Journal of Chromatography, 592 (1992) 3–12
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2455

Separation techniques for biotechnology in the 1990s

Mark Roman and Phyllis R. Brown*

Department of Chemistry, University of Rhode Island, Kingston, RI 02881 (USA)

ABSTRACT

Scientists are constantly looking for better and cheaper separation techniques to replace or complement the current technology. Over the past few decades, and in particular the last 10 years, new separation techniques or modifications of existing techniques have become available for separating compounds from complex sample matrices. There are many areas, however, where the separation technology is not sufficient to achieve high purity and yield while remaining cost effective. In the area of biotechnology, separation techniques are urgently needed to meet demands for ultra-high purity and yield. Thus, a variety of techniques are being developed to address these needs. Generally, biological compounds for the pharmaceutical and biotechnology industries must be obtained at greater than 99.9% purity (sometimes >99.99%) while maintaining high yield. In any area of chemistry this degree of purity would cause problems; in biotechnology it is even more difficult to achieve because of the complex sample matrices. In addition, the compounds of interest may be very similar to impurities or contaminants in the sample matrix, and the compounds could be denatured (or even destroyed) by certain solvents and/or high temperature. In particular, three areas of biotechnology have presented scientists with problems in separations: cell separations, DNA–RNA separations, and protein–peptide separations. The current technology available and possible future trends in these areas are discussed, and also problems to be solved in the future.

CELL SEPARATIONS

Cell separations are unique and challenging. As cells are living organisms, separation conditions are required which ensure the continued activity and function(s) of the cells. The conditions which may affect the viability of the cell include analysis time, temperature, pH and salt content of the sample solution and the presence of organic solvents.

There are may applications for cell separations. Cells which have been genetically altered by recombinant DNA must be separated from the unaltered host cells in order to propagate the genetically engineered cells and study their behavior. In cancer and AIDS research, the diseased cells must be separated from the healthy cells to investigate the abnormal behavior of the diseased cells. In addition, for treatments such as bone-marrow transplants, the red blood cells from the donor must be purified and transplanted within 2 h of removal. Other applications exist for cell separations, and novel techniques have been developed to separate various types of cells.

Two problems are encoutered with cell separations. First, cells are large and bulky, and have about the same diameter. Therefore, it is difficult to separate them by traditional high-performance liquid chromatography (HPLC) or size-exclusion chromatography (SEC). Second, because the cells must be intact and functioning after they have been separated, neither organic solvents nor extremes in pH can be used in the separation process. Hence the mobile phase must consist of aqueous buffer solutions in a narrow pH range. Nevertheless, several techniques have been developed to address the problem of cell separations [1].

As cells generally have densities ranging from 1.005 to 1.110 g/ml [2], one class of separation techniques takes advantage of different cell densities. These techniques include sedimentation at unit gravity, centrifugation, elutriation and field-flow fractionation separations.

Centrifugation methods are the most common methods of separating cells. In this method, the sedimentation rate of the cell is largely dependent on its size; however, the system will reach equilibrium

0021-9673/92/$05.00

when the cell density and the surrounding solution density are the same. Therefore, each cell will migrate to its respective solution density. This technique, in which the cells are allowed to migrate to their respective densities in the density gradient, is called isopycnic centrifugation. Because cell densities are often close to one another, and because of non-uniform density gradients, density isopycnic centrifugation is not a very selective technique and usually can only be used as a preliminary step in cell separations.

Velocity sedimentation at unit gravity works on the same basic principles as centrifugation, but no centrifugal force is applied to the cells. Instead, a constant cell velocity due to gravity is attained for each type of cell depending on the size and density of the cell, in addition to the density and viscosity of the medium. When the force of gravity is balanced by the resistance to movement, constant velocity is reached [3]. Other related techniques, such as centrifugation elutriation [4] and buoyant density centrifugation [5], work on similar principles; all these methods, however, provide enrichment of the cells and not complete purification. Thus, cell separation techniques based on centrifugation or sedimentation are often not adequate to isolate cells of extremely high purity.

Field-flow fractionation (FFF) is another technique used to separate particles based on size [6]. There are many different types of fields which can be utilized in FFF, such as thermal, electric, magnetic and gravitational. When cells are being separated, cross-flow field-flow fractionation (CFFF) is usually used. In this technique, a thin column is sandwiched between two membranes. Solvent molecules can penetrate this membrane but large macromolecules cannot. The cells are transported along the column while a cross-flow acts perpendicular to the column. The cells separate according to their weight, and the separation is governed by their diffusion coefficients. An interesting phenomenon is that zone broadening decreases with increasing retention, hence longer run times are used to achieve adequate resolution. Increasing the amount of time the solute spends in the field prior to initiation of the flow allows the solutes to reach equilibrium in the channel.

Another method developed to complement cell separations based on size and density is the partitioning of the cells between two aqueous polymer solutions [7]. Polyethylene glycol (PEG) has been used as one aqueous polymer and dextran as the other. The upper phase was 5–10% PEG in water and the lower phase was 10–20% dextran in water. In addition, salts, simple sugars and low-molecular-weight compounds which distribute equally between the two phases can be added to optimize the separation. The separation of the cells takes place according to the surface properties of the cells. These properties include surface charge, hydrophobicity and specific receptors. The type and molecular weights of the polymers used can affect the separation, as does pH and the overall composition of the phase system. Because the separation is based on the surface properties of cells, the results obtained are complementary to those of centrifugation; combining the two methods can result in highly selective separations. The main drawbacks of partitioning between two polymer phases are that it is difficult to optimize the separation and sometimes repeated phase separations are necessary to isolate the cells of interest.

