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Review

Preparative chromatography of biomolecules

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ABSTRACT

An overview of preparative chromatography is given with a description of both theoretical and practical aspects. Owing to the large-scale production of recombinant proteins in various hosts, the requirements for speed, recovery and purity are ever increasing. The introduction of new gels with higher stability, a better understanding of the adsorption process and significant improvements in equipment such as injectors, pumps, fractionation devices and valves have transformed chromatography from an art to science and technology. The rules for scale-up are well understood (constant height, constant height-to-diameter ratio, dynamic similarity) and theoretical solutions including computer programs are available to minimize experimental work.

CONTENTS

1. INTRODUCTION

The requirements for amount, purity and final concentration of particular biomolecules may influence the purification strategy, the technique of scale-up of column liquid chromatography and the selection criteria for packings, etc. The requirements for scale-up also depend on the

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intended use of the material, *i.e.*, is it only for research purposes, for clinical trials or for marketing as a fine chemical? In this paper, an attempt is made to give an overview of these requirements (Table 1). Detailed instructions depend on the particular case, but general guidelines for preparative chromatography can be applied for all of the purposes listed in Table 1.

The degree of purity, composition of starting material, stability of product and amount are the most important parameters in the design of a preparative chromatography protocol. These parameters influence the complexity and the selection of unit operations. For optimization of a total process, the cloning of the protein product with the appropriate expression system and host, the fermentation strategy and the purification must integrate well. For an understanding of these aspects, this paper will focus on the influence of the preparation of starting material on chromatography at a later time.

Conventional laboratory purification procedures for protein consist of a sequence starting with clarification by centrifugation (often in connection with flocculation), precipitation, fol-

TABLE 1

REQUIREMENTS FOR PURITY, HOMOGENEITY, AND RELATED PROPERTIES, OF PROTEINS FOR RESEARCH AND DEVELOPMENT

Intended use	Amount required	Purity criteria
Immunization	Depends on immunogenicity, less than 100 mg per animal, even for large animals such as goats	>95% for conventional immunization; crude material for sensitizing spleen cells used for hybridoma production
Animal experiments	Depends on experiment, number of animals, etc.	Avoid denaturation for long-term studies; avoid bacterial endotoxins
Clinical trials with humans[a]	Depends on experiment, potency of substance, etc.	>99.9% purity; less than 10 (100) pg DNA per dose; avoid adventitious agents, *e.g.*, viruses, incomplete virus particles, bacterial endotoxins
Protein characterization		
Crystallization	10–100 mg	99%
NMR studies	10–100 mg	High concentration necessary, >1 mg/ml
Spectorscopic studies, *e.g.*, CDR studies	1–10 mg	Avoid denaturation, may influence result
Amino acid sequencing, amino acid composition	<1 mg	Avoid amino acid buffers in final purification steps; partial denaturation is acceptable; purity is not as critical as with amino acid sequencing
Fine chemicals		
Growth factors, inhibitors, nutrition factors, etc., especially in cell culture	Depends on situation	Purity is not most important criterion; avoid bacterial endotoxins and contaminants with mitogenic, inhibitory and growth-interfering properties
Preparation of reference materials	Depends on situation	Purity is often not most important criterion; high stability; avoid proteases

[a] Very detailed instructions are available, *e.g.*, for recombinant DNA-derived products and monoclonal antibodies.

lowed by a series of chromatographic steps [1,2]. Desalting, if necessary, is performed by dialysis. Modern procedures often use microfiltration and ultrafiltration combined with a series of chromatographic steps [3–5]. Desalting is carried out by gel filtration on small columns packed with gels of low exclusion limits such as Sephadex G-25 (Pharmacia, Uppsala, Sweden) Trisacryl (Sepracor/IBF, Villeneuve la Garrene, France) or Bio-Gel P media (Bio-Rad, Richmond, CA, USA). With a few exceptions, chromatography is not used in the initial step of protein purification procedures [6,7].

The development of affinity chromatography [8,9] in the 1960s brought a new dimension to biochemistry and related fields. Proteins could be purified to homogeneity easily and purification sequences could be greatly simplified.

In the early 1980s, when the first foundations of genetic engineering companies created a boom in biotechnology, the introduction of rigid gels and equipment such as fast protein liquid chromatography (FPLC) into the field of preparative chromatography initiated a new era in the purification of biomolecules. Fast and reproducible methods could be established by taking advantage of the high precision in flow, the simple packing and the construction of inert materials for use with buffers employed for protein chromatography. Later, the on-line monitoring of pH and conductivity converted preparative chromatography from art to science and technology. The development of a scaled-up version with the same features of the analytical process was a logical consequence as FPLC became popular in the protein chemist's world.

2. ANALYTICAL *VERSUS* PREPARATIVE CHROMATOGRAPHY

The common characteristic of both types of chromatography is the interaction and distribution of a solute between a mobile phase and a stationary phase while the mobile phase is passing through the stationary phase, hence the different solutes are separated according to their distribution coefficients and some other physical parameters with minor influences.

Protein purification is accomplished primarily by liquid chromatography (LC), therefore further considerations deal only with LC where the stationary phase is packed into a column. The different types of chromatographic procedures and the principles of action are listed in Table 2. Packings and stationary phases in preparative LC have been reviewed comprehensively several times [11–17]. Features of the material did not change, but a few packings disappeared from the market as new materials were developed.

