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Review

Chromatographic and electrophoretic characterization of protein variants☆

Karin Ahrer, Alois Jungbauer*

Department of Biotechnology, University of Natural Resources and Applied Life Sciences and Austrian Center of Biopharmaceutical Technology, Vienna, Muthgasse 18, A-1190 Vienna, Austria

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Abstract

Almost all proteins are expressed in several variants, also known as isoforms. Individual protein variants differ by modifications of the individual amino acid side chains, or the *N*- or *C*-terminus. Typical modifications are glycosylation, phosphorylation, acetylation, methylation, deamidation or oxidation. It is of utmost interest to either get a quantitative picture of the variants of a particular protein or to separate the variants in order to be able to identify their molecular structure. Protein variants are present in native as well as in recombinant proteins. In the case of protein production it is interesting, how variants are generated during fermentation, purification processes, storage, and how present individual variants influence the biological activity. This review provides a comparison of chromatographic and electrophoretic separation methods to analyze and to prepare protein variants.

Keywords: Recombinant protein; Variant; Isoforms; Microheterogeneity; Chromatography; Electrophoresis

Contents

Abbreviations: 2-DE, two-dimensional electrophoresis; AIEX, anion-exchange chromatography; BHK, baby hamster kidney; CE, capillary electrophoresis; CEC, capillary electrochromatography; CF, chromatofocusing; CHO, chinese hamster ovary; CIEX, cation-exchange chromatography; CIEF, capillary isoelectric focusing; CZE, capillary zone electrophoresis; DIGE, differential gel electrophoresis; *E. coli*, *Escherichia coli*; EOF, electroosmotic flow; EPO, erythropoietin; FSH, follicle-stimulating hormone; HFBA, heptafluorobutyric acid; hGH, human growth hormone; HILIC, hydrophilic-interaction liquid chromatography; IEC, ion-exchange chromatography; IEF, isoelectric focusing; IFN, interferon; IgG, immunoglobulin G; IL, interleukin; IPG, immobilized pH-gradient; LC, liquid chromatography; MEKC, micellar electrokinetic chromatography; RPC, reversed-phase chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; *S. cerevisiae*, *Saccharomyces cerevisiae*; TFA, trifluoroacetic acid; THAC, tetrahexylammonium chloride; wt-hPAH, human phenylalanine hydroxylase

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* Corresponding author. Tel.: +43 1360066226; fax: +43 1 3697615.
E-mail address: alois.jungbauer@boku.ac.at (A. Jungbauer).

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1. Introduction

Currently, the overexpression of recombinant proteins is state-of-the-art for production of biopharmaceuticals and diagnostics, preparation of research material to investigate protein function, and applications as fine chemicals and biocatalysts. There has been a strong emphasis on the quality of overexpressed proteins on proteins intended for human therapeutics. The first recombinant therapeutic protein was human insulin [1], which was approved in the US in 1982 [2]. Since that time, numerous protein-based biopharmaceuticals have entered the pharmaceutical market [3–5].

Depending on the nature of a therapeutic protein an adequate expression system must be chosen. Therapeutic proteins have been overexpressed in large quantities in bacteria (*E. coli*), yeast (*S. cerevisiae)*, and mammalian cell lines (CHO, BHK). Selection criteria for the appropriate expression system are cell growth, nutrient requirements, simple handling in the bioreactor, high expression rates, ability to secrete the product, when required and potential of synthesizing posttranslational modifications, such as glycosylation.

Approximately 40% of protein-based biopharmaceuticals are produced in *E. coli*. In cases where the target protein is not glycosylated in its native form, or posttranslational modifications are not required for therapeutic potency, *E. coli* is a powerful production system. *E. coli* enables rapid high level expression of recombinant proteins, and processes can be easily scaled-up. Further advantages are simple growing conditions on synthetic media and high expression rates. Typical biopharmaceuticals produced in *E. coli* are recombinant insulins and insulin analogs, human growth hormone (hGH), interferons (IFNs), and interleukins (ILs). One limitation of the *E. coli* system is deposition of the target protein in inclusion bodies in a non-native conformation which has been exploited for development of simple purification schemes. The dense protein aggregates can be easily removed from the cell homogenate by centrifugation, and the protein can be recovered in a very pure form. The simple recovery is followed by a time-consuming solubilization and refolding [6].

Several therapeutic proteins are produced by genetically engineered *S. cerevisiae* strains [5]. In particular, recombinant insulin-based products are produced by *S. cerevisiae* [7] in large quantities. One of the latest approved protein-products derived from *S. cerevisiae* by the FDA is recombinant human serum albumin for vaccine formulation [8]. The advantages of using yeast as expression systems are: high expression rates, simple scale-up of fermentation, excellent nutrient and oxygen utilization, presence of an efficient protein secretion pathway, and robust post-translational machinery. *S. cerevisiae* is capable of forming correct disulfide bonds [9]. Furthermore, subunit assembly of complex proteins in their functional form is possible [10].

The majority of therapeutic recombinant proteins are produced in mammalian cell lines, since many biopharmaceuticals require glycosylation similar to human glycan structures for their biological function [11]. Recombinant and chimeric antibodies are exclusively produced by mammalian cells, owing to the complexity of these molecules [12,13]. Compared to bacterial or yeast systems, the expression of heterologous proteins in mammalian cell culture is technically more complex. High cell densities, as in bacteria or yeast, cannot be achieved. Although alternative expression systems, such as transgenic animals, have proven to be successful [14,15], no therapeutic product has yet been approved. It is likely, that the first recombinant therapeutic protein produced using transgenic technology will soon be approved in Europe [16]. A recombinant form of human antithrombin (antithrombin III) for the treatment of patients with hereditary antithrombin deficiency (HD) has been successfully expressed in transgenic goats [17].

A major concern in pharmaceutical industry is consistency of the product. Microheterogeneity results from posttranslational modifications, enzymatic modifications, incorrect translation of the target protein, and modifications caused by processing and alteration. Each modification may affect the biological activity or stability of the final product. Therefore, high standards are placed from health authorities on purity, consistency, and potency of therapeutics. Microheterogeneity is also a concern for production, especially for downstream processing. Purification of the correct form is a challenge for process development. Characterization of the product is necessary for determining isoforms, which may be present with similar properties, and may complicate purification. In bacterial expression systems, the overexpressed protein is often deposited in inclusion bodies, which often requires times-consuming refolding procedures.

An important point in commercial production of protein therapeutics is that misfolded or aggregated forms must be separated from the correctly refolded target protein. Another point is that heterogeneity with respect to the *N*-terminus has been reported, when using *E. coli* as expression system [18]. Kikumoto et al. identified three species of recombinant IL-1β, which differ in their first amino acid residue [18]. Covalt et al. showed that it is possible to favor the expression of several *N*-terminal isoforms of a recombinant protein by adjusting fermentation conditions [19]. It is clear that differences in the *N*-terminal processing can affect the biophysical and biological properties of proteins. For IL-1β, it is known that differences in the *N*-terminus affect receptor binding activity [20].