A promising technique for cell separation is free-flow electrophoresis (FFE) [8]. This method is used to separate cells on the basis of their surface charge to size ratio. Because each type of cell has a fairly unique surface charge to size ratio, FFE has the potential of being a very selective technique (Fig. 1). As it is a continuous process, potentially large-scale separations can be carried out. The major disadvantage thus far has been that scale-up to the preparative scale is not yet possible owing to the size limitation of the instrument. This limit is imposed by the thermal convection arising from Joule heating of the solution when high voltages and currents are used.

DNA

The separation and purification of deoxyribonucleic acids (DNA), both on an analytical scale and on a preparative scale, is of extreme importance in biotechnology. With the explosive growth in recombinant DNA technology, the massive undertaking of the human genome project and the research being performed on genetic diseases, the need for pure DNA strands (such as recombinant DNA or defective genomic DNA) and accurate DNA sequences

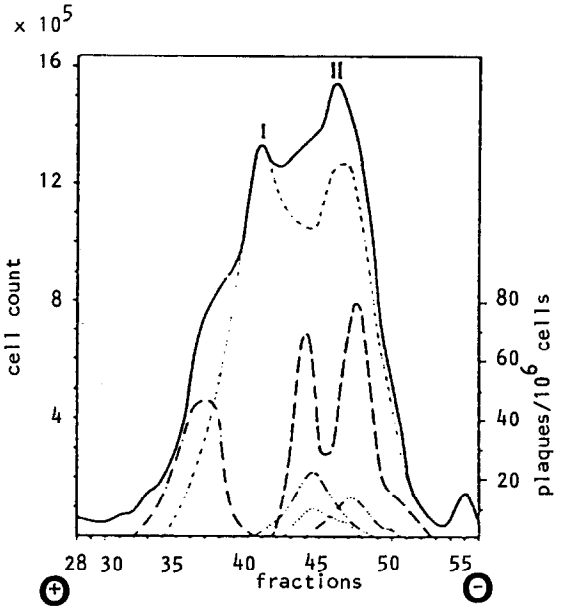

Fig. 1. Electrophoretic separation of lymph node cells. Cell isolation was carried out 82 h after injecting Wistar rat with sheep red blood cells. (—) Cell counts in the various fractions; (– – –) haemolysin-producing cells; (- - -) lymphocytes (I is the T cell region; II is the B cell region); (- · · · -) erythrocytes; (- · · · · -) plasma cells; (- · · · · · · · -) blast cells; (·····) plasmocytes. Separation medium, 15 m*M* triethanolamine–4 m*M*potassium acetate–240 m*M* glycine–11 m*M* glucose, adjusted to pH 7.2 with acetic acid and 310 mOsm with sucrose; conductivity, 900 μmho; Electrode medium, 75 m*M* triethanolamine–20 m*M* potassium acetate; voltage gradient, 100 V/cm. From ref. 8.

has increased tremendously over the past few years.

Two types of DNA separations are needed in biotechnology: (1) the separation of whole DNA strands (or large fragments) from the serum matrix, and (2) the analysis of the individual deoxyribonucleotides which make up DNA. Strands of pure DNA, free from foreign DNA and RNA, are needed for the study of recombinant DNA and for gene mapping. The analyses of nucleotides are used in gene sequencing, the study of nucleic acid metabolism and investigations of disease processes.

Separating DNA stands from blood serum is not difficult. A number of chemical precipitation techniques can be used to accomplish this task in high yield and fairly high purity [9]. In the past, serum was deproteinized with undesirable organic solvents such as phenol or chloroform. To avoid the use of these solvents, proteins may be precipitated by treatment with a saturated NaCl solution, followed by centrifugation at 2500 rpm for 15 min. The proteins precipitate as a pellet at the bottom of the centrifuge tube, leaving the DNA in the supernatant [10]. Another technique utilizes the enzyme proteinase K, which digests protein. When incubated at 65°C, the enzyme digests the proteins and then becomes inactivated after 2 h [11].

Chemical precipitation methods are only useful, however, for bulk precipitation of DNA. The technique cannot be used to precipitate certain DNA strands of interest selectively. The DNA obtained by chemical precipitation must then be further purified if it is to be used in biomedical investigations. For example, several types of DNA may be present in the matrix solution; if the DNA of interest is a recombinant DNA, it may be only a small fraction of the total DNA present. Because DNA molecules are often very large, the separation of different types of DNA is difficult. A recombinant DNA strand may differ from the bulk DNA by only a few nucleotides; thus the two different kinds of DNA possess very similar chemical properties.

Electrophoresis is probably the most common technique for separating DNA on an analytical scale. Gel electrophoresis is commonly used to separate DNA molecules and to determine their size and conformation. Conventional gel electrophoresis on polyacrylamide gels (PAGE) in a static electric field is only suitable for DNA molecules up to 1000 base pairs; however, DNA molecules of very similar sizes can be separated. Very large polynucleotides are not separated easily in gels, because their radius of gyration is often larger than the pore size of the gel [12]. Much larger DNA molecules (up to $3 \cdot 10^7$ base pairs) can be separated on agarose gels, but poorer resolution is obtained [13].

