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TANDEM SEPARATION SCHEMES FOR PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

ANN L. LEE, ABRAHAM W. LIAO and CSABA HORVÁTH*
Department of Chemical Engineering, Yale University, P.O. Box 2159, New Haven, CT 06520 (U.S.A.)

SUMMARY

Preparative chromatography of protein mixtures was carried out by tandem separation schemes involving frontal chromatography followed by stepwise desorption or displacement. In this way, with columns and instruments generally employed in analytical high-performance liquid chromatography, proteins could be purified in quantities similar to those typically separated by a preparative-scale system. A mixture of β-lactoglobulin A and B was loaded onto an anion-exchange column, and, in the process, a large fraction of the less-retained β-lactoglobulin B was recovered in pure form. The column was then flushed with the carrier, and subsequent desorption of the substances bound on the stationary phase was carried out by single-step desorption, two-step desorption, or displacement. With this mixture, the last two methods yielded approximately the same results in terms of the amount of product obtained per unit column volume. Whereas stepwise desorption is a simpler technique than displacement, the latter is required for the separation of components having similar adsorption behavior. In another set of experiments, a protein mixture obtained by heat treatment of human growth hormone was fractionated on a reversed-phase column. After loading the column by frontal chromatography, which separated a large fraction of the main product from the other components retained by the column, four desorption steps were applied to recover the individual components. These separation schemes offer an approach to preparative chromatography of proteins that is superior to conventional linear elution in terms of column load capacity, low mobile phase consumption, simultaneous separation and concentration, as well as enrichment of trace components.

INTRODUCTION

Over the past ten years, much progress has been made in extending the scope of high-performance liquid chromatography (HPLC) to the separation of proteins, both at the analytical and preparative levels[1]. Recently, HPLC has also found increasing employment in biotechnology as a purification step in downstream processing[2]. Preparative HPLC separations are typically carried out by linear elution, and wide experience with this mode of chromatography has facilitated method development in the laboratory and subsequent scale-up. Although linear elution chromato-

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graphy is eminently suitable for analytical work, it utilizes the stationary phase inefficiently and results in dilution of the product. As a consequence, preparative separations by elution require relatively large columns and large amounts of mobile phase.

It behooves us, therefore, to explore the potential of other chromatographic techniques, such as displacement development, frontal separation, and stepwise desorption, which, particularly when using the highly efficient columns and sophisticated instrumentation developed for HPLC, appear to be superior to elution in the preparative chromatography of proteins. The techniques are not new: stepwise desorption has been used since the dawn of chromatography; displacement and frontal chromatography were described by Tiselius as early as 1943[3].

Although such non-linear chromatographic techniques have been used in the purification of proteins by traditional chromatography[4], linear elution has been employed almost exclusively in the HPLC of proteins. The high selectivity and sensitivity to small changes in the mobile phase compositions, which characterize protein retention[5] and prompted Tiselius to put forward the "all or nothing principle"[6], however, render frontal chromatography and stepwise desorption particularly attractive for protein separation. The latter approach has also been referred to as "on off" chromatography[7]. Very recently, the practicability of displacement was demonstrated for protein separations[8], and this non-linear multicomponent separation process is likely to emerge as a powerful method for the preparative/process chromatography of proteins exhibiting similar adsorption behavior.

The present study was carried out to demonstrate the advantages of these preparative techniques in protein chromatography by using columns and instrumentation of analytical HPLC. Tandem schemes with frontal chromatography as the loading step, followed by either displacement development or stepwise desorption were employed for the separation of β-lactoglobulin A and B by ion-exchange chromatography and for the fractionation of a human growth hormone mixture by reversed-phase chromatography. The results illustrate that the approaches proposed here may offer significant gains over linear elution in terms of column load capacity, low mobile phase consumption, simultaneous separation and concentration, as well as enrichment of trace components. *Modi operandi*, based on schemes described here, are expected to displace classical elution chromatography in the process-scale separation of proteins.