During refolding of recombinant proteins from IBs, different configurations are present in the refolding solution. During each refolding procedure, side reactions, which lead to misfolded forms or the formation of aggregates, are present. Separation of different protein conformational species is necessary during production of recombinant proteins, since protein species with

mispaired disulfide bonds or random polypeptide chains have no biological activity.

Glycoproteins derived from mammalian cell culture often have a strong polymorphism concerning their glycan moieties. Variations in the oligosaccharide chain structure present on glycoproteins can significantly affect many protein properties such as solubility, specific activity, circulatory half-life, antigenicity, resistance to protease attack, and thermal denaturation [21].

Rapid, reliable, and quantitative analytical methods are needed to resolve several variants/isoforms of a protein. Thus, chromatographic and electrophoretic methods are the preferred analytical tools for resolution of protein variants to learn about the microheterogeneity of recombinant protein preparation. Conventional HPLC and isoelectric focusing (IEF) are classical methods for this purpose. Although these methods are robust and provide sufficient information for process development or a rough overview (distinct protein pattern), they may not be able to resolve all protein variants. When a higher resolution is required, capillary zone electrophoresis (CZE), micro-liquid chromatography (μ-LC), and two-dimensional electrophoresis (2-DE) electrophoresis are additional options.

Several reviews have been previously published concerning each special analytical method [22–28]. The review presented here provides a summary of chromatographic and electrophoretic techniques, which can be applied for characterization of recombinant proteins concerning their microheterogeneity. An overview about possible analytical tools, which can be adapted to characterize recombinant protein variants, and a comparison of electrophoretic methods with chromatographic methods regarding the ability to separate protein variants, are also provided.

2. Separation of variants/isoforms of proteins

2.1. Chromatographic methods

2.1.1. Ion-exchange chromatography

In ion-exchange chromatography (IEC), electrostatic interactions play the major role in protein retention. Boardman and Partridge [29] formulated a general model of how compounds interact with ion exchangers. The relative retention (k') of a compound defined as:

$$k' = \frac{V_R - V_0}{V_0} \quad (1)$$

where V_R is the retention volume and V_0 the void volume, is related to the salt concentration by a power law:

$$k' = aI^{-b} \quad (2)$$

where I is the salt concentration, and a and b are empirical parameters, which are related to the equilibrium binding constant and number of charges of the compound interacting with the ion exchanger. This formalism has been expanded by Kopaciewicz et al. [30] for proteins. They named it "stoichiometric mass action model", treating the adsorption of a protein like a quasi-chemical reaction. For mono-valent salt ions, the reaction can be written as:

$$C + zI_s \Leftrightarrow q + zI_m \quad (3)$$

where C is the protein concentration in the mobile phase, q the protein concentration in the stationary phase, z the number of charges of the protein, I_s the salt concentration in the stationary phase, and I_m the salt concentration in the mobile phase. The equilibrium binding constant (K_f), according to the law of mass action, is:

$$K_f = \frac{qI_m^z}{CI_s^z} \quad (4)$$

The number of interacting charges can be easily obtained from pulse response experiments at different salt concentrations as shown in Fig. 1 [31]. The steeper the curve, the more protein molecules interact with the ion exchanger. Brooks and Cramer [32] postulated the steric mass action model (Fig. 2), taking into account the number of charges of the ion exchanger shielded by the protein. Jin et al. [33] modified the stoichiometric mass action model to consider the charges, which are not accessible for the protein. These charges are located in pores with a diameter smaller than that of the protein. Shen and Frey [34] have developed a formalism taking the effect of pH into account. Protein properties such as charge distribution and molecular geometry can significantly influence retention behavior on IEC. Variants of recombinant proteins often differ only in a few amino acid residues or even in a single residue [19,35]. A single amino acid substitution can already be sufficient for separation on IEC [36,37], if the exchange causes significant modifications in the charge distribution on the surface of the protein molecule. In 1989, Chicz and Regnier [36] worked with several genetically engineered subtilisin variants, with multiple amino acid substitutions as well as with single amino acid substitutions, to describe the role of charged and uncharged amino acid residues in the different retention behavior of these protein isoforms on a strong cation-exchange material (GE Healthcare Mono-S column). Yao and Lenhoff [38,39] investigated the influence of electrostatic effects on the retention behavior within the protein-surface system of different proteins (cytochrome *c*, lysozyme, subtilisin, fibroblast growth factor) on three conventional cation-exchange materials (Toyopearl SP-650 C, Toyopearl SP-550 C, CM Sepharose FF), and explored how individual residues contribute to protein retention in IEC. In the model used for describing the protein-adsorbent surface interaction, the geometry and charge distribution of the protein were explicitly included [38]. Electrostatic modeling was found to explain slight differences in the retention behavior of protein variants with small structural variations.

Deamidation of side chains of asparagine and glutamine residues in proteins is often responsible for charge heterogeneity [40,41]. The uncharged side chains of these amino acids are modified to an iso-glutamate and iso-aspartate residue or to a glutamate and aspartate residue. Therefore, an additional charge is introduced to the protein per modification. Perkins et al. [42] showed that the deamidation of two asparagine residues was responsible for variants of a murine monoclonal antibody, which could be detected by cation-exchange chromatography