A very active area of research in gel electrophoresis is pulsed-field gel electrophoresis (PFGE). In PFGE, the electric field is alternately applied in different directions, resulting in a two-dimensional separation [14]. This technique extends the size range of the technique to several million base pairs [15]. Variations of this technique exist that differ in the manner in which the alternating electric field is applied; however, all of these techniques work on the same basic principle. The main advantage of these techniques is the high resolution of DNA. The complex instrumentation and long separation times

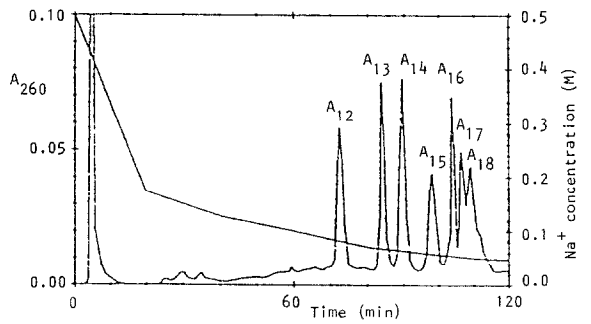

Fig. 2. Separation of oligoadenine mixture by high-performance affinity chromatography. Chromatographic conditions: column, 30 mm × 4.6 mm I.D.; stationary phase, octadecamer of thymidylic acid with primary amino group on the 5′-terminal phosphate group covalently coupled to 300 Å pore macroporous silica; temperature, 35°C. Buffer A, 0.01 *M* sodium phosphate (pH 6.5)–0.49 *M* sodium chloride; buffer B, 0.01 *M* sodium phosphate–0.09 *M* sodium chloride; buffer C, water. The gradient, shown on the right-hand ordinate, was linear between the following times and percentages: 0 min, 100% A; 20 min, 18% A–82% B; 40 min, 7% A–93% B; 60 min, 100% B; 80 min, 70% B–30% C; 100 min, 60% B–40% C; 140 min, 50% B–50% C. From ref. 17.

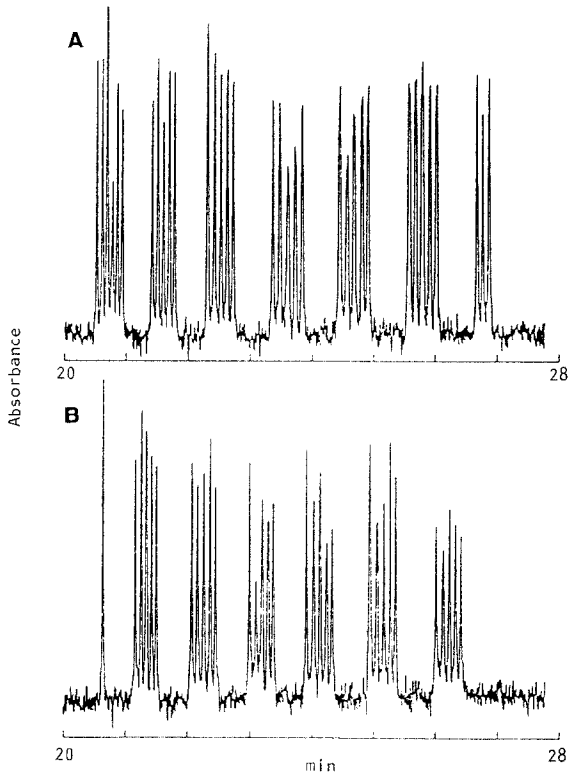

Fig. 3. Electropherogram of chain-termination sequencing reaction products. Template, TEM80.2 = 5′-$T_3A_5T_5A_5T_5A_5T_5A_5$-$T_5A_5T_5A_5T_5$ACAACGTCGTGACTGGG-3′. Primer, JOE-PRM18.1 = 5′-JOE-TCCCAGTCACGACGTTGT-3′. (A)dA reaction: primer was extended by Sequenase 2.0 in the presence of ddATP. (B) dT reaction: primer was extended by Sequenase 2.0 in the presence of ddTTP. Sequence reaction and electrophoretic conditions: column, fused-silica capillary packed with polyacrylamide gel, 75 μm I.D., 375 μm O.D., effective length = 540 mm. Injection was performed by dipping the cathodic end of the capillary tube into the sample and applying 10 kV for 10 s. Applied field, 300 V/cm; buffer, 0.1 *M* Tris–borate–2.5 m*M* EDTA–7 *M* urea (pH 7.6); analysis time, 30 min. From ref. 25.

are disadvantages. As an analytical tool, PFGE and related techniques work very well. Because of heat dissipation problems in addition to problems with sample recovery from the gel, large-scale separations cannot yet be achieved with this type of system.

Whereas low-pressure open-column affinity chromatography has been used for many years to separate DNA polynucleotides [16], HPLC has rarely been used in the separation of whole DNA molecules because of the large size of DNA. Recent innovations in affinity packings may make high-performance affinity chromatography (HPAC) a very attractive solution for large-scale DNA separations, as it is reported that DNA molecules differing by only one base pair can be separated with good selectivity [17] (Fig. 2).

Nucleotide mapping of DNA molecules is now a vital area of study. Nucleotide mapping involves enzymatic degradation of DNA by either the Sanger method [18] or the Maxam–Gilbert method [19], and then separation and analysis of the individual fragments which are created. The separations can be obtained with two-dimensional PAGE [20] or HPLC [21]. Two-dimensional gel electrophoresis gives a unique fingerprint for each type of DNA, which can then be used in forensic chemistry for positive or negative identification of a suspect. HPLC has also been used for analytical- and preparative-scale separations of nucleotides and nucleosides [22] and of derivatives of these compounds, which are being investigated as possible anti-AIDS drugs [23].

Capillary electrophoresis (CE) is being extensively studied as a method for separating DNA and RNA. The main advantages of CE over HPLC or

gel electrophoresis include its phenomenal resolving power (potentially up to 10^6 plates/m), rapid analysis time and the use of simple buffer solutions as a carrier fluid. CE has been used for the separation of nucleotides, oligonucleotides and polynucleotides with over 10^3 base pairs [24]. Capillary gel electrophoresis coupled with laser-induced fluorescence detection has been used to determine DNA sequence reaction products [25] (Fig. 3). There is currently a great deal of research on separating long-chain DNA and DNA reaction products by CE [26] (Fig. 4). The drawbacks with CE are that it is difficult to detect the extremely small amounts of analytes and that injection techniques are not routinely reliable and reproducible; however, when these problems are overcome, CE will be a valuable analytical research tool in biotechnology.