EXPERIMENTAL

Instruments

The apparatus for separation of β-lactoglobulins by frontal chromatography and stepwise desorption was the same as described previously for displacement chromatography[8]. Fractions were collected every 4 min and analyzed by a liquid chromatograph, assembled from a Model 110A pump, a Model 420 controller (Beckman, Fullerton, CA, U.S.A.), a Model 7010 injector (Rheodyne, Berkeley, CA, U.S.A.) and a Model SF 770 detector (Kratos, Ramsey, U.S.A.).

The instrument for fractionation of the heat-treated human growth hormone (hGH) by frontal chromatography and stepwise desorption was based on a single metering pump (Model 302) (Gilson, Villiers Le Bel, France), and its diagram is

shown in Fig. 1. After equilibrating the column with the carrier, the feed solution was pumped into the column, and the effluent was monitored by rapid HPLC until the breakthrough of the front of interest. The three-way valve was then turned, and the lines and the column were flushed with the carrier. Subsequently, the carrier flow was directed via a four-way valve to a 10-port valve (Valco, Houston, TX, U.S.A.) having two 4-ml loops, A and B, which were filled by a syringe with the desorbents used in the first two desorption steps. The carrier was pumped through loop A to introduce desorbent I into the column, and thus, to carry out the first desorption step. Thereafter, the valve was rotated to direct the carrier flow through loop B to carry out the next desorption step with desorbent II. In the meantime, loop A was filled with the desorbent of the next desorption step, and the process was repeated. The effluent was monitored at 280 nm by a Spectroflow 773 detector (Kratos) and a Model BD-41 dual-pen strip-chart recorder (Kipp & Zonen, Delft, The Netherlands) and directed to a Universal Fraction Collector (Eldex Labs., Menlo Park, CA, U.S.A.). A Model HP 1080 liquid chromatograph with integrator (Hewlett-Packard, Avondale, PA, U.S.A.) was used for the analytical work associated with this study.

Columns

A TSK DEAE 5-PW (75 × 7.5 mm I.D., Toyo Soda, Tokyo, Japan) anion-exchange column was used for the preparative separation of β-lactoglobulins and a 30 × 2.2 mm I.D. column, packed with DEAE-silica, prepared from 5-μm Nucleosil (Machery Nagel, Düren, F.R.G.) in our laboratory, was used in the analytical work[9].

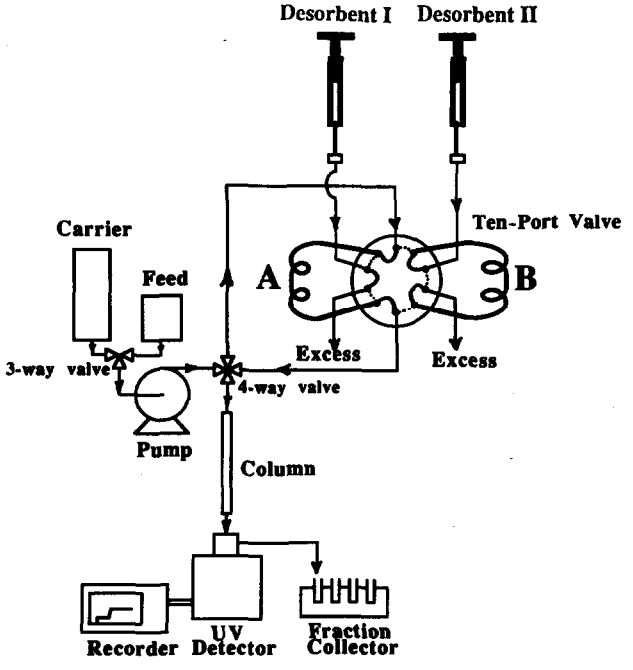

Fig. 1. Diagram of the apparatus used for frontal chromatography and stepwise desorption in the fractionation of the hGH mixture.

In the fractionation of the hGH mixture, a 200 × 1 mm I.D. column, packed with 5-μm, 300-Å, Vydac C_4 silica (The Separation Group, Hesperia, CA, U.S.A.) was used, whereas the pertinent analytical work was carried out with 250 × 2.9 mm I.D. and 50 × 2.9 mm I.D. columns, packed with the above stationary phase.