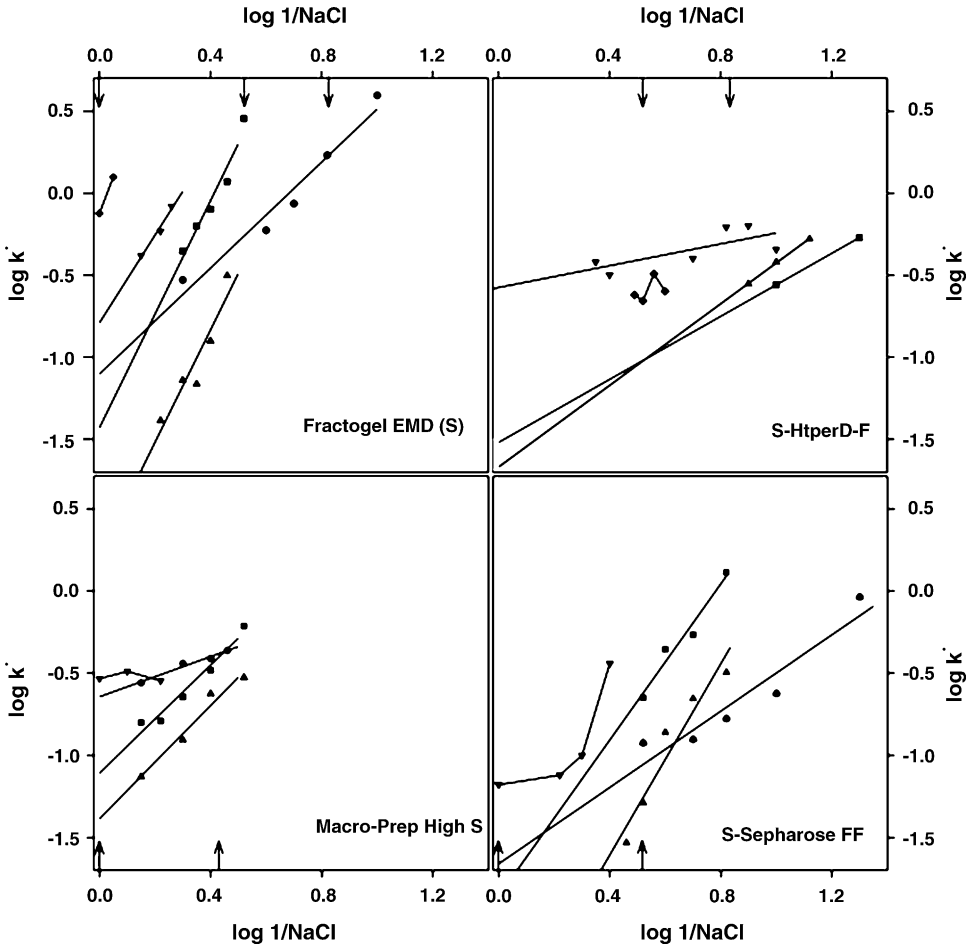

Fig. 1. Summary of isocratic runs with α-lactalbumin (●); β-lactoglobulin (■); IgG (▲); lactoperoxidase (▼) and lactoferrin (♦). The pure proteins were chromatographed on a 10 cm × 0.5 cm I.D. column at 150 cm/h. Samples (100 μl) containing 2 mg protein/ml were injected. Arrows indicate the salt concentrations at which stepwise elution was carried out [31].

(CIEX) (GE Healthcare, Mono S column). Harris et al. [43] separated seven isoforms of a therapeutic recombinant antibody caused by charge heterogeneity due to deamidation by CIEX (BakerBond CSX column). The incomplete removal of *C*-terminal lysine residues from immunoglobulin G (IgG) heavy chains is another reason for charge heterogeneity of antibodies derived from mammalian cell culture [44,45]. Such antibody variants containing none, one, or two lysine residues can be

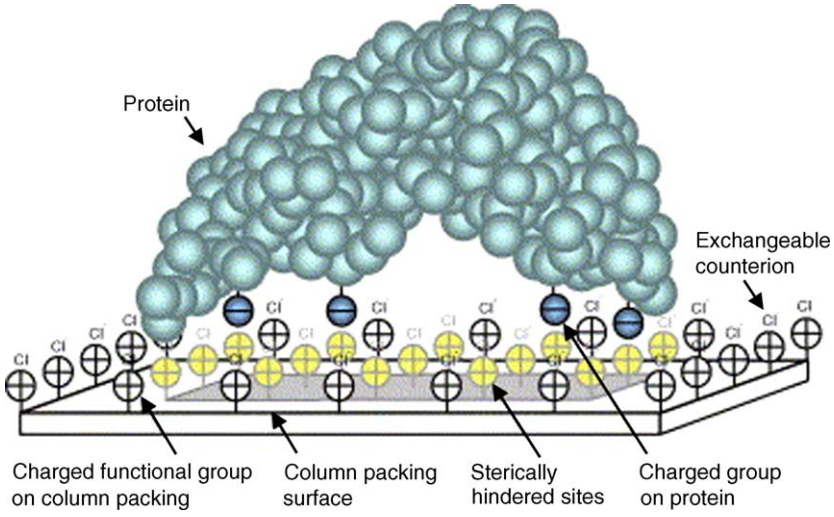

Fig. 2. Illustration of a protein having four negatively charged groups which interact with four positively charged groups on the column packing. The chloride counter-ion forms undissociated ion pairs with the sites underneath the protein in the gray area, while the chloride ion is an exchangeable counter-ion outside the gray area. The total number of functional groups on the column packing within the gray area is the steric factor σ. (Figure from Shen and Frey [34].)

resolved by CIEX as shown by Weitzhandler et al. [46]. In their work CIEX was performed on polymer grafted stationary phases (Dionex ProPac WCX-10 column and ProPac SCX-10 column). The same material was also successfully applied for separation of native ribonuclease A and two of its deamidation products, and for separation of variants of cytochrome *c* and hemoglobin [46].

Miniaturization of separation systems has rapidly advanced recently, leading to shorter analysis times and smaller sample volumes. In addition, successful separation of closely related recombinant protein variants on μ-HPLC anion-exchange chromatography (AIEX) using a non-porous grafted chromatographic support (fused silica capillary packed with 10 μm Dionex ProPac SAX-10 beads) has been demonstrated in 5 min, applying only 2 μl sample [47].

Another method to perform fast separation is the usage of monoliths as stationary phase. Most of the recent work with monolithic supports has been carried out with methacrylate-based materials [48,49]. This chromatographic support enables convective transport and prevents pore diffusion resistance [50,51]. Hahn and Jungbauer [52] showed that separation of proteins can be achieved in less then 10 s. Therefore, limits of methods using monoliths are speed of detection and liquid handling, but not the separation as such.

Charge heterogeneity of protein variants can also result from modifications such as glycosylation. An analytical method for the separation of glycoforms, such as human serum transferrin, differing in their amount of sialic acid residues by AIEX (Dionex ProPac PA1 column) was shown by De La Calle Guntiñas et al. [53]. In addition to the commonly applied salt-gradients in IEC, pH-gradient elution is an indispensable method for the separation of variants due to charge heterogeneity. When pH-gradient elution is applied, proteins elute roughly in order of their p*I* values. Similar to chromatofocusing (CF), which is discussed in the next section, proteins are focused in narrow bands where the pH is close to their p*I* values. Compared to salt-gradients, generation of reproducible, linear pH-gradients is more complex. pH-gradient-elution can be combined with salt-gradient elution. Kaltenbrunner et al. [54] were successful in separating isoforms of a human monoclonal antibody with a narrow p*I* range by CIEX (GE Healthcare, Mono S HR5/5 and Mono S HR16/10 columns) using a linear ascending pH-gradient combined with a linear descending salt-gradient (Fig. 3). pH-gradient elution on small monolithic columns could be applied for rapid separation of related protein variants [55]. Variants of manganese peroxidase were separated by a combined pH-salt-gradient on a strong anion-exchange monolithic support (CIM QA disk, BIA Separations), where elution was completed in less than 2 min.

