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SEPARATION OF PROTEINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

II. OPTIMIZING SAMPLE PRETREATMENT AND MOBILE PHASE CONDITIONS

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SUMMARY

The effects of separation variables such as temperature, pH and composition of the mobile phase (including additives such as chaotropes, ion-pairing agents and surfactants), sample size and sample pretreatment for reversed-phase high-performance liquid chromatography (RP-HPLC) of proteins is examined. Experimental optimization of these parameters using the preferred instrumental and column conditions described previously[1] lead to well behaved chromatographic performance for most proteins. This allowed us to achieve the required level of performance for the first dimension (RP-HPLC) separation of most protein samples by the chromatophoresis process.

INTRODUCTION

The preceding paper[1] has described the goals of the present study. These can be summarized as (a) the determination of optimal conditions for the separation of most protein samples by means of reversed-phase (RP) gradient-elution high-performance liquid chromatography (HPLC), and (b) the application of these findings to the special requirements of the chromatophoresis process. The role of the column, the HPLC system and extra-column effects in maximizing resolution and minimizing separation problems were examined[1]. In this paper we will consider other experimental variables: (a) mobile phase conditions such as temperature, pH, choice of solvents, buffers, additives, flow-rate, gradient program, etc., and (b) sample pretreatment.

BACKGROUND

Problems associated with the reversed-phase separation of proteins are: (a) excessive band broadening (compared to theoretical predictions for "ideal" separa-

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tion), (b) tailing or misshaped bands, (c) low recoveries, (d) ghosting (carryover from a previous injection) and (e) the appearance of one protein in two or more distinct bands. Two major contributions to these problems are[1]: (a) secondary-retention effects and (b) changes in protein conformation during separation. Secondary-retention effects are caused by the stationary phase or column and have already been discussed[1]. Problems that arise from changes in protein conformation have been extensively documented and several remedies have been suggested (as noted below).

Protein conformation and separation

The general problem under consideration arises from the possible existence of different protein conformers. In the simplest case this may be the native protein and the completely denatured (random coil) molecule; however conformations of intermediate structure are also possible[2]. Reversed-phase systems are inherently denaturing; the retention of the solute molecule on the hydrophobic surface of the column packing favors a rearrangement of the protein conformation so as to expose its (originally internalized) hydrophobic residues for maximum interaction with the hydrophobic surface. Likewise, the presence of organic solvents in the mobile phase further favors protein denaturation.

There has been considerable debate as to the relative roles of the mobile and stationary phases in promoting protein denaturation, but there is little disagreement that their combined effects generally result in solute denaturation during reversed-phase separation. Often (at least in the case of peptides and small proteins) the denatured molecule spontaneously refolds to the native structure following HPLC separation and a change in protein environment (replacement of the aqueous–organic mobile phase by an aqueous buffer). Such short-lived changes in protein conformation during reversed-phase separation may be described as transient denaturation.

The existence of the protein molecule in either one conformation or another (*e.g.*, native or transiently denatured) has little effect on bandwidth or peak capacity. That is, effective chromatography is possible as long as the protein remains in a single (discrete) conformation. This is evidenced by the fact that acceptable protein separations are commonly observed when either ion-exchange (non-denaturing) or reversed-phase (denaturing) HPLC is used. Problems due to changing protein conformation arise mainly because of the existence of different species during the separation. This is analogous to reaction of a small molecule during separation, where the parent compound and various reaction products are partially separated.

An early example of changing protein conformation during RP-HPLC separation was provided by Cohen *et al.*[3]. They showed that ribonuclease gave narrow, well shaped bands at 37°C, while band shape progressively deteriorated as the temperature was lowered (Fig. 1). In this case, it could be demonstrated[3,4] that the protein is fully (but reversibly) denatured at 37°C but is in slow equilibrium with its native conformation below this temperature.

A quantitative mathematical description of the consequences of sample reaction (including conformational changes) during separation has been presented by Melander *et al.*[5]. The application of these principles to the multiple conformational states of peptides and proteins has been described in detail by other workers[2,4,6,7]. The practical conclusion from these various theoretical and experimental studies is that denaturing conditions will generally favor "ideal" RP-HPLC and minimize the