Reagents

A mixture containing equal quantities of the two proteins, β-lactoglobulin A and B (LAC A and LAC B), from bovine milk, and the chondroitin sulfate from shark cartilage, were purchased from Sigma (St. Louis, MO, U.S.A.). The heat-treated human growth hormone mixture was from Genentech, (South San Francisco, CA, U.S.A.). Reagent-grade sodium dihydrogenphosphate and trifluoroacetic acid (TFA) were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.) and HPLC-grade acetonitrile was obtained from Fisher (Pittsburgh, PA, U.S.A.). Water was deionized with a Nanopure unit (Barnstead, Boston, MA, U.S.A.).

Separation of β-lactoglobulins

The column was first equilibrated with either 5 m*M* (pH 8.0) or 25 m*M* (pH 7.0) phosphate buffer as the carrier. An equimolar solution of LAC A and B, containing 7.6 mg/ml protein in the appropriate carrier buffer, was then pumped into the column until the front of the second component, LAC A, appeared in the effluent. Thereafter, the column was flushed with the carrier.

Displacement was performed, with the exception of the feed introduction, as reported previously[8] by using 10 mg/ml chondroitin sulfate in the carrier as the displacer. The regeneration of the column was also carried out according to the same procedure. After separation of LAC B by frontal chromatography, the proteins remaining on the column were removed with 3.8 ml of 500 m*M* phosphate buffer (pH 7.0) in a single desorption step. In another procedure, two desorption steps were used with 5.6 ml of a 25 m*M* (pH 8.0) and 1.6 ml of 500 m*M* (pH 7.0) phosphate buffer as desorbents I and II, respectively. In both cases, more than 99% of the protein were recovered according to HPLC analysis of the fractions.

Fractionation of hGH mixture

The column was first equilibrated with the hydro-organic carrier: acetonitrile–water (10:90, v/v). Then, 11.5 ml of the feed solution containing 4.5 mg of the protein per ml of the carrier was loaded onto the column by frontal chromatography at a flow-rate of 0.08 ml/min. At the breakthrough of the front corresponding to the fourth peak in the analytical chromatogram of the feed in Fig. 2, the flow of feedstock into the column was halted, and subsequently, weakly sorbed components were removed by flushing the column with the carrier. In the following, the proteins bound to the stationary phase were fractionated by four successive desorption steps with hydro-organic desorbents of increasing acetonitrile concentration. The flow-rate used for the stepwise desorption was 0.10 ml/min. Finally, the column was washed with 100% acetonitrile and reequilibrated with the carrier appropriate for the next frontal analysis.

Rapid HPLC analysis of the effluent by gradient elution was aimed at finding the breakthrough of the component corresponding to peak 4, which was the first peak retained under the gradient conditions used. At a flow-rate of 2.5 ml/min, one

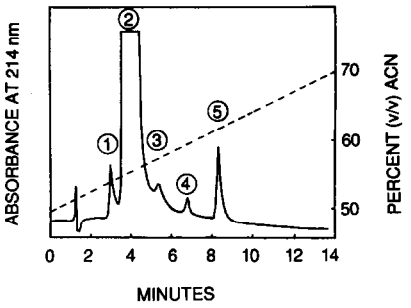

Fig. 2. Typical analytical chromatogram of the heat-treated human growth hormone mixture. Column, Vydac C_4, 5 μm, 300 Å, 250 × 2.9 mm I.D.; eluents, (A) 0.1% aqueous TFA, (B) 0.1% TFA in acetonitrile (ACN); sample, 90 μg of heat-treated hGH in 20 μl of water.

analytical run with a short column took 3 min, the time interval of fraction collection.

In order to determine the recovery of the components, first, the feedstock was analyzed by HPLC, and the weight fractions of the protein components were estimated from their peak areas, assuming that components had identical specific absorbance and ignoring non-proteinaceous substances eluted in the column void volume. Thereafter, different amounts of the feed stock were chromatographed under identical conditions, and from the measured peak areas, calibration curves were prepared for each component.

RESULTS AND DISCUSSION

Separation of β-lactoglobulins

Although high-performance displacement chromatography has been successfully used for the separation of low-molecular-weight substances[10–15], its applicability to proteins by using a single displacer was only recently demonstrated for the separation of LAC A and B[8]. In this study these proteins were also used to investigate preparative separation schemes involving frontal chromatography, flushing of the system, and subsequent stepwise desorption or displacement.