In analytical chromatography, a small sample

TABLE 2

TYPES OF CHROMATOGRAPHY DISTINGUISHED ACCORDING TO THE NATURE OF RETARDATION FORCES AND THE PRINCIPLE OF SEPARATION

Modified from Jungbauer and Jansen [10]. All listed types are used for purification of biomolecules.

Name	Action principle	Separation according to
Adsorption chromatography	Surface binding	Molecular structure
Ion-exchange chromatography	Ion binding	Surface charge
Molecular sieve chromatography (gel filtration)	Steric exclusion	Molecular size and shape
Affinity chromatography	Biospecific adsorption/desorption	Molecular structure
Hydrophobic (interaction) chromatography	Hydrophobic complex formation	Hydrophobicity and hydrophobic patches
Covalent chromatography	Covalent binding	Functional groups
(Metal) chelate chromatography	Coordination complex formation	Complex formation with transition metals
Reversed-phase liquid chromatography	Hydrophobic complex formation	Hydrophobicity

is applied to the column and maximum resolution is sought by exploiting the characteristics of the different phases of the system. In preparative chromatography, maximum throughput at a defined purity is the principal consideration. Therefore, an adsorption/desorption mode is preferred. The process can be divided into several steps:

equilibration	→ adjusting mobile and stationary phase to binding conditions
sample application (loading)	→ loading of protein solution on to column
washing	→ removal of unbounded material, often several steps
elution	→ desorption of desired compound by stepwise or continuous change of mobile phase composition
regeneration/ cleaning	→ desorption of very sticky impurities, *e.g.*, colours, endotoxins, lipids
preservation/ sanitation	→ change of mobile phase to avoid microbial contamination during storage

Elution can be carried out be instantaneously either by changing the mobile phase (stepwise elution of step gradient) or by continuously changing the mobile phase (linear gradient).

The use of buffers and materials for different steps cannot be generalized, as they depend greatly on the separation method chosen. With a few exceptions [hydrophobic interaction chromatography (HIC), reversed-phase LC (RPLC)] aqueous buffers are used as mobile phases. One should use caution in using oxidizing agents for cleaning columns as most packing materials are very sensitive to these agents. Sodium hydroxide solutions combined with detergents are compatible with most gels and sufficient cleaning may be achieved. Hydroxyapatites and silicas are very sensitive to alkaline solutions [18–20]. Storage of columns in 20% ethanol or 2-propanol avoids microbial growth in most instances. Some columns also withstand practical mistakes such as pumping air into into them [21].

2.1. *Theory of adsorption/desorption mechanism*

Velayuahan and Horvath [22] generalized the adsorption/desorption phenomena for all types of chromatography by introducing the term mobile phase modifier (MPM), which is responsible for desorption, *e.g.*, salts in ion-exchange chromatography, chaotropic agents in affinity chromatography, acetonitrile or methanol in RPLC.

The interaction of a solute with the stationary phase expressed as the retention factor or distribution coefficient (K) is plotted against the MPM concentration in the mobile phase (Fig. 1). The biomolecule is adsorbed at low MPM concentrations and desorbed at high concentrations, as the retention factor becomes very high at low salt and very low at high salt concentrations. Several types of interactions are observed, *e.g.*, a strong dependence of the retention factor which coincides with the K value of MPM and a weak dependence of MPM. The relationship between

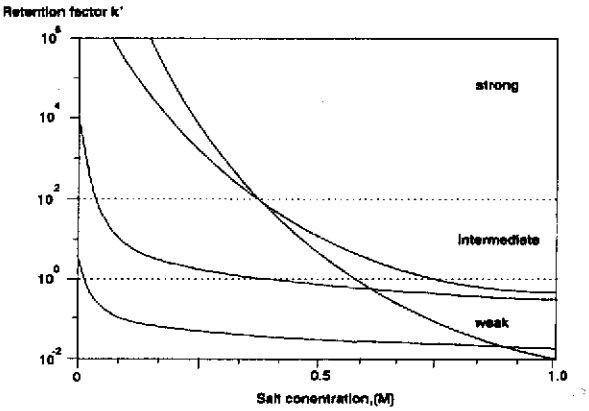

Fig. 1. Distribution coefficient (K) or capacity factor (k') *versus* salt concentration generalized as mobile phase modifier (MPM) concentration according to Velayudhan and Horváth [22]. A protein located in the strong binding domain has a tendency to be retained in the column. Changing the MPM retention will continuously alter the retention. For adsorption/desorption an on–off mechanism is desirable

the available K value and the retention factor k' is expressed by the equation

$$k' = (v_e - v_0)/v_0 \quad (1)$$

and

$$K = (v_e - v_0)/(v_t - v_0) \quad (2)$$

where v_e is the elution volume, v_t is the total column volume and v_0 is the void volume. The term $(v_t - v_0)$ is the dimensionless fraction of stationary phase, which can also be expressed by $1 - \varepsilon$, where ε is the void fraction. Inserting eqn. 2 in eqn. 1 leads to

$$k' = K(1 - \varepsilon) \quad (3)$$

The relationship between k' and salt for an isocratic retention model in ion-exchange chromatography (IEX) can be approximated by the equation

$$\log k' = m \log(1/c) + \log a \quad (4)$$

proposed by Kopaciewicz *et al.* [23], where c is the MPM concentration and m and a are constants characteristic for a given separation system describing the involvement of charged groups in the adsorption/desorption process. The curves shown in Fig. 1 can be fitted well by this equation.