Due to cost and time, miniaturization poses a challenge in analytics. Andersen et al. [56] described the separation of two genetic variants of β-lactoglobulin using a weak anion-exchange material (GE Healthcare, Mono P particles) as well as a strong anion-exchange material (Polymer Labs PL-SAX particles) packed into fused silica capillaries. The separation was achieved using a pH-gradient from 6.8–4.3. A baseline separation could be achieved on both materials, in which the capillary packed with the PL-SAX particles provided a two-fold decrease

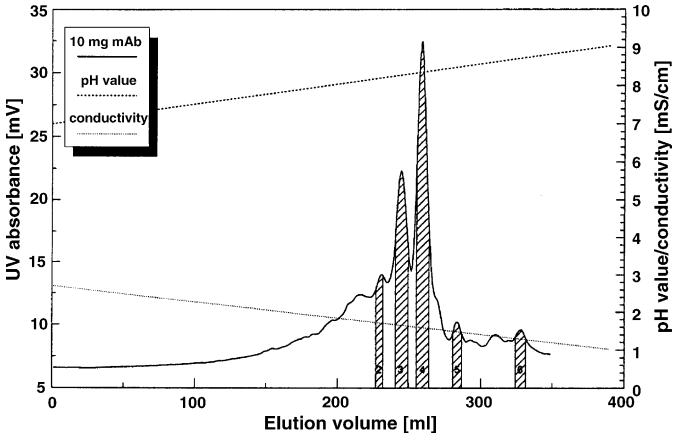

Fig. 3. Preparative separation of isoproteins of human monoclonal antibody "Virgil" by IEC on Mono S column (HR 16/10). Ten milligrams of monoclonal antibody "Virgil" were dissolved in 23.0 ml starting buffer and loaded on a 20-ml column; starting buffer: 5 mM borate, 45 mM mannitol, 20 mM NaCl (pH 7.0), $\kappa = 2.8$ mS/cm; elution buffer: 5 mM borate (pH 9.1), $\kappa = 0.9$ mS/cm. Elution was effected with a linear pH-salt-gradient over 20 column volumes at a flow rate of 2 ml/min [54].

in peak width compared to the capillary packed with the Mono P material.

Displacement chromatography [57] is another method which can provide resolution of closely related protein variants [37,58]. Kundu and Cramer [58] separated bovine and horse heart cytochrome *c* by CIEX (Waters SCX-column) using *N*-α-benzoyl-L-arginine ethyl ester (BAEE) hydrochloride as a protected amino acid displacer. Their work underlined that displacement chromatography offers high-resolution separation in a preparative mode, which is important for purifying recombinant proteins.

2.1.2. Chromatofocusing

CF, a variant of IEC, was first described by Slyterman et al. [59,60]. Frey and colleagues further developed the theory of CF advancing numerical simulation methods for the calculation of the internal pH [61,62] and creating simple buffers systems to generate a quasi-linear pH-gradient in the column [63]. During CF, proteins are separated according to differences in isoelectric points. A linear pH-gradient is generated within the column by specifically designed and matched amphoteric buffers, containing mixtures of polyampholytes with different p*K*a values to achieve buffering capacity over the entire pH range used for separation. The retained pH-gradient travels slowly through the column compared to the unadsorbed eluate. CF is performed by titration of a weak ion-exchange column in contrast to pH-gradient elution, where strong ion-exchange materials are used. Thus, proteins with different p*I*s migrate at different rates down the column as the pH-gradient develops, and are continually banded and dissociated while being focused into narrow bands. The formation of the pH-gradient is slow compared to the migration velocity of unadsorbed eluates.

An example for CF is the separation of protein variants of mAB (Fig. 4) and recombinant human superoxide dismutase using weak anion-exchange Mono P columns (GE Healthcare)

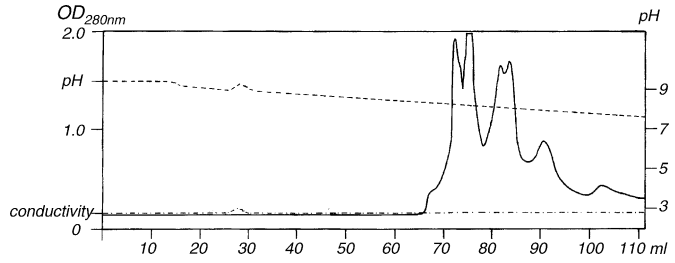

Fig. 4. Separation of isoproteins of a human monoclonal antibody by CF on a Mono P column (HR 10/30). Ten milligrams of lyophilized human monoclonal antibody were dissolved in 2 ml of equilibration buffer and loaded onto a 23.0 ml column. Starting buffer: 25 mM diethylenamine, pH 9.5; elution buffer: Polybuffer 96 (GE Healthcare), pH 7.0. Elution was effected with a linear pH-gradient from 9.5–7.0 at a flow-rate of 1 ml/min [64].

[64]. The peaks shown in Fig. 4 were fractionated and analyzed with IEF showing that isoforms of the antibody with a varying p*I* were present in the single peaks (data not shown).

Gradient CF is a newer technique [65,66] with simple low-molecular mass buffer substances compared to conventional CF, which allows the generation of linear or other function pH-gradients. Shan and Anderson [67] compared the resolution of β-lactoglobulin A and B using gradient CF and salt-gradient chromatography on the same chromatographic material (Waters Protein-Pak DEAE column) and could show a significant increase of resolution using gradient CF. Compared to conventional CF, a larger range of buffer concentrations can be applied in gradient CF. Similar work by Kang and Frey [62] show the applicability of commercially available micropellicular ion-exchange columns (Tosoh Biosep TSKgel DEAE-NPR column, Dionex ProPac WAX-10 column) performing gradient CF for the separation of hemoglobin variants. Cation-exchange CF is rarely used for the separation of protein variants compared to anion-exchange CF. Recent work by Kang and Frey [68] demonstrates the use of high-performance cation-exchange column packings (Dionex ProPac WCX-10 column) in CF, where similar resolution was achieved using this material compared to on anion-exchange CF. In addition, some separations were found to show better or worse resolution on anion-exchange CF compared to cation-exchange CF.

2.1.3. Reversed phase chromatography

Reversed phase chromatography (RPC), especially RP-HPLC, is used extensively for the analysis of proteins. In RPC, silica or polymeric matrices are functionalized by a high density of alkyl groups (e.g., C4, C8, C18). Protein variants exhibit differences in hydrophobicity and can, therefore, be separated by RPC. Important factors for separation are column temperature, solvent strength, and structure of the ligand. Purcell et al. [69] investigated the chromatographic behavior of four insulin variants on RP-HPLC depending on temperature (5–85 °C), column residence time (10–60 min), and ligand structure (C_{18} and C_4). Under certain chromatographic conditions, resolution of these four variants, differing in the amino acid residues A8-A10, can be achieved. Their work concludes that these three amino acid residues are directly involved in the chromatographic contact area of insulin. Improvement of the resolution of protein variants has been reported by adjustment of the right column temperature by Dillon et al. [70], peak shape and recovery of a monoclonal IgG1 on RP-HPLC (Zorbax SB300 C_8 column) were found to improve by increasing column temperature to 70 °C. Additionally, this work showed that a combination of two ion-pairing agents (trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA)) in the mobile phase was beneficial for separation of charge variants and antibody fragments. Karlsson et al. [71] separated oxidized and deamidated hGH variants by an isocratic RP-HPLC method (Vydac C_{18} column) at neutral pH and column temperature of 55 °C. Berti et al. [72] used RPC (C_4 column) for the successful separation of oxidized forms of recombinant human cystatin C. The different peaks resolved by RPC were found to contain non-, mono- and dioxidized forms. Bondoc et al. [73] used RP-HPLC (Vydac C_8 column; 35 °C column temperature) to characterize recombinant human IL-10 variants derived from *E. coli* inclusion bodies, and found that the right form could be separated from three variants resulting from acetylated lysine residues. Previous work from George-Nascimento et al. [9] deals with the characterization of protein variants of recombinant human epidermal growth factor, which is expressed in yeast. In the study, two oxidized forms were found to be resolved by RP-HPLC (Vydac C_4-column). Additionally, isoforms with variances at the *C*-terminus could be separated, in which the main component contained a leucine residue at the *C*-terminus and a second component lacked this leucine residue. Guo and Feng [74] showed by RP-HPLC (C_8 column) that engineered insulin variants, where one or two cysteine residues essential for disulfide bond formation were substituted with serine residues, have different structural properties due to different chromatographic behavior. RP-HPLC has also been adopted for the monitoring of product quality during production of recombinant antibodies [75], where an automated system, consisting of an affinity chromatographic step with a subsequent RP-HPLC step (Poros R220, Vydac C_4), was used. Degraded forms of the product could be detected with this analytical system. Furthermore, the influence of temperature (40–70 °C) and flow-rate (0.5–3 ml/min) on peak shape and retention time was investigated concerning RP-HPLC [75].