The results obtained by frontal chromatography and a single desorption step are shown in Fig. 3. The feed solution contained 3.8 mg/ml of each protein in 25 m*M* phosphate buffer (pH 7.0), and 76% of the LAC B was recovered in pure form by

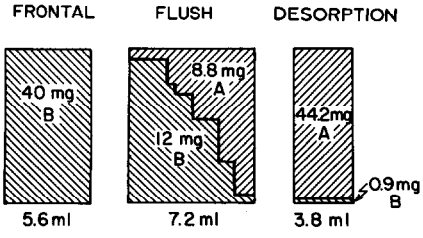

Fig. 3. Separation of β-lactoglobulin A and B by frontal chromatography and subsequent single-step desorption. Column, TSK DEAE 5-PW, 75 × 7.5 mm I.D.: carrier, 25 m*M* phosphate buffer (pH 7.0); desorbent, 0.5 *M* phosphate buffer (pH 7.0); flow-rate, 0.1 ml/min in frontal chromatography and 1 ml/min in stepwise desorption; feed, 109 mg of LAC in 14.3 ml of carrier.

frontal chromatography in a solution containing 7.1 mg/ml of protein. In the desorption step with 500 m*M* phosphate buffer (pH 7.0) as the desorbent, 83% of the LAC A was recovered at a purity of 98% in a solution containing 11.6 mg/ml of protein.

Frontal anion-exchange chromatography was a surprisingly simple method for isolating LAC B in pure form. The remarkably efficient separation suggests that under conditions of strong binding and high protein loads employed here, the adsorption of LAC B is strongly suppressed by LAC A. The theoretical basis of the phenomenon and its implications in preparative chromatography have recently been discussed[16].

However, a single desorption step did not suffice to recover LAC A in pure form as seen in Fig. 3. Therefore, another experiment with two desorption steps was carried out to remove the proteins bound to the column in the loading step by frontal chromatography with 5 m*M* phosphate buffer (pH 8.0). The results are illustrated in Fig. 4. The low ionic strength and high pH of the carrier as compared to those in the previous experiment, resulted in even stronger binding of the proteins onto the chromatographic surface and concomitantly higher load capacity of the column. In the first desorption step, with 25 m*M* phosphate (pH 8.0) as the desorbent, LAC B was eluted preferentially, albeit contaminated with LAC A. In the second desorption step, where the desorbent was 500 m*M* phosphate (pH 7.0), 61% of the LAC A was recovered in pure form in a solution containing 28.1 mg/ml of protein.

It is seen that, with the expedient of an additional desorption step, the second separation scheme yielded not only pure LAC B but pure LAC A. However, the conditions were not optimal, and some of the LAC A was lost in the first desorption step. The use of a slightly weaker desorbent in the first step may have resulted in complete separation of LAC B without any contamination by LAC A. The selection of the appropriate strength of the desorbent would be facilitated by the knowledge of the competitive adsorption isotherms of all components, however, such data are usually not available in practice.

The results of frontal chromatography, followed by displacement, are shown in Fig. 5. In this tandem scheme, LAC A and B, which remained on the column after frontal chromatography, were separated in the course of displacement. The initial conditions and the result of the frontal separation were nearly identical with those

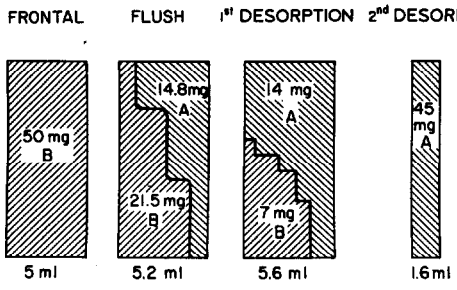

Fig. 4. Separation of β-lactoglobulin A and B by frontal chromatography, followed by two desorption steps. Column, TSK DEAE 5-PW, 75 × 7.5 mm I.D.; carrier, 5 m*M* phosphate buffer (pH 8.0); desorbent I, 25 m*M* phosphate buffer (pH 8.0); desorbent II, 500 m*M* phosphate buffer (pH 7.0); flow-rate, 0.1 ml/min in frontal chromatography and 1 ml/min in stepwise desorption; feed, 152 mg of LAC in 20 ml of carrier.