As a consequence of the MPM concentration-dependent change of k' or K, several types of elution are observed. IEX is used as an example for the explanation of this behaviour.

Using a stepwise elution protocol, a sharp peak is often eluted together with the wave of the salt. This type of elution is also called fronting or type I [24]. When the salt concentration in the elution buffer is decreased, a broader peak will be observed. The substance is travelling down the column behind the salt front (Fig. 2). The elution profiles are called type II elution [24]. Of course, type I elution is preferred, as the protein is highly concentrated in contrast to that observed with type II and profiles generated by linear gradients (Fig. 3). The sample volume (loading volume) does not influence the peak width (Table 3). In a purification experiment for human monoclonal antibodies we obtained a 40-ml peak fraction from a

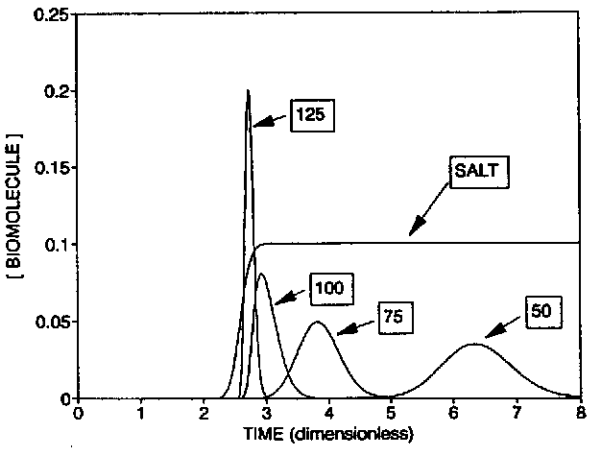

Fig. 2. Types of elution profiles in stepwise elution. Type I or fronting is characterized by elution of the protein together with the salt front and type II by elution of the protein behind the salt front. Calculated values of an IEX system described in ref. 64. Calculation was carried out with different salt concentrations.

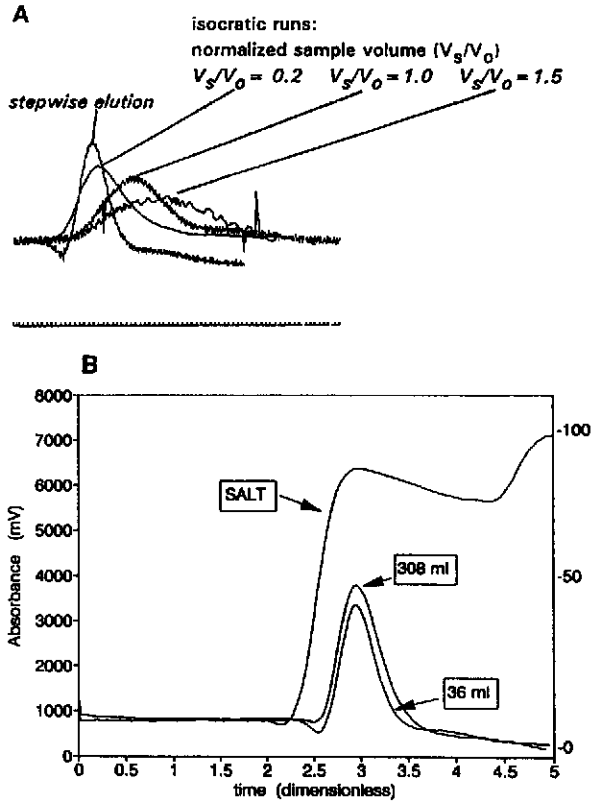

Fig. 3. Influence of loading volume on peak volume. (A) Experimental results of an ion-exchange purification of SOD on DEAE-Sepharose fast flow; (B) elution profile of the same purification system with stepwise elution.

TABLE 3

DEPENDENCE OF PEAK VOLUME ON LOADING VOLUME IN STEPWISE ELUTION GENERATING TYPE I ELUTION PROFILES

The model was purification of SOD with a DEAE-Sepharose fast flow using 100 m*M* NaCl for desorption. The detailed experimental conditions for loading, washing and desorption are described elsewhere [27]. v_s = Sample volume, v_p = peak volume and v_0 = void volume of the column.

Dimensionless sample volume, v_s/v_0	Dimensionless peak volume, v_p/v_0
0.2	1.5
1.1	0.65
10.7	0.6
11.3	0.6

10-ml CM-Sepharose fast flow column loading with 2150 ml of desalted culture supernatant [25]. In the scale version, 100 l were percolated over a 1000-ml column and the protein was recovered in less than 1 l, indicating a concentration factor higher than 100. Malm [26] reported a monoclonal antibody purification, in which concentration factors up to 200 were achieved. These examples indicate that optimization should be directed to type I elution profiles, when a salt gradient is defined. A simple approach is to load the sample under optimum binding conditions and conduct the elution procedure with a series of consecutive elution steps [25,27].