Normally, RPC is used for rapid analytical analysis. Work by Sabharwal and Chase [76] shows that RPC (Whatman BioPrep C_4 particles) can also be applied for the production of recombinant proteins. They presented the separation of two closely related insulin variants in a process-scale and outlined that the applied system is suitable for theoretical modeling for optimization and scale-up. Sunasara et al. [77] used reversed phase displacement chromatography for the purification of recombinant brain-derived neurotrophic factor from its variants, where tetrahexylammonium chloride (THAC) was used as a displacer. Operationally, displacement chromatography is similar to step gradient chromatography, with the difference lying in the fact that the displacer has the highest affinity for the stationary phase compared to all other solutes. Therefore, the displacer stays behind the protein zones and back mixing is avoided. Sunasara et al. [77] were also able to scale-up this separation step from an analytical column (Phenomenex Jupiter C_4 column, Vydac C_4 column) to a pilot-scale system (Phenomenex Jupiter 10 μm

C_4, 250 mm × 50 mm). The system was successful in removing an oxidized protein variant and a protein variant containing norleucine instead of leucine from the native form of recombinant brain derived neurotrophic factor.

2.1.4. Hydrophilic interaction liquid chromatography

The characteristic of hydrophilic interaction liquid chromatography (HILIC) is the separation of solutes in a hydrophobic mobile phase using polar stationary phases [78]. Although HILIC is not a commonly applied separation method, examples exist concerning the separation of protein variants, especially variants of histones that have been characterized with HILIC. Lindner et al. [79] used a weak cation-exchange column (SynChropak CM300 column) and an increasing sodium perchlorate gradient in the presence of 70% acetonitrile to separate acetylated forms of histone H2A and H4. Mizzen et al. [80] applied this method for the separation of histone H1 variants. By HILIC (PolyCAT A column), resolution of six known non-allelic variants of histone H1 was achieved. In addition, allelic variants which co-migrate in 1D electrophoresis could be separated by this technique. In a recent study by Sarg et al. [81] eight forms of human histone H1 were resolved by HILIC (PolyCAT A column). These variants had differences in amino acid sequence and differed in the degree of phosphorylation. HILIC can be considered an alternative method for separation of protein variants, when IEC and RPC fail or do not provide sufficient resolution. For very basic proteins, this mode of interaction appears to be well-suited.

2.2. Electrokinetic methods

2.2.1. Isoelectric focusing

A technique commonly used for determining charge heterogeneity of proteins is IEF in slab gels. Two main principles for generation of a pH-gradient can be applied in (a) immobilized, non-amphoteric buffers (IPGs), and (b) soluble, amphoteric buffers also known as carrier ampholytes. IPGs were introduced in 1982 by Bjellqvist et al. [82], and are now the method of choice since higher resolution and reproducibility are achievable. An important aspect of IEF is prevention of protein precipitation during focusing, otherwise artefacts are generated and false positive or negative charge variants are obtained. The separation of recombinant human erythropoietin (EPO) variants [83–86], caused by different glycan patterns, is a prime example for the resolution power of IEF (Fig. 5). A more commonly known use of IEF is in doping control of top athletes. In this validated analysis, recombinant human EPO is separated from endogenous EPO [85]. Other impressive examples of human glycoproteins separated by IEF, are IgGs [87,88], human recombinant follicle stimulating hormone [89], human transferrin [90], human α-1-acid glycoprotein [91], recombinant human interferon-β-1a [92], recombinant tissue-type plasminogen activator [93], and recombinant human follistatin [94]. Tsai et al. showed that charge heterogeneity of antibodies derived from mammalian cell culture must not mainly result from different glycan pattern [95], and attributed four major species of a distinct antibody separated by IEF to sequential deamidation products of the IgG heavy chain.

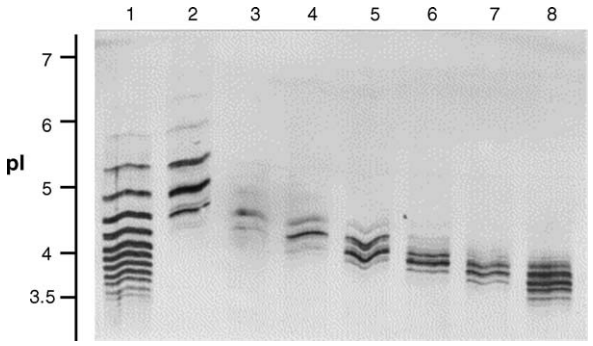

Fig. 5. IEF of rhEPO. Fractions obtained by DEAE–Sephacel chromatography: (1) starting material; (2) unadsorbed material; (3) material eluted with 0.015 M; (4) 0.03 M; (5) 0.06 M; (6) 0.15 M; (7) 0.35 M and (8) 1 M NaCl. (From Ref. [83] with permission.)

A new approach to increase resolution of IEF is two-dimensional IEF [96]. The same IEF separation technique was used consecutively in two directions of the same gel. This method was successful in separating two isoforms of β-lactoglobulin, which could not be resolved with conventional IEF. Today, conventional IEF on slab gels for analytical purposes is often replaced by capillary IEF (cIEF), which is discussed in Section 2.2.3.

IEF also has application in preparative protein fractionation. We have also applied IEF for preparative separation of recombinant human superoxide dismutase [97], where IEF was performed in 0.5 cm thick preparative IPG gels. After separation the individual isoforms were recovered by cutting the corresponding bands and by overnight elution in water. In general, IEF suffers from the low solubility of proteins at the isoelectric point. This especially poses a problem in preparative modes, since higher protein concentrations are applied. Addition of salt improves solubility, but as a consequence too high current would be obtained and heat dissipation could not be handled anymore.