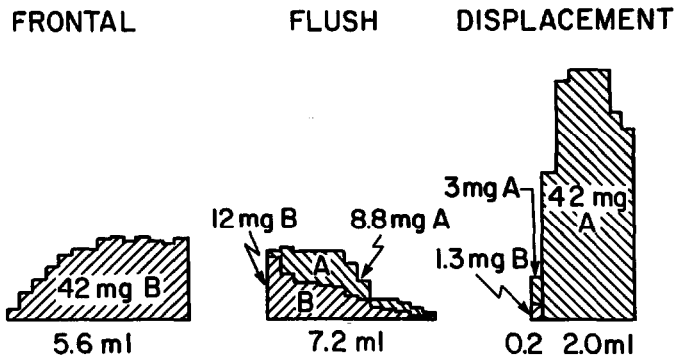

Fig. 5. Separation of β-lactoglobulins by frontal chromatography, followed by displacement. Column, TSK DEAE 5-PW, 75 × 7.5 mm I.D.; carrier, 25 m*M* phosphate buffer (pH 7.0); displacer, 10 mg/ml of chondroitin sulfate in the carrier; flow-rate, 0.1 ml/min; feed, 109 mg of LAC in 14.3 ml of carrier.

shown in Fig. 3. Displacement development resulted in a mixed band of the two proteins, followed by a band of pure LAC A, containing 21 mg/ml protein, as illustrated in Fig. 5. The front of displacer, not shown in Fig. 5, appears directly after the LAC A band.

TABLE I

RECOVERY OF THE TWO β-LACTOGLOBULINS BY THE VARIOUS SEPARATION SCHEMES, AS MEASURED BY THE AMOUNT, CONCENTRATION AND YIELD OF THE COMPONENTS IN THE FRACTIONS

The feed was 7.6 mg/ml of an equimolar mixture of LAC A and LAC B in the appropriate carrier. In the schemes described in Figs. 3 and 5, 109 mg of the feed in 14.3 ml of carrier was used, and in the scheme of Fig. 4, 156 mg of the feed in 20.5 ml of the carrier was used. The conditions are as given in Figs. 3–5.

Fractions	*LAC B*			*LAC A*		
	mg	*Conc. (mg/ml)*	*% of feed*	*mg*	*Conc. (mg/ml)*	*% of feed*
Frontal chromatography						
I, see conditions in Fig. 3	40.0	7.1	37.8	–	–	–
II; see conditions in Fig. 4	50.0	10.0	32.8	–	–	–
III; see conditions in Fig. 5	42.0	7.5	38.5	–	–	–
Single step desorption (Fig. 3)						
Flush	12.0	1.7	11.3	8.8	1.2	8.3
Desorption step	0.9*	0.2	0.8	44.2*	11.6	41.7
Two step desorption (Fig. 4)						
Flush	21.5*	4.1	14.1	14.8*	2.8	9.7
Desorption step I	7.0*	1.25	4.6	14.0*	2.5	9.2
Desorption step II	–	–	–	45.0	28.1	29.5
Displacement (Fig. 5)						
Flush	12.0*	1.7	10.9	8.8*	1.2	8.1
Displacement						
Fraction 1	1.3*	6.5	1.2	3.0*	15.0	2.7
Fraction 2	–	–	–	42.0	21.0	38.5

* Mixed fractions.

In Table I, the results obtained in the separation of LAC A and B are summarized. The recovery data show that no measurable loss of protein occurred in any of the schemes due to irreversible binding to the column. Comparison of the three methods (*cf.*, Figs. 3–5 and Table I) shows that displacement gives the best results. However, it is more time-consuming and requires an appropriate displacer as well as column regeneration. Two-step desorption is simpler and is a suitable alternative for the preparative separation of the two proteins under consideration. It is noted that the binding characteristics of the column were altered slightly by repeated displacement and frontal chromatography, including rather harsh regeneration procedures in a period of over six months[8].