The different types of elution profiles should not be mixed with the concentration-dependent behaviour of adsorption isotherms, which are normally described as Langmuir adsorption isotherms (Fig. 4). As a consequence of this physical behaviour, the bulk of the solute is eluted earlier than traces. Therefore, one cannot transfer analytical resolution data to preparative chromatography under conditions of overloading biomolecules. Analytical chromatography should be carried out in thc linear range, whereas preparative chromatography should be carried out in the saturated range. Divergences of Langmuir plots in the shape of sigmoid curves have also been reported [28], but may have minor

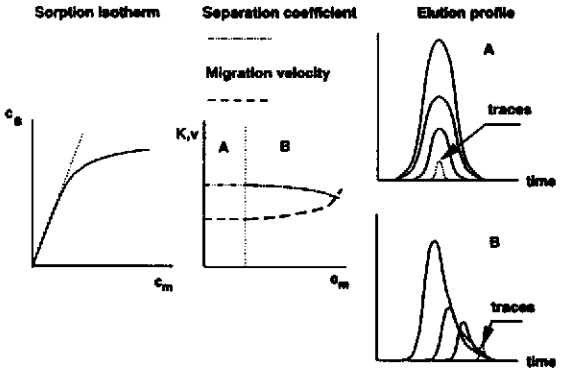

Fig. 4. Influence of sorption isotherm on the elution profile and migration velocity. K = distribution coefficient described by the slope of the sorption isotherm; v = migration velocity, c_m and c_s = mass concentration of solute in mobile and stationary phase, respectively.

consequences for the elution profile than chromatography under non-equilibrium conditions.

A preparative chromatographic system is sufficient characterized by a three-dimensional plot of *K versus* MPM concentration and concentration of the solute (Fig. 5). For practical work it is not necessary to know the exact relationship between *K*, MPM concentration and solute concentration, but one has to keep in mind the type (weak, moderate or strong) of elution behaviour when the gradient is designed.

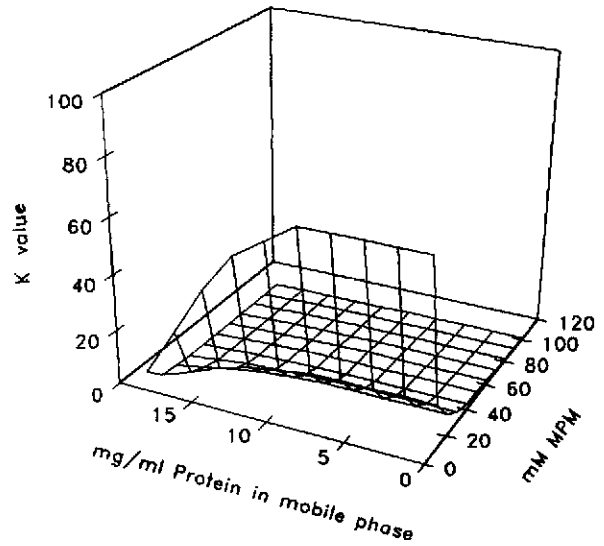

Fig. 5. Three-dimensional plot of *K versus* MPM concentration and protein concentration in the mobile phase under equilibrium conditions.

The adsorption/desorption conditions are also influenced by the ligand density [29]. Therefore, one cannot transfer the conditions from one matrix to another. Also, it should be noted that the ligand density influences the peak profile; the lower the density, the more pronounced is the band broading.

2.2. Adsorption/desorption in hydrophobic interaction chromatography

HIC has received increasing attention as it is an effective step that can be directly linked to IEX, affinity chromatographic (AC), gel filtration (gel permeation) chromatographic (GPC) or non-chromatographic purification steps. The effect of salt at a significantly high ionic strength (m) can be described as

$$\log(k'/k'_0) = \lambda m \quad (5)$$

where λ is a parameter that measures the retentive strength and is similar to the salting-out constant [30]. An early report from Pahlman *et al.* [31] noted that the protein may undergo a conformational change at the high salt concentrations used (up to 3 M) in HIC. Additionally, HIC is very sensitive to the temperature of the loading step and to the residence time in the column. Detailed instructions for the adjustment of retention behaviour in HIC were given by Melander *et al.* [30]. By modifying the hydrophobic ligand, the selectivity of the salt-promoted interaction (as named by Porath [32]) can be increased.

2.3. Adsorption/desorption in affinity chromatography

As the adsorption in AC is biospecific and driven by the interaction of a protein or a biomolecule with a ligand with a certain molecular structure [33], adsorption cannot be generalized. Adsorption also can occur at both low and high salt concentrations and in a broad range of pH values. The effects of ligand density on retention behaviour and capacity are comparable to those seen with other types of chromatography [34]. Desorption can be performed by addition of free ligand or of a mimic of the ligand to the mobile phase (affinity elution [35]) by changing the pH or ionic strength of the mobile phase. Electrophoretic elution and high-pressure elution have also been described, but are not broadly applied [36–38]. Chaotropic ions are very well suited for desorption. They disrupt the structure of the binding between the ligand and the biomolecule; often the recovery is low owing to denaturation during desorption. Group-specific adsorbents such as protein A [39], protein G, lectins [40,41] and triazine dyes [42] with a broad binding spectrum are very popular. This interaction is based on a particular protein domain which is conserved during evolution or is a random event as with lysine-Sepharose used for the isolation of plasminogen, plasminogen activators, rRNA and dsDNA.

Immunoaffinity chromatography is the most universal technique especially considering the monoclonal antibodies as ligands. Immunoaffinity chromatography has industrial applications for purifying materials such as interferon, FIX, FVIII and EPO and for vaccine purification. Ligand leakage is still an unsolved problem in AC. Binding chemistry and molecular orientation of the ligand are largely responsible for the leakage and loss in performance. The immobilization chemistry (reviewed by Narayanan and Crane [43]) is directed to immobilization of high-molecular-mass biomolecules with a single-point attachment, whereas small molecules are immobilized with a spacer with an optimum length.