At present, the best method for the large-scale purification of small DNA molecules is HPLC. In addition, it is a very good analytical technique for nucleotides and oligonucleotides. Oligonucleotides up to 500 base pairs can be readily separated by HPLC. The DNA which is frequently made by synthesis of restriction fragments is usually separated on ion-exchange columns. High throughput is the major advantage of HPLC over capillary and gel electrophoresis. Although HPLC can achieve a similar resolution as electrophoresis of similar DNA fragments, extremely large DNA molecules are difficult to work with in HPLC because they tend to clog the column frits. Nevertheless, at present HPLC is the only method available for high-purity, large-scale separations.

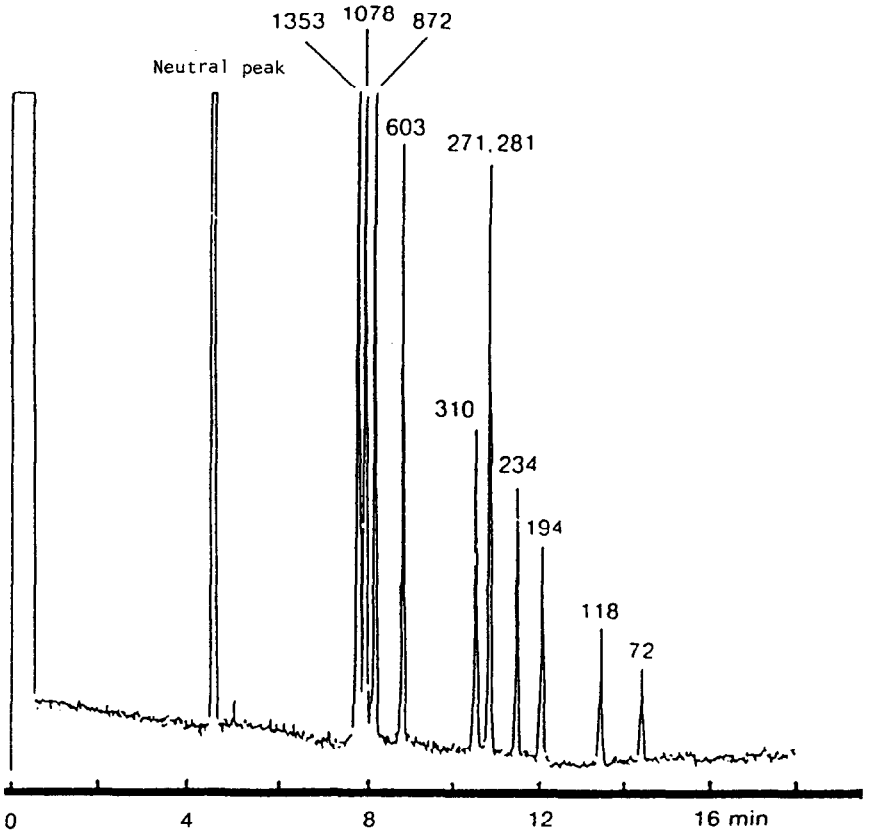

Fig. 4. HPCE (counter-migration) separation of double-stranded DNA fragments (Phi X174/Hae III fragments). Capillary, 42 cm × 50 μm I.D.; effective length, 21 cm; field 140 V/cm; detection, $\lambda = 260$ nm; buffer, Sepragene 500. From ref. 26.

PROTEINS AND PEPTIDES

As with DNA, the separation of proteins and peptides is a challenging problem. Protein–peptide separations also fall into two categories: (1) the separation and purification of entire proteins or peptides for either analytical purposes or for use in biotechnology and (2) the identification of protein–peptide amino acid sequences. Because of the large number of possible protein products, such as interleukins, interferons and antibodies, a great deal of effort has been expended on developing methods for purifying proteins in high yield [27]. Like DNA, proteins exist in a complex matrix, making high-purity, high-yield separations difficult.

HPLC has been used extensively in protein separations. Several different modes can be used for protein–peptide separations. Traditionally, SEC and ion-exchange chromatography (IEC) have been used, often in sequence. Thus, the proteins can be separated first according to size by SEC and then according to ionic species by IEC. A new size-exclusion technique based on the use of aligned fibers made of porous silica has been developed for work