Other possibilities for preparative IEF are techniques where the proteins are recovered in solution [98–100]. The Rotofor is a device in which IEF is performed in free solution [101]. A preparative-scale Rotofor (total volume up to 55 ml) and a mini-Rotofor (total volume 18 ml) are available. The sample chambers of the Rotofor are separated by liquid-permeable nylon screens. Since this focusing method is based on the use of carrier ampholytes, the p*I* accuracy is relatively poor. Ayala et al. [102] discussed the advantages and disadvantages of using the Rotofor regarding complex protein solutions. Development of the multi-compartment electrolyzer based on Immobiline membranes was a considerable improvement concerning preparative IEF in free solution [99,103]. The single chambers of this special device are separated by isoelectric membranes. In an electrical field, proteins can migrate from one chamber to another chamber until they have the same p*I* as a particular membrane. Then, the proteins are captured and can be simply harvested. Owing to this membrane technology, the pH-gradient decay which is typical of IEF using conventional carrier ampholytes is fully eliminated. Conductivity and pH constancy are guaranteed in all flow chambers for running periods of more than 11 days (160,000 V h) [99]. Wenisch et al. [104] demonstrated that this

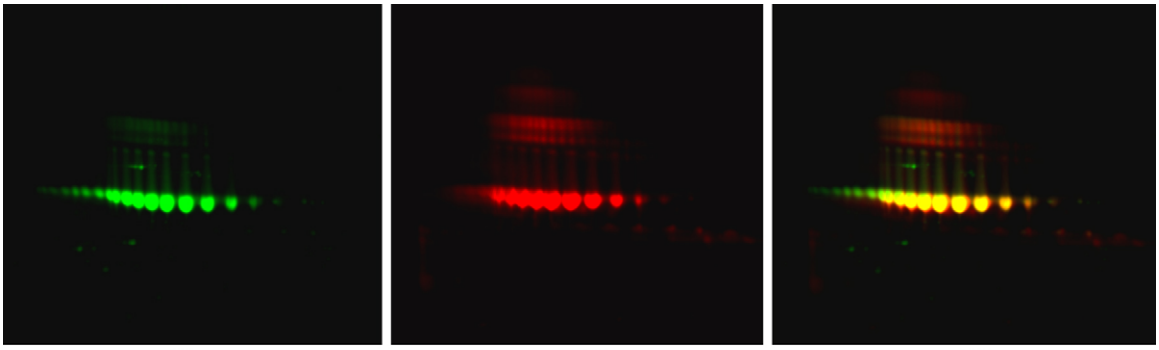

Fig. 6. Analysis of a recombinant protein before and after purification analyzed with DIGE technology. (A) Starting material labeled with Cy3. (B) Purified material labeled with Cy5. (C) Overlay of Fig. 5A and B. (Kindly provided by Grzeskowiak.)

system allows processing of large sample volumes with protein loads in gram-scale regarding the purification of a human monoclonal antibody. Furthermore, resolution of protein isoforms as close as 0.001 in p*I* difference was found to be possible with the multi-compartment electrolyzer. This method has also been successfully applied for separation of protein variants of interleukin-6 mutein [105]. A recent development concerning IEF is the possibility of protein fractionation based on "off-gel IEF" [100], where proteins are fractionated depending on their p*I* values in a 96- or 384-multiwell devices, where the separated proteins are directly recovered in solution. The principle of this separation method is to place the sample in a liquid chamber which is positioned atop an IPG gel. Therefore, the gel is able to buffer a particular layer of the solution in the liquid chamber. Upon applying an electric field perpendicularly to the liquid chamber, charged species (p$I \neq$ pH of the IPG gel) move into the gel. When the separation is complete, only the neutral species (pI = pH of the IPG gel) remain in solution and, therefore, the sample compounds can be easily recovered in solution.

These IEF methods, where proteins are recovered in solution, also have a potential application in proteomics [106,107], since generated fractions of discrete p*I*-intervals allow the analysis of narrow segments of a proteome. Optimally, high abundance proteins should be removed, allowing low-abundance proteins to be applied at sufficient concentrations for the subsequent analysis, such as 2-DE or LC–MS [108,109].

2.2.2. *Two-dimensional electrophoresis*

2-DE is currently the method of choice for separation of complex protein samples and is often used in combination with mass spectrometry in order to identify the individually separated proteins. In 2-DE, IEF (as a first dimension) and SDS-PAGE (as a second dimension) are combined. Depending on the size of the gel and the pH-gradient applied, more than 5000 proteins can be separated simultaneously. The basic protocol for the first dimension in 2-DE using IPGs is still considered state-of-the-art [110]. The first dimension is performed on IPG strips, which are later equilibrated with SDS buffer containing agents such as glycerol, urea, and iodoacetamide. The standard procedures for 2-DE have been extensively reviewed [24,111].

2-DE is not commonly used for routine analysis and quality control of recombinant proteins, due to cost and long experimental time. The main advantages of this method are resolution and ability to identify isoforms of recombinant proteins with respect to charge and mass heterogeneity within one run. For process development and optimization for the production of recombinant proteins, 2-DE is a worthwhile analytical tool. Especially with the help of differential gel electrophoresis (DIGE) [112], slight differences in the protein pattern of recombinant proteins can be identified, which may result from different construct designs, fermentation strategies, or purification steps. Conventional 2-DE suffers from gel-to-gel variations, making quantitative comparison complicated. DIGE overcomes these problems, because different protein samples, which are labeled with structurally similar but spectrally distinct fluorophores, can be merged after labeling and this mixture can be separated within one 2-DE gel [113]. Since three CyDyes (fluorescent dyes) are commercially available, current 2-D DIGE methods enable separation of up to three samples under identical electrophoretic conditions in one 2-D gel. After scanning the gel with the appropriate excitation and emission wavelengths for each dye, the images of the two samples can be easily superimposed and image analysis is facilitated (Fig. 6). The modern detection techniques in 2-DE have been previously reviewed [114–116]. Fluorescence labeling of different samples is not the only method of analyzing multiple protein samples on one 2-D gel. Differential electrophoresis can also be performed by radiolabeling of different samples with distinguishable isotopes, such as ^{14}C and ^{3}H [117]. This detection technique has been improved and is called differential gel exposure [118].