The purification power of AC in the field of biomolecules is ranked the highest among all types of chromatography for its effectiveness in retaining biological activity.

3. SCALE-UP OF COLUMN LIQUID CHROMATOGRAPHY

Examples are discussed where the same particles are used for the separation of proteins on a different scale. Owing to the incompressibility of modern gel particles, the different packing density of various column heights also can be neglected. If particle types are changed during scale-up, the situation becomes complicated.

3.1. Starting material

Although it seems trivial, altering the starting material during processing can greatly change the characteristics of the system. Fermentation conditions may change for several pragmatic reasons (cheaper medium or ingredients) or the sampling of tissues or biological fluids is changed. To illustrate this problem, disintegrated *Escherichia coli* produced by different methods used in the experiment are described in Fig. 6. The composition changed dramatically.

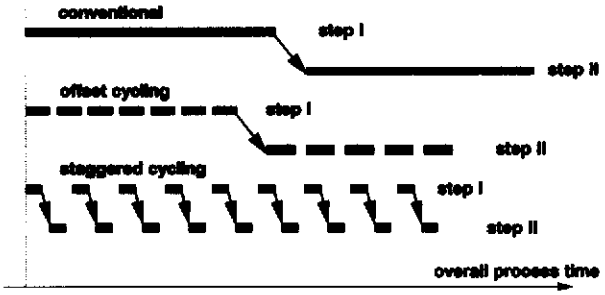

Fig. 7. Time profile of two subsequent chromatographic steps using conventional scale-up, offset cycling and staggered cycling, according to Fulton *et al.* [45].

3.2. Repeated cycles

Repeating cycles also may appear trivial unless one considers the details. When a purification procedure consists of several steps, one has to calculate carefully whether offset cycling or staggered cycling should be used (for definition, see Fig. 7). Fulton *et al.* [45] studied several cases and concluded that staggered cyclings were superior to offset cyclings. Offset cycling is simpler than staggered cycling and therefore applied more often on the laboratory scale.

One also has to take into account fouling of columns [46] and ligand leakage [47,48]. Fouling at the top of the column and ligand leakage change the peak profile. The peak becomes broader and tailing increases. For safety reasons and for obtaining reproducibly high yields, preparative chromatography is not carried out at the capacity limit in practice. Therefore, ageing of columns caused by loss of ligands cannot be detected from decreased yields. Ageing only affects the purity of the eluted biomolecule; especially non-proteinaceous contaminants will increase.

As long as the column is not clogged, backflushing after several steps is advisable to increase the column lifetime. Lifetimes of more than 1000 cycles have been reported for preparative gel filtration columns [49,50] and HIC columns [51]. The earlier a column is used in the process scheme the shorter the lifetime will be.

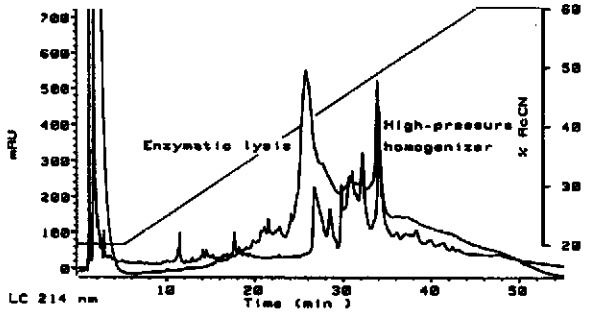

Fig. 6. Analytical reversed-phase liquid chromatography of an *E. coli* homogenate produced by enzymatic lysis with lysozyme (laboratory-scale method) and with a high-pressure homogenizer (pilot- and technical-scale method). The experimental conditions are described in ref. 44.

3.3. Column overload and multiple injections

In practice, column overload with solute molecules is the first attempt to purify more material. Loss in resolution caused by column overloading was described in detail by Gareil *et al.* [52]. In this particular situation, the shape of the isotherm plays an important role. Under isocratic conditions the throughput can be optimized [53,54] more easily than under gradient conditions [55–58]. Recently an analytical solution for volume-overloaded gradient elution chromatography was described [59]. A detailed treatment of the problem would exceed the scope of this paper, but one should bear in mind that overloading can be achieved by application of a more concentrated sample (concentration overload) or by a larger volume (volume overload).

Especially in RPLC and HIC, multiple injections are in common use. Proteins are not soluble or stable in the mobile phases such as

50% saturated ammonium sulphate or 0.1 *M* trifluoracetic acid. Multiple injections of proteins dissolved in a suitable buffer followed by mobile phase circumvent this problem. In Fig. 8A, the pilot-scale purification of recombinant single-chain antibody by means of HIC is shown using the technique of multiple injections.