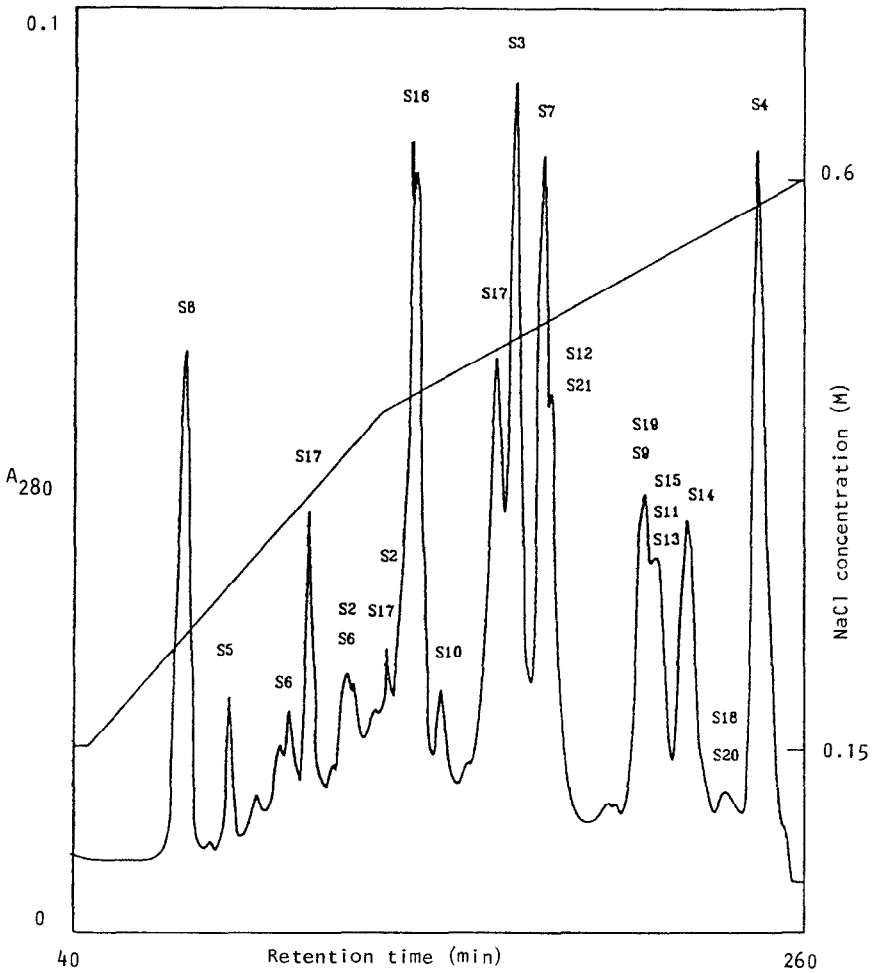

Fig. 5. HPIEC on LKB Ultropac TSK CM-3SW (150 × 7.5 mm I.D.) of TP30 under non-denaturing conditions. 50–200 μg of TP30 in 5–10 ml of 0.05 *M* ammonium acetate (buffer A)–0.15 *M* NaCl were applied to the column. Following a 30-min equilibration time with buffer A–0.15 *M* NaCl, gradients were applied from 0.15 to 0.4 *M* NaCl over 30 min and from 0.4 to 0.6 *M* over 140 min. Note that the absorbance scale was changed to 0.2 a.u.f.s. after 145 min (elution time of S16). From ref. 30.

with proteins and polymers [28]. This technique could increase the efficiency of size separations. Although SEC–IEC often requires multiple separations, good separations can be achieved in a single experiment with new higher performance (HP) high-pressure ion-exchange columns [29]. Because the conditions of HPIEC may denature the proteins, ion-exchange separations under non-denaturing conditions utilizing salt gradients have been developed [30] (Fig. 5). The disadvantages of this technique are that a prefractionation step is needed to separate a large number of proteins and the run times are very long, sometimes over 4 h.

Affinity chromatography, reversed-phase chromatography (RP-HPLC) and hydrophobic-interaction chromatography (HIC) are gaining popularity in protein separations. In affinity chromatography, a ligand which binds strongly but selectively to a certain protein (or group of proteins) is covalently attached to the solid support in the column. The sample is then passed through the column with buffer as the mobile phase. Only those proteins which selectively bind to the ligands will be retained on the column. All other components are washed off the column with the mobile phase. After all unwanted components have been removed, the column is treated with another solution (often a salt or acidic solution), which then releases the desired protein from the stationary phase and elutes it [27]. These columns are expensive, but the purity of the desired protein is usually $\geq 99.99\%$ after dialysis has been used to remove the buffer salts. Unfortunately, if the concentration of the protein of interest is low, as is usually the case, the solution must be passed through the column several times for high adsorption efficiency, making affinity chromatography very time consuming. A method in which the analyte is adsorbed in a batch mode in a slurry tank and eluted through a fixed-bed column has been developed which greatly reduces the time for a complete separation [31].

In RP-HPLC the separations are based on the hydrophobic character of proteins [32]. Selective separations are possible, but sometimes no separation will occur if the hydrophobic areas of the proteins are not accessible to the stationary phase. In addition, denaturation of proteins can occur if there is too much organic modifier in the stationary phase or as a result of the interaction of proteins with the stationary phase. Therefore, both the stationary phase and the mobile phase must be carefully selected and controlled to ensure good separation, purification and maintenance of biological activity of the proteins. The proteins must be soluble in the mobile phase; as a result, the mobile phase is usually a water–acetonitrile gradient.

HIC is similar to RP-HPLC in that both utilize differences in the hydrophobic nature of the solutes to achieve separation. Whereas the stationary phase is usually dense and very hydrophobic in RP-HPLC, HIC utilizes less dense, less hydrophobic stationary phases so that the adsorption of proteins to the stationary phase is more gentle. The mobile phase is an aqueous solution, usually of high ionic strength at the beginning and gradually decreasing in the applied gradient, and very little if any organic modifier is used. Therefore, denaturation due to stationary phase and mobile phase interactions is minimized, while the mode of retention is essentially the same. PEG-bonded stationary phases are now available which preserve the full biological activity of proteins while yielding a mass recovery of $>90\%$ for some proteins [33].

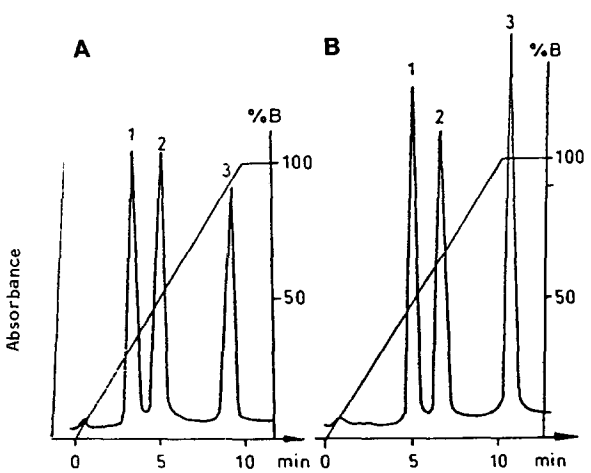

Fig. 6. Protein separation by HPLC on short capillary columns. (1) Trypsinogen, (2) chymotrypsinogen and (3) lysozyme separated by means of microcolumn cation-exchange HPLC. Column, 150 × 9.5 mm I.D.; flow rate, 10 μl/min. (A) Mono S sorbent (Pharmacia); (B) sorbent MPG-350 polyol-sulfopropyl (Lenchrom, USSR). Sample mass, 10 ng of each protein; detection, fluorescence (excitation wavelength 230 nm, emission wavelength 375 nm). From ref. 34.