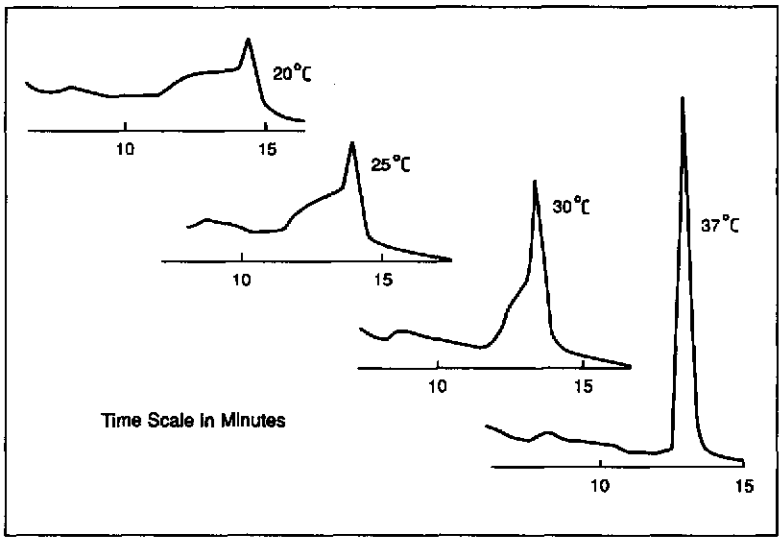

Fig. 1. Changes in band shape *vs.* temperature for ribonuclease in RP-HPLC. Propanol–water gradient, 300-Å pore, C_4 column. Reprinted from ref. 3 with permission.

separation problems noted above. The reason is that most proteins will exhibit some degree of transient denaturation during reversed-phase separation; if this is the case, then the complete denaturation of each protein to its random-coil conformation is the most promising approach for obtaining a single molecular species for each compound as it moves through the column.

Other separation artifacts

It is clear that the slow interconversion of protein conformers can lead to broad and/or misshaped bands as in Fig. 1. If the formation of two or more conformers occurs initially (*e.g.*, following injection), and further change in conformation during separation is slow, two or more separate bands will be observed (as has been reported by Cohen *et al.*[8]).

The underlying causes of low sample recoveries and ghosting are somewhat more complex in their origins, but we believe that these problems are also related to changes in protein conformation, *i.e.* due to on-column denaturation in the highly hydrophobic environment within the pores of the packing. Under the conditions of sample injection (including high concentrations of the protein on the column-packing surface), it is probable that more than one conformation will be present for many proteins (*i.e.* other than random coil). The native protein is often more water-soluble, because hydrophobic groups are concentrated within the interior of the protein molecule, while (external) hydrophilic groups can interact strongly with the aqueous environment. During denaturation of the protein, these various groups are free to rearrange themselves and to interact with corresponding groups on adjacent protein molecules. This leads to aggregation and precipitation of the protein.

Precipitated aggregates are highly insoluble and are probably irreversibly bound to the column-packing surface, resulting in lower protein recoveries (and de-

creased column life). It is also conceivable that in some cases these aggregates partially dissociate into more soluble, less interactive random-coil species during the latter part of the separation, so that in the next gradient the released molecules chromatograph normally. This would explain ghosting effects.

Some denaturing reagents (*e.g.*, chaotropic agents, detergents) encourage protein solubilization. This suggests that such conditions will reduce ghosting, favor sample recoveries and promote narrow, well-shaped elution bands.

Conditions favoring protein denaturation and "ideal" RP-HPLC

Protein denaturation is favored by a number of different factors, summarized in Table I. These conditions can be applied prior to injection of the sample (sample pretreatment) and/or during separation (*e.g.*, via additions to the mobile phase). If sample pretreatment is used, the sample must be held under denaturing conditions long enough to allow denaturation to proceed to completion. If denaturing conditions are used during the separation, the sample must be denatured prior to injection (sample pretreatment). If not, the denaturing conditions during separation must then favor rapid denaturation of the sample (before migration of the sample through the column).

Conditions that favor RP-HPLC separation of proteins were first discovered in the 1970s, *e.g.*, low pH, ambient or higher temperatures and the use of acetonitrile or propanol as organic solvents[12]. It should be noted that these conditions generally favor protein denaturation. Several workers have reported on the selective use of one or more of these denaturing conditions as summarized in Table I. This paper details our efforts to systematically evaluate all of the options of Table I for sample pretreatment and mobile phase modification in an attempt to achieve our goals for good chromatographic behavior of most proteins by RP-HPLC.