3.4. Increase column size by diameter

The conventional way to scale up preparative chromatography is to increase the column diameter using the adsorption/desorption mode, if the bead size or bead size distribution is not changed from the laboratory to the preparative

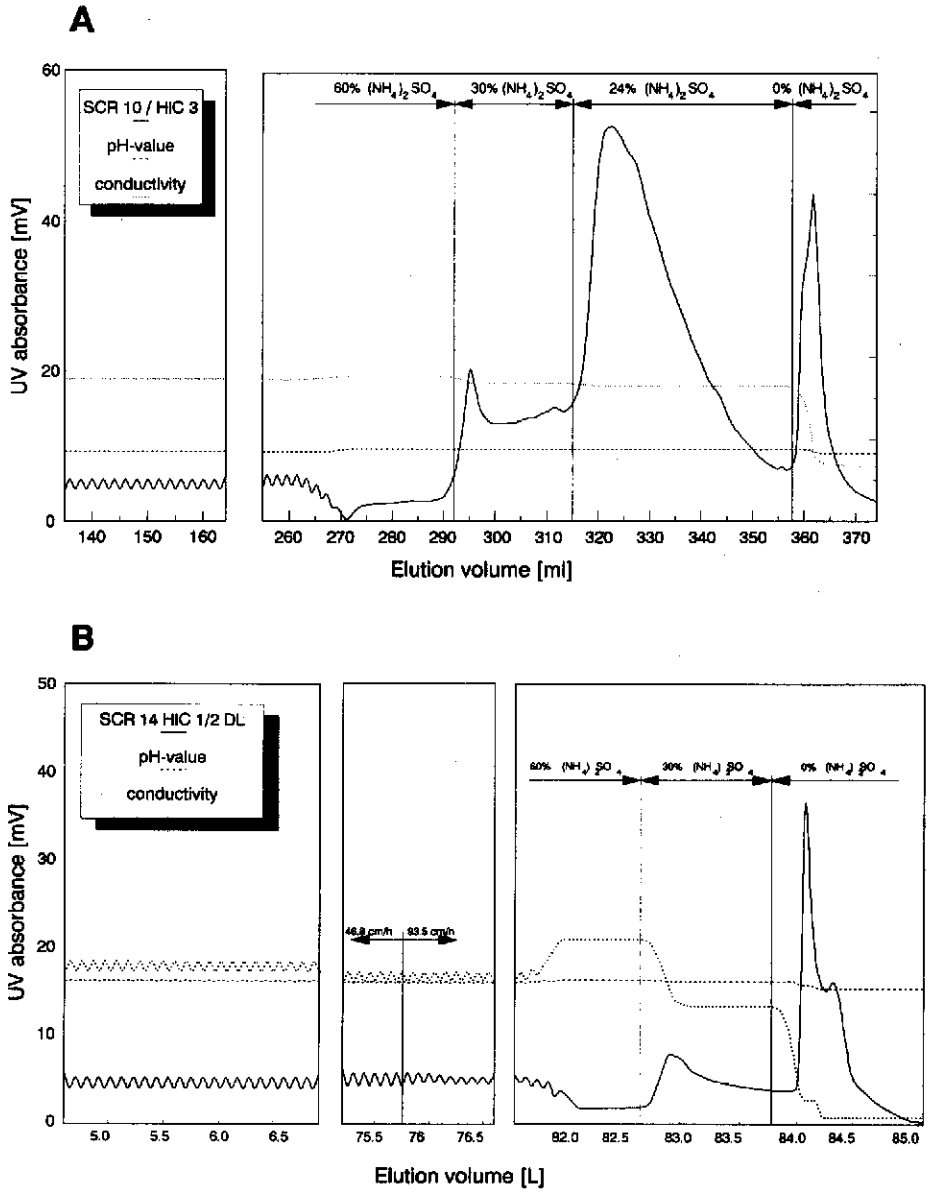

Fig. 8. Pilot-scale purification of a recombinant single-chain antibody by phenyl-Sepharose fast flow using repetitive injections of protein solution at low salt concentration followed by 60 m*M* ammonium sulphate buffer. (A) Laboratory-scale experiments; $H = 13.5$ cm and $D = 0.9$ cm. (B) Pilot-scale experiment; $H = 14$ cm and $D = 7$ cm. Solid line, UV absorbance; dashed line, pH; dotted line, conductivity.

TABLE 4

SCALE-UP OF DEAE-SEPHAROSE FAST FLOW CHROMATOGRAPHY OF HUMAN SOD FROM CLARIFIED ERYTHROCYTE LYSATE [27]

Elution was effected stepwise with 100 m*M* salt. v_t = Total column volume; *H* was kept constant for both pilot- and large-scale runs.

Run	Column volume (l)	Loading volume (l)	Peak volume (l)	Peak volume (% v_t)	Recovery (%)
Pilot 1	20	50	4.0	20	90
Pilot 2	20	57	3.8	19	92
Pilot 3	20	69	2.7	13.5	95
Large 1	159	576	26.3	17.5	89
Large 2	150	500	37.0	24.6	96