Capillary HPLC columns have been developed for protein analyses where the proteins of interest are present in very low concentrations (< 10 ng/ml). These columns are made of PTFE and have an inner diameter of 500 μm. In addition to the ability to separate and detect much lower concentrations of proteins, separations on these columns require considerably less solvent than standard 4.6 mm I.D. columns. These columns can be used in the ion-exchange, RP-HPLC or HIC mode [34] (Fig. 6). The use of fast atom bombardment mass spectrometry (FAB-MS) for detection in capillary HPLC could prove to be a very powerful technique for protein and peptide analyses [35]. With conventional HPLC columns, time-of-flight mass spectrometry (TOF-MS) is a useful detection method [36].

Both gel and capillary electrophoresis are used for analytical-scale separations of proteins and for protein mapping. The methods utilized are very similar to electrophoretic separations of DNA–RNA; in fact, proteins have been analysed simultaneously with DNA–RNA [37]. CE is rapidly gaining popularity in protein separations because of its speed and resolving power. In addition, CE is much less labor intensive than gel electrophoresis [38]. Mass spectrometry has been coupled to CE for the analysis of proteins and peptides, resulting in an extremely powerful analytical technique. This technique (CE–MS) can provide not only quantitation of amino acids after enzymatic digestion, but also on-line peptide sequencing [35,39] (Fig. 7). FFE, which is a continuous process, has potential for preparative-scale separations of proteins. A major problem in FFE is the conductive heating which prevents scale-up of the technique. On a small scale, in which the bed thickness is between 0.5 and 2 mm, FFE has been used to separate proteins continuously [40]. Hoffstetter-Kuhn and Wagner [41] have achieved a sample throughput of 2.75 g/h of crude yeast extract protein and a 4.7-fold purification of alcohol dehydrogenase from crude protein by FFE (Fig. 8).

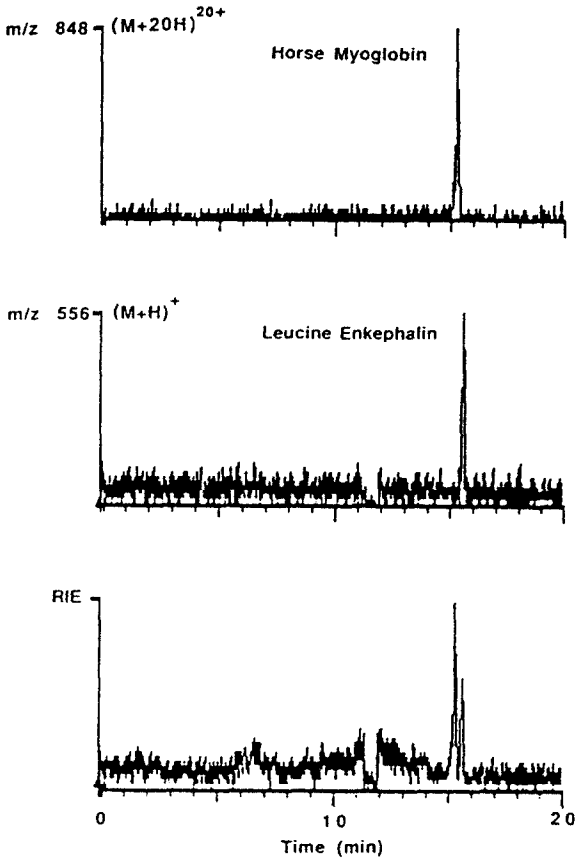

Fig. 7. CZE–ESI-MS separation–detection of horse myoglobin and leucine enkephalin (Tyr–Gly–Gly–Phe–Leu) in a 125 cm × 50 μm I.D. fused-silica capillary at 30 kV (17 μA). Buffer, 20 mM Tris solution adjusted to pH 8.25 with HCl–KCl solution. Electromigration injection for 10 s at 15 kV. From ref. 39.

Immunoglobulins

Immunoglubulins are a special class of proteins which are of extreme importance in biotechnology. Five classes of immunoglobulin antibodies are produced by the body. Immunoglobulin G (IgG) is the most abundant of all immunoglobulins; it has molecular weight of about 150 000 dalton. Immunoglobulins are composed of four chains. Two chains are heavy chains (called "H" chains) and two are light chains (called "L" chains). These four chains, which are held together by –S–S– bridges, comprise three different areas or domains on the antibody: two antigen-binding sites and one non-antigen-binding site is called the Fc part. Enzymatic cleavage and fractionation of immunoglobulin into its three separate domains does not deactivate the binding sites but greatly reduces the ability of the antibody to remove foreign antigens [42].