TABLE I

CONDITIONS THAT FAVOR PROTEIN DENATURATION AND GOOD CHROMATOGRAPHY IN RP-HPLC

Variable	*Application in HPLC*	*Ref.*
Higher temperature	Ribonuclease	2
	Papain	8
	Synthetic peptide	9
	Collagen	10
Low pH (< 3)	Papain	8
	Cytochrome *c* variants	11
Organic solvent	2-Propanol	12,13
	Sulfolane	14
Chaotropic agents	Bovine serum albumin*	15
	Glycopeptide	16
Surfactants	Various proteins	17
Ion-pairing reagents	Various proteins	12

* Used in sample pretreatment.

EXPERIMENTAL

Apparatus

The instrumentation used in these studies is described in ref. 1.

Columns

PLRP-S polymeric HPLC packing-material (5-μm diameter, 300-Å pores) was obtained from Polymer Labs. (Church Stretton, U.K.). The microbore column hardware was obtained from Upchurch Scientific (Oak Harbor, WA, U.S.A.). All HPLC columns were packed in our laboratory.

Reagents

Protein standards used were obtained from Sigma (St. Louis, MO, U.S.A.). HPLC grade acetonitrile, 2-propanol, tetrahydrofuran and water were obtained from EM Science, (Cherry Hill, NJ, U.S.A.). Phosphoric acid, formic acid and dioxane were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Trifluoroacetic acid (TFA), triethylamine, heptafluorobutyric acid (HFBA) and dithiothreitol (DTT) were obtained from Pierce (Rockford, IL, U.S.A.). β-Mercaptoethanol (BME) and urea were obtained from BioRad, (Richmond, CA, U.S.A.). Sulfolane, butanol, pentanol, hexanol, heptanol and octanol were obtained from Aldrich (Milwaukee, WS, U.S.A.) Physiological buffers [2-(N-morpholino)ethanesulfonic acid (MES) and 3-(cyclohexylamino)propanesulfonic acid (CAPS)] and detergents [sodium dodecyl sulfate (SDS), cetyl trimethyl ammonium bromide (CTAB), Triton X-100, *n*-octyl glucoside and Zwittergen C8, C10, C12, C14, C16] were obtained from CalBiochem Behring (La Jolla, CA, U.S.A.).

Echerichia coli preparation

E. coli was grown on Difco nutrient broth at 37°C to early stationary phase growth. Cells were harvested by centrifugation, washed once with 10 m*M* Tris–HCl (pH 7.5) and broken by grinding with alumina at 4°C. Cellular debris was removed by centrifugation, and the supernatant was used in this study without further purification.

Gel electrophoresis

The second dimension of the chromatophoresis separation employed a polyacrylamide gel with a single buffer chemistry as suggested[18] for the electrophoresis of SDS–protein complexes. The pH of the gel buffer is adjusted so that SDS–protein complexes migrate as a stack in the 4-cm high, non-restrictive 6%T/5%C_{bis} gel at the top of the slab. They subsequently unstack during migration through a 12-cm high, 12%T to 23%T exponential gradient gel. A number of different buffer compositions have been tried. Results reported here were obtained with 0.6 *M* Tris–lactate (pH 8.8). The catholyte solution was 0.036 *M* Tris–0.11 *M* glycine–0.03% SDS, and the anolyte solution was 25 m*M* Tris–HCl (pH 8.1). The power supply was set at a constant voltage of 300 V and the catholyte and anolyte chambers were maintained at 8°C.

RESULTS AND DISCUSSION

After optimization of the HPLC column and system hardware, as detailed in ref. 1, it became obvious that major improvements in the RP-HPLC separation were still required. The recovery and band characteristics of several proteins (Table II of ref. 1) were still unacceptable. This prompted a matrix investigation of a number of variables associated with sample pretreatment, mobile phase composition and gradient conditions. As mentioned in the ref. 1, it was important to check each variable under optimal conditions for the remaining variables in the matrix. This was done for all 33 proteins, after each variable was approximately optimized in earlier studies with a smaller number of proteins.

In the case of several experimental variables (Table II and Table III), the same condition (*e.g.*, added chaotrope) could be used in both the pretreatment and separation steps. We initially assumed that denaturation during sample pretreatment (*e.g.*

TABLE II

SUMMARY OF THE PARAMETERS INVESTIGATED RELATIVE TO SAMPLE PRETREATMENT AND AN OVERVIEW OF THE RESULTS OBTAINED

(O) No effect compared to consensus conditions, (−) negative effect compared to consensus conditions, (+) positive effect compared to consensus conditions.