It is revealing to compare our results with those reported for the separation of LAC A and LAC B by conventional elution chromatography. According to one report[17], the protein mixture was separated by using a 100 × 18 mm I.D. column, packed with DEAE Selectacel, Type 40, and a linear gradient from 50 m*M* phosphate (pH 5.8) to 80 m*M* sodium chloride in the same buffer. The amount of feed was 8.25 mg/ml of column volume. Furthermore, over 500 ml of mobile phase was required for elution, resulting in inevitable dilution of the products. In another example[8], elution chromatography of the LAC proteins on the same TSK DEAE column used here resulted in a maximum sample load of 6 mg of protein per ml of column volume under isocratic conditions with 130 m*M* phosphate buffer (pH 7.0) and 10 mg of protein per ml of column volume by gradient elution from 25 to 263 m*M* phosphate buffer (pH 7.0). In contrast, the tandem schemes described here resulted in the separation of 33 mg (Figs. 3 and 5) and 46 mg (Fig. 4) of protein per ml of column volume. In each case, the products were recovered at a concentration higher than the feed concentration. Comparison of the results in the literature to those presented here suggests that the tandem schemes involving frontal chromatography, followed by stepwise desorption or displacement development, are superior to conventional elution chromatography in terms of throughput and product concentration.

Fractionation of hGH mixture

Human growth hormone had been subjected to heat treatment; the components of the protein mixture thus obtained are referred to by the corresponding peak numbers in the analytical chromatogram of the feed mixture in Fig 2. Our goal was to evaluate the expediency of frontal chromatography with subsequent stepwise desorption for fractionation of the mixture, in general, and to isolate the substance corresponding to peak 5, which was tentatively thought to be a dimer, in particular. We shall show that by this approach, tens of milligrams of "dimer" can be isolated with columns and equipment typically used in analytical HPLC.

Reversed-phase[18–22], ion-exchange[23] and size-exclusion[24] chromatography have been used to separate the variants of hGH, a 22-kilodalton (kD) hydrophobic protein. In our study, reversed-phase chromatography was found to offer convenient operation and high selectivity for the separation of the protein mixture obtained by heat treatment of hGH. The separation of the least-retained components, represented by peaks 1–3 in Fig. 2, was not an aim of this investigation, and their mixture was considered as the main product. Preliminary results indicated that frontal chromatography would be an effective means of isolating the main product from the mixture, while concomitantly loading the column with the more retained substances, repre-

sented by peaks 4 and 5 as well as "higher aggregates", not seen in the chromatogram in Fig. 2. After completion of frontal chromatography, they were selectively desorbed from the column in a series of desorption steps. As a first approximation, the composition of the desorbents used in these steps was taken as the mobile phase composition at which the peaks emerged from the column in the analytical gradient elution chromatogram. Subsequently, the organic modifier contents of the desorbents were adjusted by trial-and-error to establish the four-step desorption scheme, presented in Fig. 6. This was necessary because mutual interference by the components at high-load conditions differs from that at low-load conditions. In fact, peak 2 was eluted at 55% acetonitrile in the analytical run by gradient elution, but stepwise desorption with 45% acetonitrile was sufficient to remove the substance bound to the stationary phase under conditions of high load. Generally, stepwise desorption of a component required slightly lower concentration of the mobile phase modifier than that at which it was eluted from the column by gradient elution. It should be noted that the mobile phase contained 0.1% TFA in analytical chromatography, but not in preparative chromatography.

The individual fractions obtained in this separation scheme (see Fig. 6) were analyzed by HPLC and their chromatograms, together with that of the feed mixture, are shown in Fig. 7. The purity of the protein (peak 4) obtained by the second desorption step was 82%, although the elution scheme was not optimized for this product. The desired product, the compound in peak 5, was recovered in the third desorption step with a purity in excess of 99%, according to HPLC analysis under the conditions shown in Figs. 2 and 7. However, rechromatography of the "pure" product under isocratic conditions yielded two partially resolved peaks, probably