As immunoglobulins are being extensively studied as possible cancer therapeutic agents, good separation and purification techniques are urgently

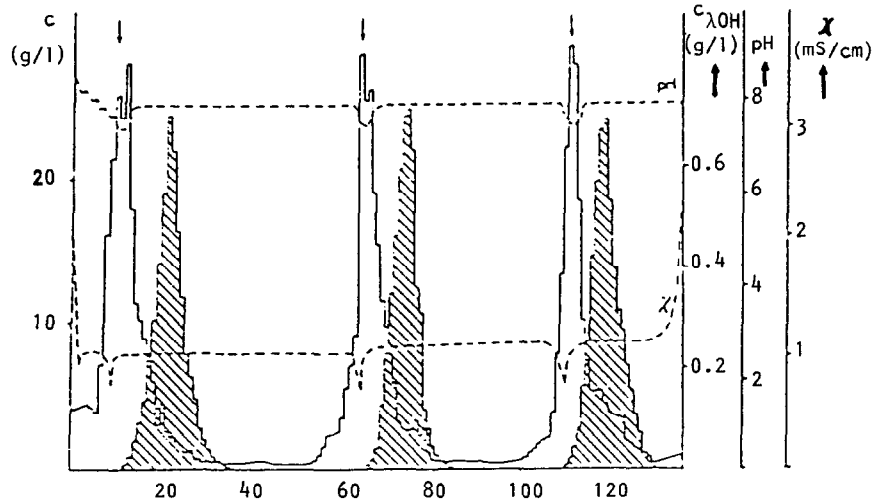

Fig. 8. Free-flow zone electrophoresis of yeast extract with a simultaneous threefold sample dosing in 0.02 *M* Tris, adjusted to pH 8.0 with HCl. Sample dialyzed yeast extract, containing 45.8 g/l of total protein and 1.53 g/l of ADH. Conditions 1000 V; residence time, 5 min; sample flow-rate, 20 ml/h; electrode solution, 0.2 *M* Tris, adjusted to pH 8.0 with HCl; electrophoretic apparatus, ElphorVap 22 (Bender and Hobein), 100 cm length × 15 cm width × 1 mm thick. From ref. 41.

needed to obtain extremely pure (>99.99%) products. It has been estimated that between 40% and 90% of the overall cost of processing immunoglobulins is in the separation and purification steps [43].

The most common method of purification of immunoglobulins is by precipitation with ammonium sulfate, followed by anion-exchange chromatography on a DEAE-cellulose column [44]. This procedure results in an IgG purity of >90%; to achieve higher purity, a high-performance ion-exchange column must be used. In either case, at least two steps are necessary to achieve the purification of IgG. A third method of purification involves coupling RP-HPLC with anion-exchange chromatography, further purifying the IgG, but adding another step to the purification process [40].

In a non-chromatographic technique, albumin and other serum proteins were precipitated by octanoic acid, leaving IgG in the solution. IgG was then precipitated by addition of ammonium sulfate. About 80% of rabbit serum IgG was recovered by this method, in high purity (comparable to that obtained by anion-exchange chromatography) and at low cost. Increased purity was obtained by repeating both steps, but the IgG yield was decreased to about 50% [45].

One of the most promising methods of IgG sep-

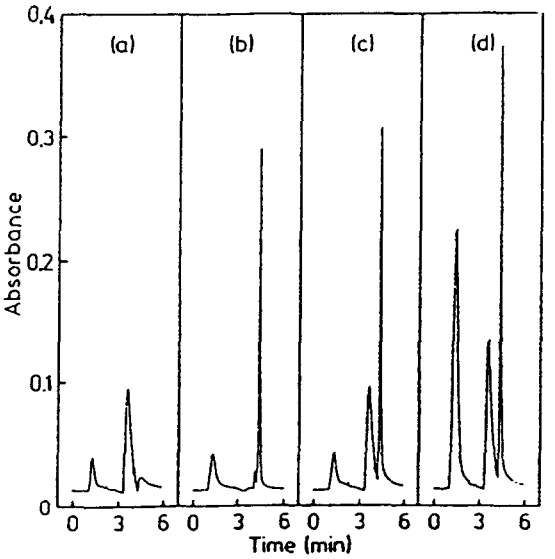

Fig. 9. Dual-column determination of human serum albumin (HSA) and immunoglobulin G (IgG) in serum by high-performance affinity chromatography. (a) HSA; (b) IgG; (c) HSA plus IgG; (d) normal serum in the dual-column system. Chromatographic conditions: column 1 protein A column (protein A coupled diol-bonded LiChrospher Si-500), 6.4 mm × 4.1 mm ID; column 2, anti-HSA column (anti-HSA antiserum coupled to LiChrospher Si-4000), 12.8 mm × 4.6 mm I.D. Sequence of events: 0 min, switch from pH 3.0 phosphate buffer to pH 7.0 phosphate buffer; 0.50 min, sample injection; 2.25 min, protein A column switched off-line, switch to pH 3 buffer; 4.00 min, protein A column switched on-line. From ref. 46.

aration is HPAC. Hage and Walters [46] separated albumin and IgG simultaneously from serum by using dual columns (Fig. 9). One column contained albumin-specific antibodies, immobilized on the stationary phase, and the other column contained IgG-specific protein A. This technique has been used for the determination of IgG and albumin, and it is widely used for preparative and semi-preparative purifications.

A new technique geared towards large-scale purification is affinity cross-flow filtration (ACFF). This technique combines aspects of biospecific adsorption with cross-flow filtration. The antibody of interest is reversibly bound to an adsorbent coated with antigen "ligands". When the antibody is bound to the adsorbent, the molecular size is greatly increased, making separation by filtration possible. Washing with buffer removes the antibody from the adsorbent. A drawback to this technique is the low recovery of IgG (about 50%) [44].

Gel electrophoresis has been used a great deal for the qualitative determination of IgG. A method of continuous free-film electrophoresis has been developed which can overcome the problems of heat dissipation associated with the scale-up of electrophoresis. Its major disadvantage is that it introduces a 40-fold dilution into the fractionation [47].