In addition to the frequent application of 2-DE and DIGE in proteomics, these methods have also been frequently applied in process biotechnology. Rinas and Bailey [119] analyzed inclusion bodies derived from *E. coli* fermentation containing recombinant β-Lactamase with 2-DE and detected truncated variants of the target protein which may result from proteolysis or erroneous protein translation. Oswald et al. [120] focused on the influence of different *N*-terminal fusion domains on the product heterogeneity of a recombinant protein. Several fusions of recombinant restriction endonuclease produced in *E. coli* were analyzed with 2-DE. Depending on the fusion-tag used, fragmentation of the target protein was observed [120]. Another application of 2-DE regarding the characterization of protein variants was shown by Solstad et al. [121,122]. They sepa-

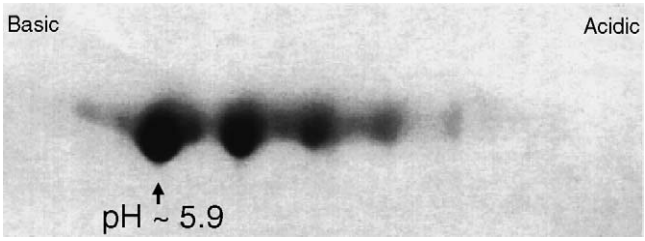

Fig. 7. 2D-electrophoresis pattern of full-length wt-hPAH obtained as a fusion protein after 24 h of induction in *E. coli* at 28 °C. Approximately 30 μg of enterokinase cleaved fusion protein MBP-$(D_4K)_{ek}$-hPAH was subjected to 2D-electrophoresis and stained with Commassie Brilliant Blue. The multiple molecular forms of the protomer (denoted hPAH I-IV [121]) differed in pI by 0.0.1 pH unit, but shared the same apparent molecular mass of ≈50,000 g/mol. (From Ref. [122] with permission.)

rated four to five isoforms of recombinant human phenylalanine hydroxylase (wt-hPAH) expressed in *E. coli* and found that heterogeneity was caused by labile asparagine residues which were prone to nonenzymatic deamidation (Fig. 7).

2.2.3. Capillary electrophoresis

In many applications, capillary electrophoresis (CE) has been proven to be the analytical method of choice to characterize protein variants, since the use of capillaries greatly reduces sample volume and analysis time compared to conventional gel electrophoresis. Additionally, it can be fully automated and allows for quantitative data evaluation. The basic set-up of a CE-system was described by Jorgenson and Lukacs [123]. In CE, electrophoretic migration and electroosmotic flow are combined for the separation of positively charged, negatively charged, and uncharged species through silica capillaries. One of the major problems associated with protein separation by CE using untreated fused-silica capillaries is the adsorption of proteins on the negatively charged silanol groups of the capillary wall. This can be circumvented either by chemical additives [124,125] to eliminate wall effects, capillary coating to shield the silanol groups chemically [126–129], or by lowering the pH [124,125,130] to decrease the charges present at the capillary wall. Denton and Harris [131] investigated the advantages of coated capillaries over non-coated capillaries by analyzing human serum albumin variants.

The role of CE as analytical tool for recombinant proteins has been extensively reviewed [132–136]. One major application is the characterization of glycoprotein variants [137,138]. A set of different microscale techniques of CE exists for analyzing recombinant glycoproteins. CZE is the general method in this field for analyzing recombinant protein variants, such as human EPO [139,140], human chorionic gonadotropin [141], human tissue plasminogen activator [142], human granulocyte–macorphage colony stimulating factor [143], and human blood coagulation factor VII [144]. All of these examples were performed in uncoated silica-fused capillaries. Coated fused-silica capillaries have frequently replaced the traditional silica-fused capillaries. Many different hydrophilic polymeric coatings [145–148] have been developed to improve the performance of CE, and several pre-coated capillaries are commercially available. The advantage of these modified capillaries has been demonstrated [149–151].

A special technique of CE is cIEF, which offers an alternative to conventional IEF with slab gels. This technique was first described by Hjertén and Zhu [152], in which a mixture of ampholytes and sample are filled in a capillary. Upon applying an electrical field, a pH-gradient is formed by the ampholytes, and the proteins are focused in discrete zones at positions according to their p*I*. For detection, the focused zones of protein are transferred through the column either by pressure or electrophoretic means [153]. One major problem is the negative effect of salts on the performance of cIEF, since high salt concentrations in the sample destroy the generated pH-gradient. Therefore, samples must be desalted either off-line or on-line [154].

Similar to conventional IEF, the separation of glycoforms of proteins derived from cell culture is a major field of application. Cifuentes et al. [155] compared the performance of cIEF (laboratory-made polyacrylamide-coated capillary with an effective length of 20 cm) with conventional CZE and flat-bed IEF using recombinant human EPO as a model protein. They concluded that this cIEF method had a shorter analysis time with similar resolution; therefore, the method is well-suited for rapid quality control. The results of the study also show that conventional CZE had better reproducibility and robustness, and is therefore the method of choice for strict quality control. This cIEF method for analyzing recombinant human EPO was enhanced by Lopez-Soto-Yarritu et al. [156]. They investigated different types of coated capillaries (laboratory-made, polyacrylamide coated capillary with an effective length of 20 cm; Beckman eCAP neutral capillaries with an effective length of 40 and 20 cm). In this study, the influence of several parameters – such as capillary length, range of pH of ampholytes, internal standard selection, pH and composition of electrolytes, and salt content of sample – were investigated regarding the performance of cIEF. They underlined that cIEF is a precise method, also for routine analysis, when these parameters are carefully optimized.

Tang et al. [157] also introduced a rapid and highly-reproducible cIEF method with a μSIL DB-1 capillary coated with dimethyl siloxane with an effective length of 40 cm, and used this technique for the routine analysis of recombinant IgGs. They showed that profiles of cIEF for different recombinant IgG preparations were comparable with densitometric scan profiles of gel-based IEF. The resolution and reproducibility could be improved using a "two-step cIEF", meaning that proteins are mobilized after focusing by applying low pressure.

A special separation technique of CE is micellar electrokinetic chromatography (MEKC) [158,159]. Dönges et al. [160] characterized the high molecular mass glycoprotein antithrombin III by means of MEKC (polyimide coated capillary from Supelco). With a cyclodextrin-modified MEKC using SDS for micelle formation, the analysis time for baseline-separation of antithrombin III α and β could be shortend compared to conventional CZE. β-Cyclodextrin was added to the running buffer, to introduce selectivity behavior [161]. Goldman et al. [162] described the separation of glyco-variants of human interferon γ derived from CHO cells with the help of MEKC (uncoated fused

Table 1
Separation efficiencies (number of theoretical plates (*N*) per meter) for several analytes of CZE and CEC capillaries

Capillary	N_f/m	Analyte
CZE		
Polyacrylamide coated capillary [185]	464000	Cytochrome *c*
Polyacrylamide coated capillary [185]	1290000	Ribonuclease A
Fused-silica capillary [186]	312796	Myoglobin
Fused-silica capillary [186]	227488	α-Lactalbumin
CEC		
Coated capillary GPTMS-PEI coating [187]	118445	Tracer peak
Monolithic capillary column VBC-EGDMA monolith GPTMS-PEI[a] coating [187]	52966	Tracer peak
Monolithic capillary column VBC-EGDMA-SWNT[b] monolith GPTMS-PEI coating [187]	102775	Tracer peak
Monolithic capillary column with C_4 functionality [186]	137440	Myoglobin
Monolithic capillary column with C_4 functionality [186]	156398	α-Lactalbumin

[a] GPTMS: 3-glycid-oxypropyltrimethxysilane; PEI: polyethyleneimine.
[b] VBC: vinylbenzyl chloride; EGDEMA: ethylene glycol dimethacrylate; SWNT: single-wall carbon nanotubes.

silica capillaries; electrophoresis buffers contained borate and SDS). This procedure has been previously described by James et al. [163]. Goldman et al. [162] resolved the three glycosylation site occupancy variants of interferon γ: one variant has two occupied asparagine sites, the second form has only one asparagine site occupied, and the third one is nonglycosylated. In addition, glycosylated forms of interferon γ were shown by cIEF (neutral coated capillaries from Beckman), to have at least 11 differently sialylated glycoforms over a p*I* range of 3.4–6.4.