Variable	*Parameter*	*Bandwidth*	*Tails*	*Recovery*	*Ghosting*	*High mol.wt.*	*Hydrophobic*
Temperature (°C)	5	O	O	O	+	+	+
	25	O	O	O	O	O	O
	60	−	O	−	−	−	−
	90	−	−	−	−	−	−
pH	2	+	+	+	+	+	+
	6	−	−	−	−	−	−
	10	−	−	−	−	−	−
Ion-pairing acid	Phosporic acid	−	−	+	+	−	+
	TFA	+	+	O	O	+	O
	HFBA	O	O	−	−	O	−
	Formic acid	−	O	−	−	+	+
Organic solvent	Acetonitrile (10%)	+	O	+	O	+	+
	2-Propanol (10%)	O	O	O	O	O	+
	Sulfolane (10%)	−	−	−	−	O	+
Detergent	Anionic	−	−	−	−	−	−
	Cationic	−	−	−	−	−	−
	Zwitterionic	O	+	O	O	+	+
	Non-ionic	O	O	O	O	O	O
Chaotrope	Urea	+	O	+	+	+	+
	Guanidine	+	O	+	+	+	+
Reducing agent	BME	−	−	−	O	−	−
	DTT	−	−	−	O	−	−
Injection	Optimum size	+	+	+	+	+	+
	Optimim volume	+	+	+	+	+	+

by urea) might require addition of urea to the mobile phase to maintain denaturation. In these cases, we studied the effect of the variable both individually and in combination, *e.g.*, use of urea in the pretreatment step and addition of urea to the mobile phase. However, as discussed in following sections, a variable that was important in the pretreatment step was sometimes of no importance in the separation step.

Table II presents a summary of our sample pretreatment study. Table III shows a similar summary of mobile phase optimization, along with information on the range of parameters investigated plus a qualitative summary of their effect on the final chromatogram. The assessment of a positive (+) or negative (−) effect of a particular parameter was based on the average result for all of the indicator proteins.

TABLE III
SUMMARY OF THE PARAMETERS INVESTIGATED RELATIVE TO THE MOBILE PHASE, AND AN OVERVIEW OF THE RESULTS OBTAINED

Symbols as in Table II.

Variable	*Parameter*	*Band-width*	*Tails*	*Recovery*	*Ghost-ing*	*High mol.wt.*	*Hydro-phobic*
Temperature (°C)	20	−	−	−	−	−	−
	40	○	○	○	○	○	○
	60	+	+	+	+	+	+
	80	−	○	−	○	○	○
pH	2	+	+	+	+	+	+
	6	−	−	−	−	−	−
	10	−	−	−	−	−	−
Ion-paring acid	Phosphoric acid	−	−	+	+	−	+
	TFA	+	+	○	○	+	○
	HFBA	○	○	−	−	○	−
	Formic acid	−	○	−	−	+	+
Organic solvent	Acetonitrile	+	+	+	+	+	+
	2-Propanol	○	○	○	○	○	+
	Tetrahydrofuran	−	○	−	○	−	○
	Dioxane	−	○	−	○	−	−
	Sulfolane	−	−	−	−	−	−
	C_4–C_8 alcohols	+	○	○	○	+	+
Detergent	Anionic	−	−	−	−	−	−
	Cationic	−	−	−	−	−	−
	Zwitterionic	○	+	○	○	+	+
	Non-ionic	○	○	○	○	○	○
Chaotrope	Urea	○	○	+	○	+	+
	Guanidine	○	○	○	○	○	○
Reducing agent	BME	−	−	−	○	−	−
	DTT	−	−	−	○	−	−
Gradient	Increasing time	−	○	−	○	−	−
	Increasing range	+	○	−	−	○	○
	Increasing delay volume	−	○	−	○	−	−
	Increasing re-equilibration	+	○	+	○	○	+