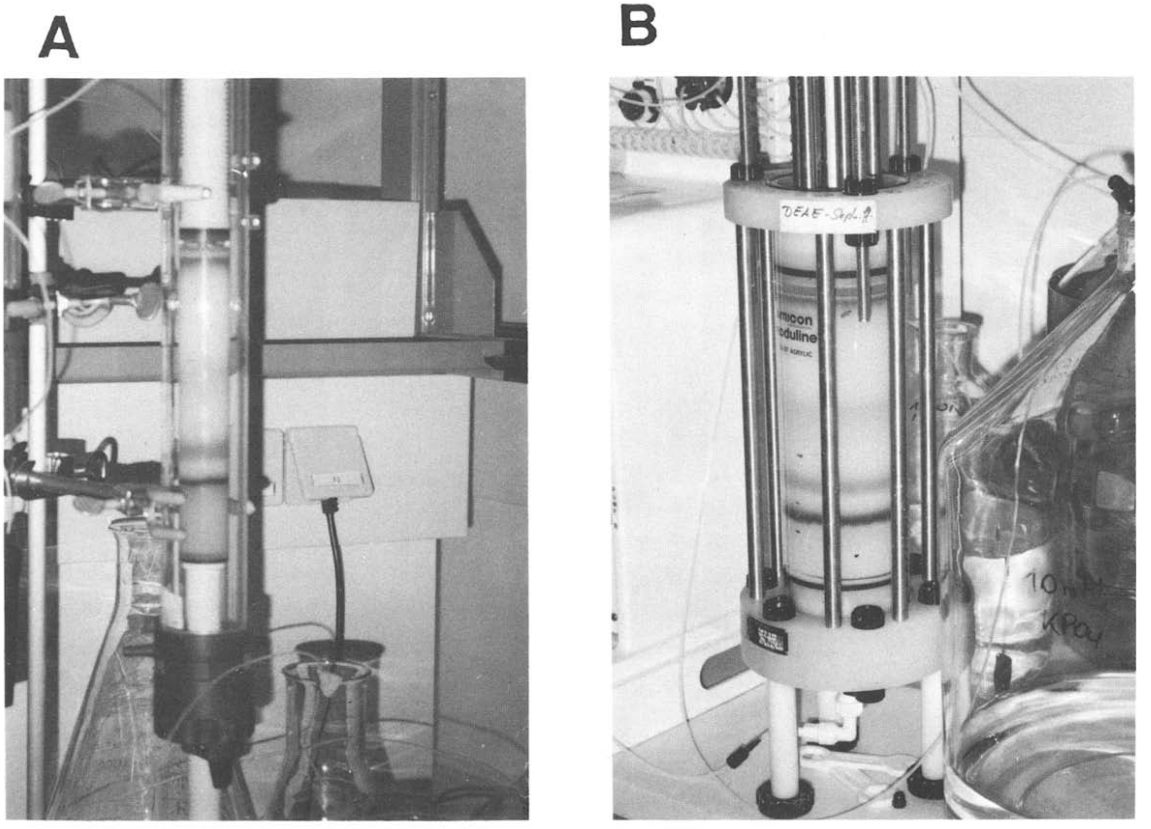

Fig. 9. Purification of SOD on the laboratory scale (18 cm × 2.6 cm I.D. and 18 cm × 9 cm I.D.). The small column was connected to an FPLC set-up and the large column to a Biopilot. A linear flow-rate of 25 cm/h was kept constant. The runs were carried out simultaneously with the same starting material. Identical patterns on the columns were observed.

scale. If different particles sizes are used on the small and large scales, additional experiments are necessary below the process size can be increased. Owing to the decrease in resolution when using larger particles or a broader distribution of particle sizes, the height also must be adjusted to the new packing material. For pilot- and industrial-scale chromatography it is necessary to optimize the resolution and height on the bench scale [60], then further scale-up is performed simply by increasing the column diameter (Table 4). In Fig. 9, scale-up by increasing the column diameter is demonstrated with a laboratory-scale and a pilot-scale column connected to an FPLC system or a Biopilot (Pharmacia Biotechnology). The resolution pattern and the peak profiles are identical with one exception: the scale.

The construction of the column inlet is an important consideration if this mode of scale-up is to be feasible. An even distribution can be achieved by nets and grids placed on the inlet adapter or by small radially arranged buffels or frit profiles [61].

IEX and AC operated with stepwise elution are very insensitive to changes in column geometry. The finding that the shape of the column has no influence on either the shape of the breakthrough curve [62,63] or the peak shape and volume indicate the use of short, wide columns to minimize the pressure drop and process time [24,64,65]. Linear gradients in conjunction with binding characteristics (Fig. 1) should be investigated more carefully prior to a change of column geometry [66]. Also, the use of the salt (often called displacer) has an influence on band broadening. The effect of the displacer ion appears to be dependent on the relative position of the ion in the lyotropic series [67,68]. The overall advantage of this mode of scale-up is a constant cycle time on all scales.

3.5. Increase column size by constant height-to-diameter ratio

In RPLC, single-point injection is commonly employed. In that event, the column size must be increased by a constant height-to-diameter ratio (H/D). In single-point injection, a certain height of a valuable packing is sacrificed for an even distribution of the sample over the cross-sectional area of the column.

Using line elution chromatography (*e.g.*, size-exclusion chromatography or frontal chromatography), an efficient scale-up is achieved by increasing the column size to a constant H/D.

In some instances, scale-up processing by just increasing the column diameter is not successful. The loss of resolution may be caused by irregularities in the bed, an unequal distribution of the sample over the gel surface or propagation of disturbances in the gel. HIC columns are more sensitive than other packings to such phenomena. Vorauer *et al.* [44] reported the purification of recombinant superoxide dismutase (SOD) as a model for scale-up of HIC with constant H/D.

The cycle time (θ_1, θ_2) increases with increasing size of the column according to

$$\theta_1/\theta_2 = H_1/H_2 \quad (6)$$

where H_1 and H_2 are the heights of the small and large column, respectively. The volumes of the columns, V_1 and V_2 change by the factor

$$V_1/V_2 = (H_1/H_2)^3 \quad (7)$$

This mode of scale-up should be avoided if at all possible.