The examples described here represent only high-performance separation of glycoforms of proteins. Conventional CE and modifications thereof can also be applied for the separation of protein variants due to other modifications. Lindner et al. [164] resolved different phosphorylated isoforms from unphosphorylated forms of histone H1 with CE. Bullock [165] emphasized the use of CZE (uncoated fused silica capillary, effective length 50 cm) for the characterization of recombinant human interleukin-4 and its degraded forms. The standard procedures for analyzing size-based protein variants are SDS-PAGE and size-exclusion chromatography. Hunt and Nashabeh [166] described an analytical method using CE for the analysis of recombinant monoclonal antibodies as a replacement for SDS-PAGE gels. First, the antibodies are labeled with a neutral fluorophore (5-carboxytetramethylrhodamine succinimidyl ester). Subsequently, the labeled sample is incubated with SDS. The complex protein-SDS is then analyzed by CE using a hydrophilic polymer functioning as a sieving matrix (fused silica capillaries from Polymicro Technologies, effective length 19.4 cm).

A recent development is chip-based capillary electrophoresis [167,168]. One instrument is the Agilent Bioanalyzer originally developed by Caliper Technologies [168]. In contrast to conventional SDS-PAGE, this system provides a reduction of analysis time by minimizing time-intensive steps such as staining, destaining, and gel scanning. The "lab-on-a-chip", or so-called microfluid technology, enables rapid and automated analysis of proteins on a chip. Proteins from 5 to 200 kDa can be analyzed with a resolution of 5–10% and much better sizing precision and quantitation capability than SDS-PAGE [169]. An example concerning recombinant proteins was the quantitation of a recombinant monoclonal IgG4 by Vasilyeva et al. [170].

2.2.4. Capillary electrochromatography

Capillary electrochromatography (CEC) is a hybrid technique that combines chromatographic selectivity with the high efficiency of electrophoresis. Solutes are driven through the capillary by the electroosmotic flow (EOF), instead of a pressure gradient as in conventional chromatography. Capillaries used for CEC can either be packed with chromatographic particles [171], monolithic support [172], or adsorbed stationary phases onto open-tubular capillaries [173–175]. In Table 1, values are listed for the efficiencies of CZE and CEC capillaries. The obtainable high peak capacities are promising for the high-speed separation of complex protein mixtures. Therefore, CEC appears to be a viable method in conjunction with mass spectrometry. In Fig. 8, a comparison of peak capacities obtained by HPLC and CEC is illustrated by Zhang et al. [176]. However, when connected to mass spectrometry, conventional HPLC methods are often preferred for the separation of complex protein samples. Reversed phase chromatography seems to be more robust and the sample matrix, in contrast to CEC, less influences retention and resolution. Mistry and Grinberg [177] emphasized the

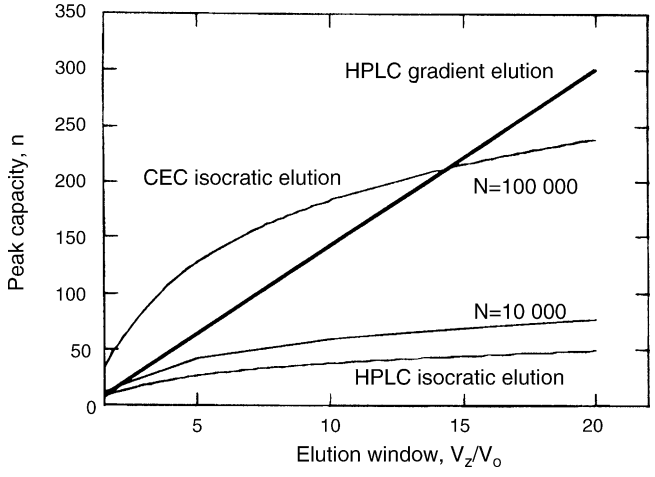

Fig. 8. Comparison of peak capacities of HPLC and CEC in isocratic mode and gradient mode according to Zhang et al. [176], reproduced by kind permission of Amercian Chemical Society.

difficulty of transferring HPLC conditions to CEC. One important point regarding CEC is that the average EOF in packed capillaries is not significantly influenced by particle diameter [178,179] compared to pressure-driven chromatography. Flow heterogeneity during CEC is mainly influenced by differences in the zeta-potential between the capillary surface and the particle surface. High excess zeta-potential has a disadvantageous effect on the column cross-sectional flow profile. The application of monolithic capillaries has been reviewed by Svec et al. [180].

CEC is not commonly used for the characterization of protein variants. Zhang et al. [176] showed a high-resolution separation of two variants of hemoglobin with CEC using fused silica capillaries packed with silica beads with strong anion-exchange functional groups, which were attached on the chromatographic support via hydrophilic spacers. This group also separated three variants of cytochrome *c* by CEC [181], using functionalized polymethacrylate microspheres with strong-cation-exchanger properties. Rehder and McGown [182] used open-tubular CEC for the separation of bovine β-lactoglobulin A and B. This work described the preparation of an aptameric stationary phase in open-tubular CEC. The attached aptamers are single-stranded oligonucleotides, which are designed through combinatorial selection and are therefore specific ligands for the target molecule. Future prospects in the field of CEC are in the field of chip-based technology, where innovative work has already been shown [183,184].

3. Conclusions

For high-resolution separations to resolve protein variants, chromatographic and electrophoretic methods are often applied. Both techniques can be principally applied for these tasks. Chromatographic methods are more robust and less influenced by the sample matrix, but have lower resolution than electrophoretic methods. Thus, chromatographic methods are preferably used for quality control, in-process control analysis, and preparative separation of isoforms. In addition, chromatographic methods can be easily automated. The same holds for the capillary electrophoretic methods. Regarding the production of recombinant proteins, rapid analysis is essential for (a) control of fermentation conditions for the improvement of product homogeneity, (b) control of the efficiency of downstream processing in purifying specific protein variants, and (c) determination of product heterogeneity caused by changes in process conditions. IEF and 2-DE have higher resolution than chromatogrpaphic methods. Therefore, these methods are popular for characterization of complex protein samples, essential tools for proteomics, and are often used in connection with mass spectrometry for identification of individual proteins.

We think that even in future both chromatographic and electrophoretic methods will be applied for protein separation. As a conclusion, no single analytical method is able to compare every aspect of protein properties, and careful considerations should be made which separation method is suitable for a particular application.

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