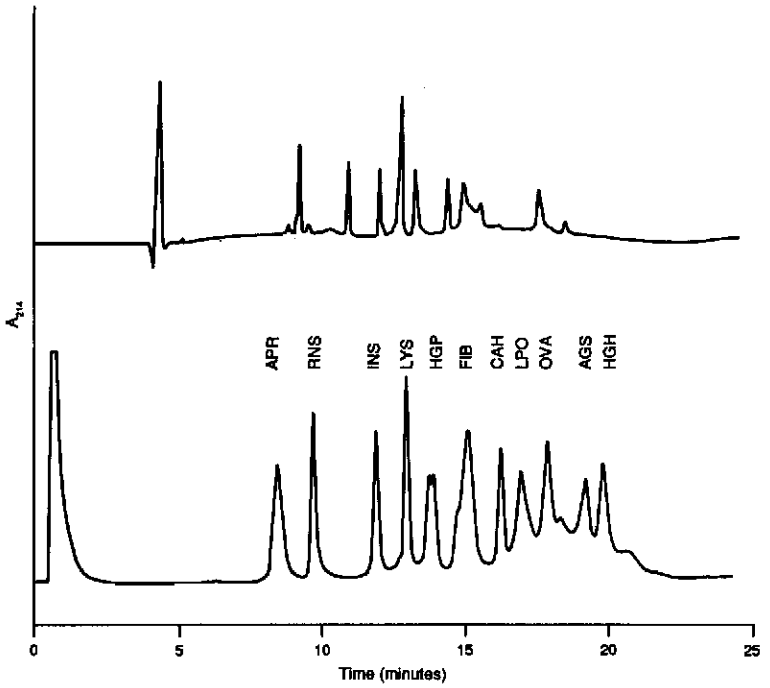

Fig. 2. Comparison of separations obtained for the indicator protein mixture from "consensus" conditions (top) to the final "optimized" conditions (bottom). The sample (abbreviations) and conditions have been described previously[1].

Therefore, the recommended final conditions may not be optimal for any specific protein. The results of these optimization experiments are detailed below and illustrated by the chromatograms in Fig. 2; these show "before and after" results for "consensus" and "optimized" conditions as determined in this study (Parts I and II).

Temperature

Sample pretreatment. Since it is well known that higher temperatures generally favor protein denaturation, this was the first variable we explored. Samples were heated to 60°C or 90°C for 2–5 min prior to analysis, in the presence of various additives, as summarized in Table II. Heated samples showed significant losses in chromatographic performance (mainly multiple peaks and lower recoveries) when compared to samples incubated at lower temperatures. Samples held at room temperature showed small, variable losses over 24 h, so storage at or below 5°C is recommended for maximum recovery. These results are summarized in Table II.

HPLC separation. Temperature affected the HPLC separation and sample pretreatment differently. Although specific proteins showed individual patterns, average recovery increased as the column temperature was increased from 20 to 60°C; this is summarized in Fig. 3. The increase in temperature had the added benefit of decreasing bandwiths and lowering backpressure without any adverse affects on the polymeric packing (stable up to 150°C). Under the same conditions (60°C), silica-based packings suffered a severe loss in performance after fewer than ten injections. Other observations regarding separation temperature are listed in Table III.

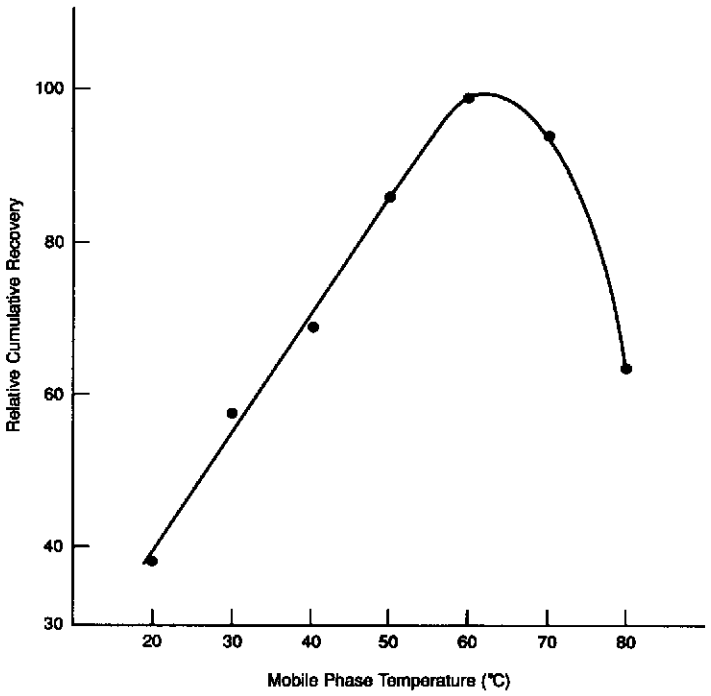

Fig. 3. Plot of average recovery (peak areas) for all test proteins *vs.* mobile phase temperature.

pH

Most RP-HPLC separations are performed on silica-based packings, limiting the useable pH range to 2–7.5. Our selection of a polymer-based packing for these separations extended the pH range to 1–14. Therefore, the effect of pH over the pH range 2–10 was examined for both sample pretreatment and the mobile phase (Tables II and III). Two separate sets of conditions were examined to test the effect of pH: (a) with physiological buffers to cover the pH range 2–10, and (b) with 0.1% TFA titrated with triethylamine to adjust the pH to above 2 (this choice of experiments was intended to distinguish between pH and ion-pairing effects). Although there were a few exceptions, the overall effect for all test proteins was a dramatic loss in chromatographic performance with increasing pH. It appears that the current use of ion-pairing acids at pH 2 provides an optimal state of denaturation for most proteins in RP-HPLC. Since this effect was observed for polymeric packings, it appears that sample denaturation is involved, rather than suppression of silanol interactions (as is assumed for silica-based columns).

