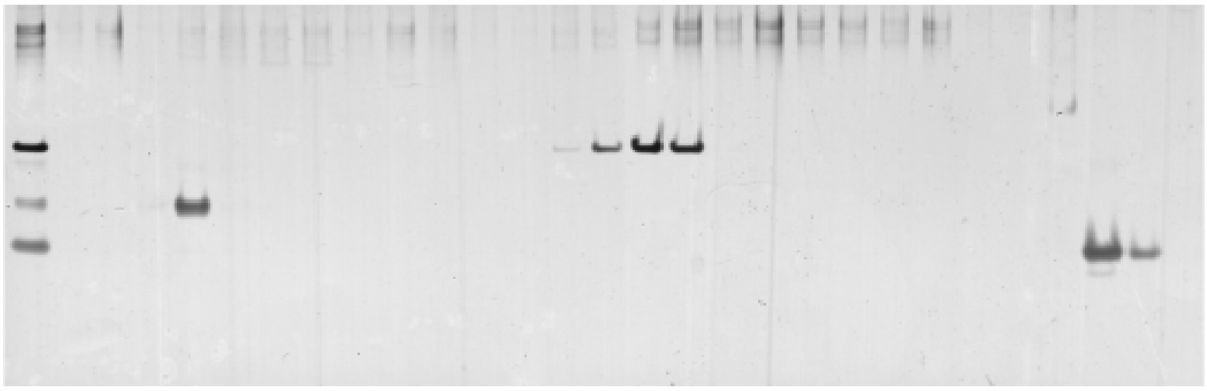

Figure 1: Silver-stained SDS-PAGE analysis of a IEF-FFE-fractionated protein mix consisting of trypsin inhibitor, carbonic anhydrase B, and cytochrome C. Lanes from left to right: Unprocessed sample; fraction 32 (pH = 4.4);33;...;58;59 (pH = 9.9). Fraction 35 contains trypsin inhibitor, fraction 45-47 contain carbonic anhydrase B, and fraction 57+58 contain cytochrome C.

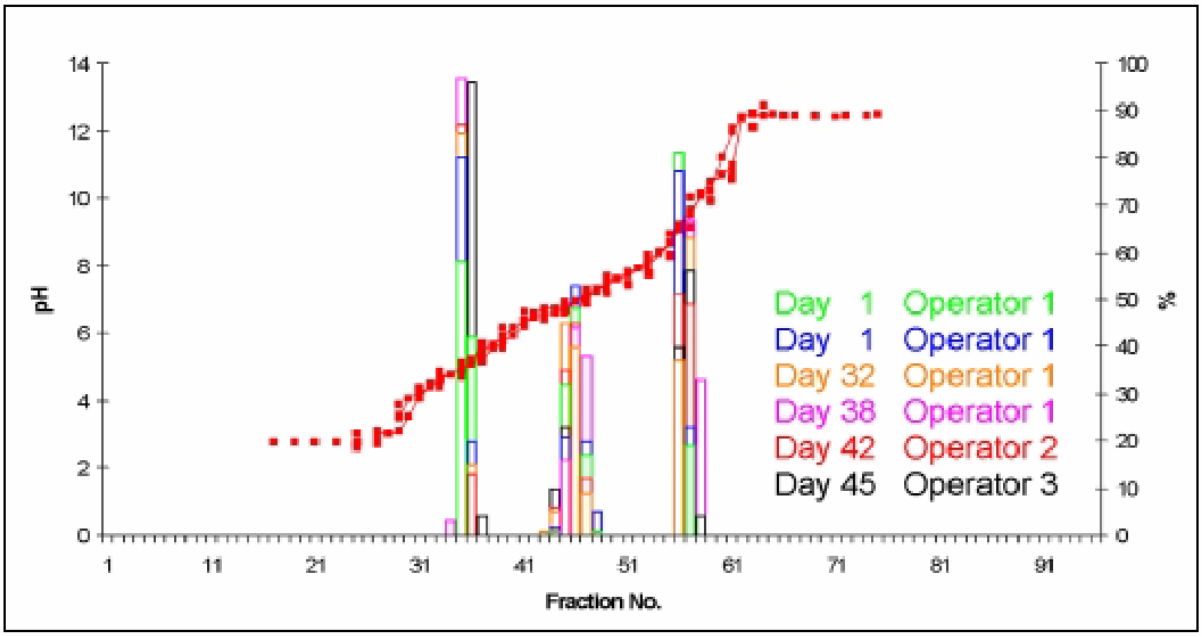

Figure 2: Excellent long-term fractionation-reproducibility of FFE: Superposition of 6 FFE-fractionations of trypsin inhibitor (left peak cluster), carbonic anhydrase B (central peak cluster), and cytochrome C (right peak cluster). pH-gradients: red squares.

Conclusion

The fractionation of a mixture of trypsin inhibitor, carbonic anhydrase B, and cytochrome C by freeflow electrophoresis in the isoelectric focusing mode using ProlytesTM-based separation media is a descriptive example of the great long-term reproducibility of the fractionation-results. Furthermore it shows the excellent resolution of the instrument even in the "narrow separation chamber" mode using limited amounts of HPMC.

References

1) Weber, G. and Bo_ek, P. (1998) Recent developments in preparative free flow isoelectric focusing, Electrophoresis 19(10) 1649-1653.