REFERENCES

1 K. Shortman, *Annu. Rev. Biophys. Bioeng.*, 1 (1972) 93.
2 T. G. Pretlow and T. P. Pretlow, *Methods Enzymol.*, 171 (1989) 462.
3 T. G. Pretlow and T. P. Pretlow (Editors), Cell Separation: Methods and Selected Applications, Vol. 1, Academic Press, San Diego, CA, 1982, p. 41.
4 M. J. Sanders and A. H. Soll, *Methods Enzymol.*, 171 (1989) 482.
5 J. Wells, *Methods Enzymol.*, 171 (1989) 497.
6 H. M. Widmer and L. Koch-Kellner, *Chimia*, 43 (1989) 388.
7 P. Albertsson, *Methods Enzymol.*, 171 (1989) 532.
8 H. G. Heiddrich and K. Hannig, *Methods Enzymol.*, 171 (1989) 513.
9 J. K. Karlinset, G. Stamatoyannopoulos and T. Enver, *Anal. Biochem.*, 180 (1989) 303.
10 S. A. Miller, D. D. Dykes and H. F. Polesky, *Nucleic Acid Res.*, 16 (1988) 1215.
11 J. Grimberg, S. Nawoschik, L. Belluscio, A. Turck and A. Eisenberg, *Nucleic Acid Res.*, 17 (1989) 8390.
12 J. Noolandi, *Makromol. Chem., Rapid Commun.*, 10 (1989) 207.
13 S. Tas, *Anal. Biochem.*, 188 (1990) 33.
14 M. V. Olson, *J. Chromatogr.*, 470 (1989) 377.
15 R. W. Whitcomb and G. Holzwarth, *Nucleic Acids Res.*, 18 (1990) 6331.
16 P. T. Gilham, *J. Am. Chem. Soc.*, 86 (1964) 4982.
17 T. A. Goss, M. Bard and H. W. Jarret, *J. Chromatogr.*, 508 (1990) 279.
18 F. Sanger, S. Nicklen and A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.*, 74 (1977) 5463.
19 A. M. Maxam and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.*, 74 (1977) 560.
20 B. W. Birren, M. I. Simon and E. Lai, *Nucleic Acids Res.*, 18 (1990) 1481.
21 P. R. Brown (Editor), *HPLC in Nucleic Acid Research*, Marcel Dekker, New York, 1984.
22 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 126 (1976) 679.
23 J. G. Turcotte, P. E. Pivarnik, S. S. Shirali, H. K. Singh, R. K. Sehgal, P. Macbride, N.-I. Jang and P. R. Brown, *J. Chromatogr.*, 499 (1990) 55.
24 V. Dolnik, J. Liu, J. F. Banks and M. V. Novotny, *J. Chromatogr.*, 480 (1989) 321.
25 A.S. Cohen, D. R. Najarian and B. L. Karger, *J. Chromatogr.*, 516 (1990) 49.
26 Y. Baba, M. Tsuhako, S. Enomoto, A. M. Chin and R. S. Dubrow, *J. High Resolut. Chromatogr.*, 14 (1991) 204.
27 L. A. Haff, *Chromatography*, March (1987) 25.
28 M. Czok and G. Guiochon, *J. Chromatogr.*, 506 (1990) 303.
29 A. M. Tsai and D. Englert, *J. Chromatogr.*, 504 (1990) 89.
30 C. Cachia, P.-J. Flamion and J.-P. Schreiber, *J. Chromatogr.*, 539 (1991) 343.
31 B. Yang, M. Goto and S. Goto, *J. Chem. Eng. Jpn.*, 22 (1989) 532.
32 B. L. Karger, F. E. Regnier and T. C. Wehr (Editors), *High Performance Liquid Chromatography of Proteins and Peptides*, Academic Press, New York, 1983.
33 N. Cooke, P. Shieh and N. Miller, *LC · GC*, 7 (1989) 954.
34 V. G. Mal'tsev, D. G. Nasledov, S. A. Trushin, T. B. Tennikova, L. V. Vinogradova, I. N. Volokitina and V. N. Zgonnik, *J. High Resolut. Chromatogr.*, 13 (1990) 185.
35 M. A. Moseley, L. J. Deterding, K. B. Tomer and J. W. Jorgenson, *J. Chromatogr.*, 480 (1989) 197.
36 R. C. Simpson, W. B. Emary, I. Lys, R. J. Cotter and C. C. Fenselau, *J. Chromatogr.*, 536 (1991) 143.
37 R. G. Hatch, *J. Chromatogr. Sci.*, 28 (1990) 210.
38 F.-T. Chen, C.-M. Liu, Y.-Z. Hsieh and J. C. Sternberg, *Clin. Chem.*, 37 (1991) 14.
39 R. D. Smith, J. A. Loo, C. J. Barinaga, C. G. Edmonds and H. R. Udseth, *J. Chromatogr.*, 480 (1989) 211.
40 J. Chmelík, M. Deml and J. Janca, *Anal. Chem.*, 61 (1989) 914.
41 S. Hoffstetter-Kuhn and H. Wagner, *Electrophoresis*, 11 (1990) 451.
42 L. Stryer, *Biochemistry*, W. H. Freeman & Co., New York, 1981, Ch. 33.
43 D. C. Herak and E. W. Merrill, *Biotechnol. Prog.*, 6 (1990) 33.
44 F. E. Regnier, *LC · GC*, 5 (1987) 962.
45 M. McKinney and A. Parkinson, *J. Immunol. Methods*, 96 (1987) 271.
46 D. S. Hage and R. R. Walters, *J. Chromatogr.*, 386 (1987) 37.
47 D. E. Austen, T. Cartwright and C. H. Dickerson, *Vox Sang.*, 44 (1983) 151.