Ion-pairing acids

TFA has long been recognized as a useful mobile phase additive for the RP-HPLC of peptides and proteins[12], not only for its low pH and protein-denaturing properties, but also because of its action as an ion-pairing agent. Guo *et al.*[19] have recently showed that varying the concentrations of TFA and HFBA leads to predictable changes in sample retention, with more positively charged peptides exhibiting

relatively increased retention as the concentrations of TFA and especially HFBA are increased. We therefore anticipated that the concentration of TFA in the mobile phase might be an important variable.

The effects of several ion-pairing acids as both sample and mobile phase additives were tested (Tables II and III). Phosphoric acid, widely used in the RP-HPLC of proteins (and the weakest ion-pairing agent tested), gave maximal recoveries for the more hydrophobic proteins but often caused significant band broadening or multiple peaks for other (less hydrophobic) proteins. For most proteins, TFA appeared to be the best mobile phase acid. It exhibited intermediate ion-pairing properties, which gave the best balance between minimizing average bandwiths and reducing losses of hydrophobic proteins.

We explored the effects of TFA concentration (0.05–0.5%) on protein separation. As expected, lower concentrations (< 0.1%) gave better recoveries of hydrophobic proteins but with increased bandwidths (similar to results obtained with phosphoric acid). Higher concentrations of TFA (> 0.3%) had the opposite effect. An interesting observation in this experiment was that changes in TFA concentrations between 0.1 and 0.5% resulted in significant selectivity changes for many of the test proteins (even more pronounced than that due to the use of different packings or different organic solvents). This is similar to the effect of TFA concentration on peptide separations[9].

HFBA, the strongest ion-pairing agent tested, gave generally increased retention, a narrower total elution range (lower peak capacities) and lower recoveries for the most hydrophobic proteins. Formic acid has been used for very hydrophobic proteins[20]. We examined its effects in both sample pretreatment and as a mobile phase modifier and found that formic acid generally gave much poorer performance than the optimal TFA system. Thus, formic acid should only be considered for very insoluble, hydrophobic proteins which do not behave well under the "optimized" conditions of Table III.

Organic solvents

Most reversed-phase separations of proteins are carried out with either acetonitrile or propanol as the mobile phase modifier. Although propanol has been cited in some cases as having advantages over acetonitrile[12,13], the advantages are not clear cut. We examined a variety of organic solvents for both sample pretreatment and as mobile phase modifiers.

Several organic solvents (Table II and III) were used in place of acetonitrile in both the mobile phase and sample pretreatment. When gradients of 10 to 60% organic solvent in water were used, these solvents gave markedly poorer chromatograms than acetonitrile, except for the case of 2-propanol; the latter gave acceptable chromatography, but generally wider bands than acetonitrile. Pure acetonitrile was generally the preferred organic solvent except for very hydrophobic proteins, which showed better chromatographic performance with 2-propanol.

Lower concentrations of various organic solvents (Table III) were also studied. In this case, eluents A and B of the gradient were each supplemented with 5% (v/v) of the organic solvent under study, while the concentration of acetonitrile went from 5 to 55% (gradient range constant at 10 to 60% of total organic solvent). Fig. 4 shows chromatograms of our indicator protein mixture under these conditions with