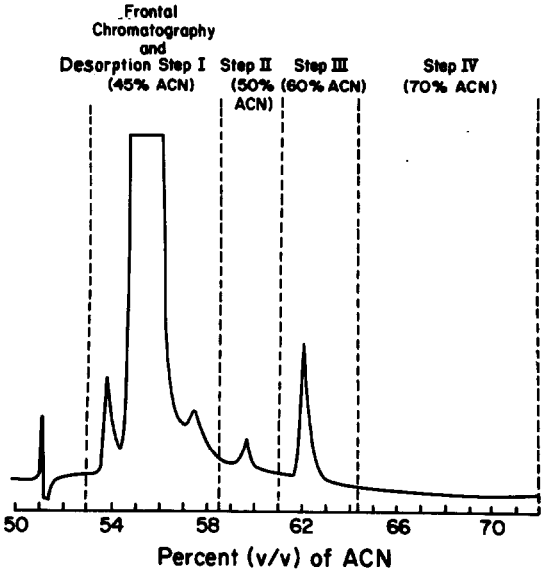

Fig. 6. Illustration of the separation steps and fractions recovered in the chromatography of the hGH mixture in conjunction with an analytical chromatogram, obtained under conditions given in Fig. 2. The horizontal scale represents the eluent composition. Acetonitrile (ACN) concentrations of the aqueous desorbents in the four desorption steps are shown at the top.

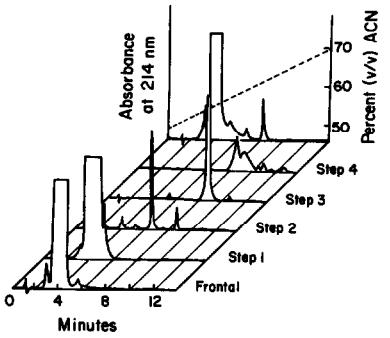

Fig. 7. Chromatograms of the hGH fractions. Conditions as in Fig. 2.

representing different forms of the dimer[25]. Trace components of the feed, which are not seen in the analytical chromatogram, were also recovered in concentrated form in the fourth desorption step as a fraction called "higher aggregates".

Table II presents an overview of the results obtained by the purification scheme: frontal chromatography *cum* stepwise desorption. The experiments were performed with a 200 × 1 mm I.D. column, having an empty-column volume of 0.16 ml, in order to keep protein consumption at a low level. The amounts of the various products recovered were first normalized for unit column volume, and for the sake of illustration, recalculated for a standard 250 × 4.6 mm I.D. analytical column, having an empty-tube volume of 4.15 ml. It can be seen that the separation scheme presented here would allow processing greater-than-gram quantities of the protein mixture by means of an analytical HPLC unit. The time estimated for the process described in Table II, assuming identical flow velocities as those used in the experiment, is less than 4 h. As shown in Table II, frontal chromatography resulted in a slight enrichment of the main product[26] and the concentrations of the other components in the fractions recovered by the subsequent desorption steps were higher

TABLE II

FRACTIONATION OF THE PROTEIN MIXTURE, OBTAINED BY HEAT TREATMENT OF HUMAN GROWTH HORMONE

The data are calculated for a 250 × 4.6 mm I.D. butyl-silica column. Feed, 1364 mg of protein in 303 ml of acetonitrile–water (10:90, v/v). Separation steps as in Fig. 6.

Separation step	*Fraction*	*Amount (mg)*	*Fraction volume (ml)*	*Recovered conc. (mg/ml)*	*Feed conc. (mg/ml)*
Frontal	Peaks 1–3	1158.0	237.9	4.8	4.35
Desorption step I	Peaks 1–3	141.9	18.8	7.5	4.35
Desorption step II	Peak 4	9.5	10.1	0.94	0.033
Desorption step III	Peak 5	42.1	3.0	14.03	0.15
Desorption step IV	"Higher aggregates"	1.4	2.0	0.7	–

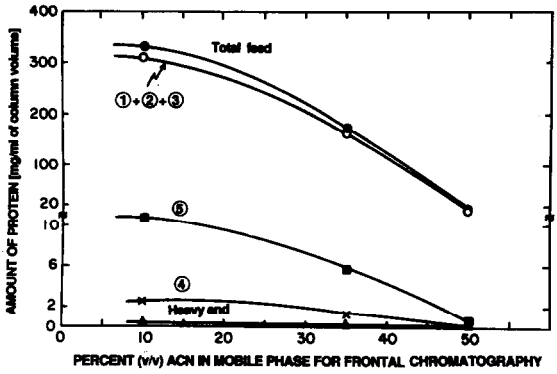

Fig. 8. Column loading by frontal chromatography and the amount of the components recovered in the ensuing separation steps as a function of the initial organic solvent concentration in the carrier. The amounts are given per unit column volume.

than in the feed. In the particular case of the fraction containing peak 5, the product was concentrated almost 100-fold.