3.6. Scale-up by dynamic similarity

Columns with constant H/d_p are called isochronic. Scale-up processing by dynamic similarity is not carried out in practice. Experiments with larger particles must be carried out on the laboratory scale to ascertain that the resolution is sufficient. For theoretical considerations and optimization of economics in a process, scale-up with constant H/d_p plays an important role. Wall effects are negligible in large-scale chromatography. On the 1–100-ml scale, wall effects influence the performance of chromatography. The larger the column, the smaller are the wall effects.

4. PRACTICAL ASPECTS OF SCALE-UP OF PROTEIN CHROMATOGRAPHY

4.1. Flow-rate, dynamic capacity

Flow-rate and dynamic capacity (defined as

adsorbed solute at a particular flow-rate) are important parameters in preparative chromatography using rigid gels, which allow a linear flow-rate up to 1500 cm/h (*e.g.*, SP-Sepharose fast flow). The mechanical stability allows a high flow-rate but the dynamic capacity decreases with increasing flow-rate. Therefore, an optimum must be found at which the column should be operated.

Conventional soft gels and rigid gels are available for a broad range of applications. The relationship between flow-rate and dynamic capacity is valid for all types listed in Table 1. Conventional soft gels continue to be used owing to their inertness. The introduction of cross-linkers also changes the surface hydrophobicity or charge of the surface of a gel particle. As a result of the enhanced mechanical stability to some extent the inertness of the gel is sacrificed.

4.2. Column packing

The packing of a soft gel with a broad distribution of particle size is very time consuming and the packing quality may differ from experiment to experiment. One rule should be obeyed: the particles must be held in motion to avoid separation of particles according to Stoke's law. Using rigid particles, the packing procedures are very simple. A slurry is poured or pumped into the column [69], then the particles are allowed to settle. Separation of particles into different sizes will not occur because the settling velocity is very high. The adapter is placed on the liquid surface and a flow is started at a higher rate than used during separation. When the gel bed does not change, the adapter is lowered on to the gel surface. For optimum distribution of the sample, the adapter should not touch the surface. This type of packing is a mixed procedure of a method called semi-constant-pressure packing and gravitational packing [70,71].

The packing quality is easily checked by a pulse response experiment, applying a small volume of salt to the column, or by the frontal curve of salt when the column is equilibrated.

4.3. Gradient formation

Reproducible gradient formation is an obvious requirement when a preparative chromatographic scheme is elaborated. For shallow gradients, one should bear in mind that continuous mixing of solutes is very imprecise at a mixing ratio of less than 1:20. If highly accurate mixing is necessary, elution buffer should be prepared beginning with 5% buffer B and elution should be completed within 95% B. The upper limit of precision was well demonstrated by Kaltenbrunner *et al.* [72]. A linear pH gradient produced with an LCC 500 gradient programmer and two P500 pumps and a 1-ml mixer varied by less than 0.1 pH/ml flow.

4.4. On-line measurement of pH and conductivity

On-line measurements of pH and conductivity are recommended, as pH and salt concentration are the most important criteria for protein chromatography. All steps can be examined quickly and accuracy is improved owing to the continuous monitoring.

4.5. Yield and productivity

The performance of different processing steps should be compared on the basis of solute productivity (P). Yamamoto and co-workers [73,74] defined productivity by

$$P = [(\text{recovery ratio})(\text{sample feed volume})]/[(\text{column volume}/\text{cycle time})]$$

Productivity has an optimum range (Fig. 10). At very high flow-rates the productivity again decreases. The recovery ratio, better known as the yield or recovery, is influenced by the operating conditions, stability of the protein, source of the protein and the presence of degrading enzymes. A general rule is that the faster the sequence of purification steps is performed, the higher is the yield. Often this rule is not too valid when considering a single step. Also, operation at 4°C does not guarantee higher recovery. Rapid oper-

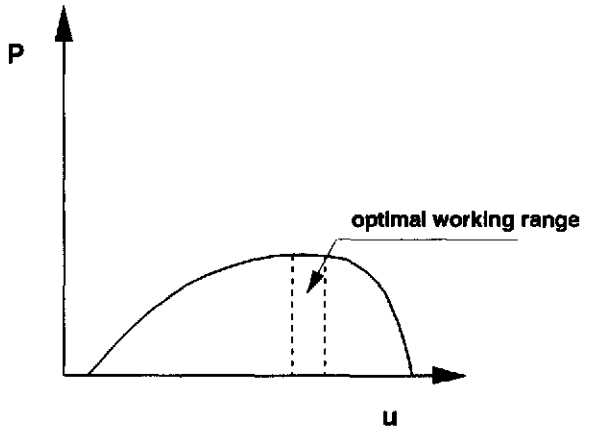

Fig. 10. Productivity *versus* velocity according to the definition by Yamamoto and Sano [73].

ation using FPLC strategies can circumvent cool room operation, however.

5. CONCLUSIONS AND FUTURE PROSPECTS

Preparative chromatography retains an indisputable position in the purification of biomolecules and the preservation of their activity. A variety of specific adsorption/desorption systems with high yield productivity and simple performance make this technique superior to others such as counter-current extraction and preparative electrophoreis. Improvements of the kinetic by the introduction of through-pores in the particle [75] or by membrane adsorption are current trends. Nevertheless, one should not forget that kinetics in preparative chromatography are not the only parameter. The overall story depends more on productivity, which includes resolution, dynamic capacity and resistance to fouling and clogging. Preparative chromatography continues to serve well "when one wants more" of precious biomolecules serving mankind.

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