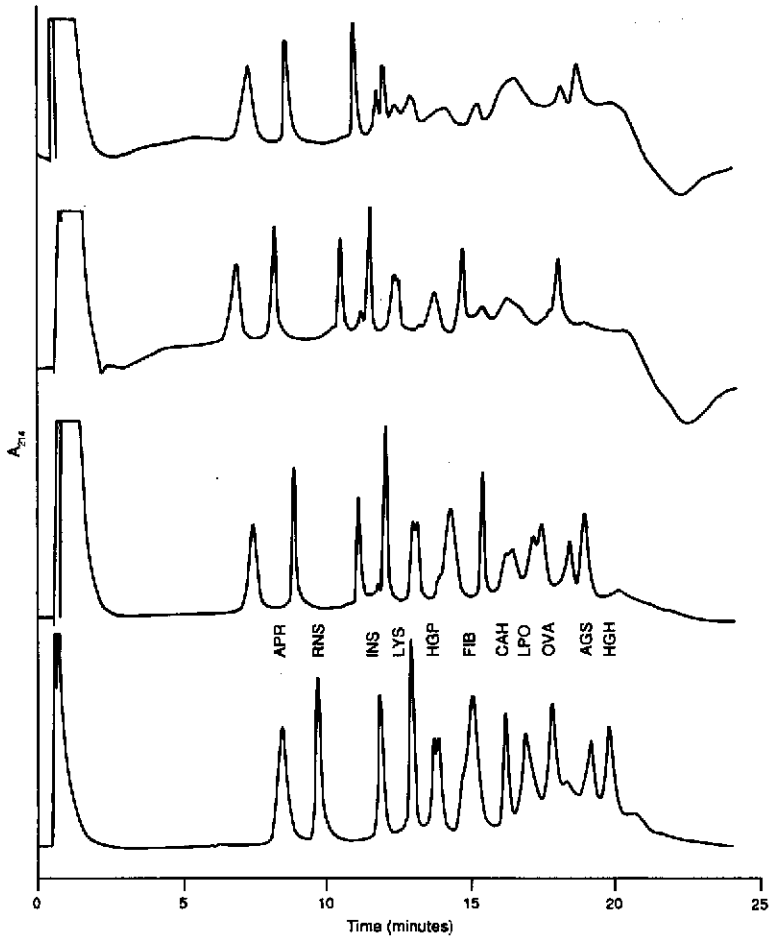

Fig. 4. Comparison of separations obtained for different mobile phases (described in text) under our optimal conditions (see Table I in ref. 1) using the indicator protein mixture and conditions described previously[1]. Organic modifiers from top to bottom: dioxane–acetonitrile, tetrahydrofuran–acetonitrile, 2-propanol–acetonitrile, acetonitrile.

2-propanol, tetrahydrofuran and dioxane as added solvents. While interesting changes in band spacing can be seen in these mixed-solvent chromatograms, the overall separation performance is poorer than in the absence of these added organic modifiers (bottom profile).

Higher alcohols (C_4–C_8) have been reported to improve protein separation when used in low concentrations as additives to the (acetonitrile–water) mobile phase[12]. Our studies showed that although some improvements in peakwidth occurred (especially for longer-chain-length alcohols), retention times for more hydrophobic proteins were also shortened. Thus, the total elution range was narrowed, which had an unfavorable effect on peak capacity. It has recently been reported[14] that sulfolane helps to solubilize hydrophobic proteins, suggesting its use as a mobile

phase modifier. For all of our test proteins (even the most hydrophobic), sulfolane adversely affected separation whether added to the sample or the mobile phase.

Detergents

Detergents (surfactants) are commonly used to solubilize proteins. There have been a few references (with mixed results) to the use of detergents as mobile phase additives[16,17,21]. Some detergents irreversibly bind to the reversed-phase packing and alter its retention characteristics[16]. In the case of heterogeneous detergents, a continuous change in retention may occur as the column is exposed to a cumulatively larger mass of detergent. This suggests that more-hydrophobic detergents should be used with caution, if at all.

The effects of the detergents listed in Experimental on protein chromatography are summarized in Tables II and III. Anionic and cationic detergents severely impaired separation performance; non-ionic detergents had little effect. Zwitterionic detergents, on the other hand, generally improved separation by reducing band-tailing of higher-molecular-weight and more-hydrophobic proteins.

Chaotropic agents

Chaotropes such as urea and guanidine are well known for their ability to denature proteins and promote the solubility of protein samples. Several references in the literature have been made to their use as mobile phase additives in RP-HPLC[17,22], but not always with an overall improvement in separation. In our work with guanidine and urea, we found that these chaotropes were quite advantageous in sample pretreatment for a wide range of proteins (Table II). Pre-treatment was carried out at room temperature for a few minutes (until dissolution of the sample), using chaotrope concentrations of 3–8 *M*.

As mobile phase additives, chaotropes had less effect on separation (Table III), although recovery was improved for the higher-molecular-weight and more-hydrophobic proteins. Because of their harsh effects on various instrumental components, addition of these chaotropes to the mobile phase is not recommended.