With increasing acetonitrile concentration in the mobile phase, less protein was adsorbed on the column in the frontal step and, as a consequence, lower amounts of the components were recovered, as shown in Fig. 8. This is in agreement with the theoretical predictions that in this kind of chromatography high separation efficiency is obtained when the feed components are strongly bound to the stationary phase, *e.g.*, the concentration of the organic solvent in reversed-phase chromatography is low[16]. However, it must be kept in mind that, with proteins, strong binding may result in conformational changes and loss of recovery. In our study, recovery of the proteins was in excess of 95%; however, possible conformational changes due to the chromatographic process are yet to be investigated.

Our results compare favorably to those reported[27] for the purification of crude hGH with a 20 × 5.5 cm I.D. anion-exchange column and a linear salt gradient at 30 ml/min for 180 min. The maximum sample load in the linear elution mode was 240 mg, so that the amount of feed separated per ml of column volume was 0.51 mg, as compared to 340 mg in the work presented here. Furthermore, the gradient volume was almost 5.5 l, and the products were recovered in solutions much more dilute than those in the present work. Although different chromatographic systems and feed compositions were used, comparison of the data suggests that frontal chromatography with subsequent stepwise desorption offers significant advantages over linear elution chromatography.

Comparison of separation schemes

Frontal chromatography alone is a useful binary separation process when the desired product is the least-retained component of the feed and its binding to the stationary phase is significantly weaker than that of the other component. In such a case, a large amount of feed can be processed and pure product withdrawn in one step before the more retained component breaks through. Alternatively, in the preparative separation of the more strongly retained feed components, frontal chromatography offers an efficient method for loading them onto the column, with the

added benefit of recovering a fraction of the first component in pure form. The other feed components that are adsorbed on the stationary phase in the process are subsequently separated by stepwise desorption or gradient elution or displacement procedures. If the separation factors are small, displacement development is the method of choice, since this multicomponent technique is best suited to the separation of closely related substances. If the separation factors are large, gradient elution or stepwise desorption is more effective. In industrial processes, stepwise desorption is preferred because of the simplicity of this operation in comparison to gradient elution.

As mentioned before, retention data from linear elution chromatography may not be useful in choosing the appropriate preparation scheme. This is because the adsorption behavior of the proteins at high concentrations is strongly influenced by interference between the feed components and may be quite different from that observed at low concentration. Further studies are required to gain understanding of these phenomena and to establish guidelines for the selection of the most suitable operating conditions and the compositions of the carrier, the desorbents and the displacer.

The techniques described in this report seemingly depart from the widespread notion of chromatography as a differential migration process that, in the strictest sense, encompasses only bona fide elution under isocratic or gradient conditions. Displacement, however, also involves differential migration of the components until the final pattern is reached. Since the front velocities of the components are different in frontal chromatography, this technique entails features of differential migration as well. Stepwise desorption is only quantitatively different from gradient elution and is often referred to as "stepwise gradient". These are sufficient reasons for considering such techniques as chromatography, notwithstanding the other terminology occasionally used for them in the field of adsorption technology[28].

There is a significant difference between elution or displacement chromatography on the one hand, and stepwise desorption or frontal chromatography on the other. The latter two techniques, which are the typical operational modes also in affinity chromatography, can be considered as single-stage processes and as such may not qualify as genuine chromatographic methods. Yet, the employment of sorbents and mobile phases that are germane to chromatography as well as the use of mobile phase flow through the column to bring about the separation of a mixture warrants the term chromatography. In the design of non-linear chromatographic techniques for the large-scale purification of proteins, both isotherm data on competitive adsorption behavior and the tools of chemical engineering will be employed. Thus, with the growing utilization of chromatography in industrial settings, a new discipline, "chromatographic engineering", may emerge.

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