Reducing agents

The addition of reducing agents such as BME or DTT to protein samples results in a rupture of inter- and intra-molecular disulfide bonds with a disruption of secondary structures. It might therefore be expected that these compounds would promote protein denaturation and improve separations by RP-HPLC. However, as seen in Tables II and III, the addition of reducing agents to either the sample or the mobile phase generally deteriorated the separation. This may be due to some non-specific reformation of disulfide bonds during separation —possibly while the protein is sorbed to the column packing.

Sample weight

Several studies have shown that resolution is independent of sample weight for small sampls, but for a sufficiently large sample the protein band begins to broaden and resolution decreases. However detection sensitivity and sample recovery are favored by larger sample weights, and in a given case some intermediate sample weight will represent an optimum compromise. A detailed analysis of resolution *vs.* sample

weight in the reversed-phase gradient elution separation of proteins is described elsewhere[23,24].

After properly pretreating the sample ("optimized" conditions of Table I of ref. 1), we found that a much wider range of sample sizes and volumes could be injected without adversely affecting the separation. For our 50 × 1.0 mm I.D. column, total protein loads of 1 mg are possible in the case of complex samples such as *E. coli*. Individual protein loads of 5 ng to 5 μg could be injected, without losses at low concentrations or additional band broadening at high concentrations. Injection volumes of 1–100 μl were tolerated by our microbore system without adverse affects.

Gradient parameters

A mobile phase gradient and its effect on HPLC separation can be defined in terms of gradient time, range and shape. The initial gradient delay (either intentional or as a result of equipment design) and column equilibration between runs are also important. Table III summarizes our results for the effect of these gradient parameters on the separaton of our indicator protein sample.

Increasing the gradient time increases overall sample resolution, but it also results in wider bands that are harder to detect. Longer analysis times caused lower recoveries of the more hydrophobic proteins, as did an increase in gradient delay time (we simulated changes in gradient delay by varying the gradient hold; note that gradient delay-time increases as the flow-rate decreases, other factors being equal). Gradient time and gradient delay each affect the time spent by the sample on the column. A related observation is that the use of wider-range gradients having the same steepness, *i.e.* increasing change in percent organic solvent while increasing gradient time proportionately, also reduces the recovery of hydrophobic proteins. For hydrophobic proteins, it appears that the less time spent in the column, the greater the recovery (this is the same as seen for longer column lengths, as mentioned in ref. 1).

Column equilibration. The time allowed for column equilibration (after each gradient run) had an interesting effect on the separation of the indicator protein sample. In the case of small-molecule separations, the main effect of inadequate column equilibration is the reduced retention of early bands in the chromatogram. For our protein sample, decreasing the equilibration period to less than five column volumes of eluent A resulted in band broadening and peak splitting for later bands in the chromatogram. Apparently an incompletely equilibrated column leads to changes in protein denaturation when a protein molecule first contacts the column packing.

Gradient shape. Gradient shape is a variable that cannot be optimized for all protein samples. Usually a linear gradient will be the best initial choice. However, for a particular sample, it may be desirable to further improve resolution, *e.g.*, to provide better resolution of critical band-pairs in the initial chromatogram. Because we have fixed most of the separation variables in the process of optimizing conditions for our indicator proteins, we are restricted to gradient shape as the best means for further improving our initial (linear gradient) separation.

A segmented multi-step gradient permits a variation in gradient steepness at specific parts of the chromatogram, without changing gradient range or time[25]. This in turn can have a significant effect on the resolution of critical band-pairs and leads to the best possible separation. The design of optimized gradient shapes for this purpose is most conveniently done with commercially available software designed for this purpose, *e.g.*, DryLab G (LC Resources).

Optimized conditions

Fig. 2 presents chromatograms that illustrate the improvement in separation of our indicator protein mixture when the final "optimized" conditions (all variables) were used instead of the original "consensus" conditions. Fig. 2 can also be compared with the corresponding Fig. 6 in ref. 1 where "optimized" conditions include improvements in the column and hardware, but not the various pre-treatment and mobile phase parameters assessed in this paper.

Another "before-and-after" comparison is offered in Fig. 5 for individual proteins from the "good", "bad" and "ugly" classes of Table II of ref. 1. Band quality is seen to be markedly enhanced by the use of fully optimized conditions compared to the use of "consensus" conditions. Thus, in the middle profile of Fig. 5, the bandwidth was decreased by a factor of two through the use of optimized conditions. Similarly, in the bottom profile of Fig. 5, the multiple bands observed with "consensus" conditions are replaced by a single band when optimized conditions are used.

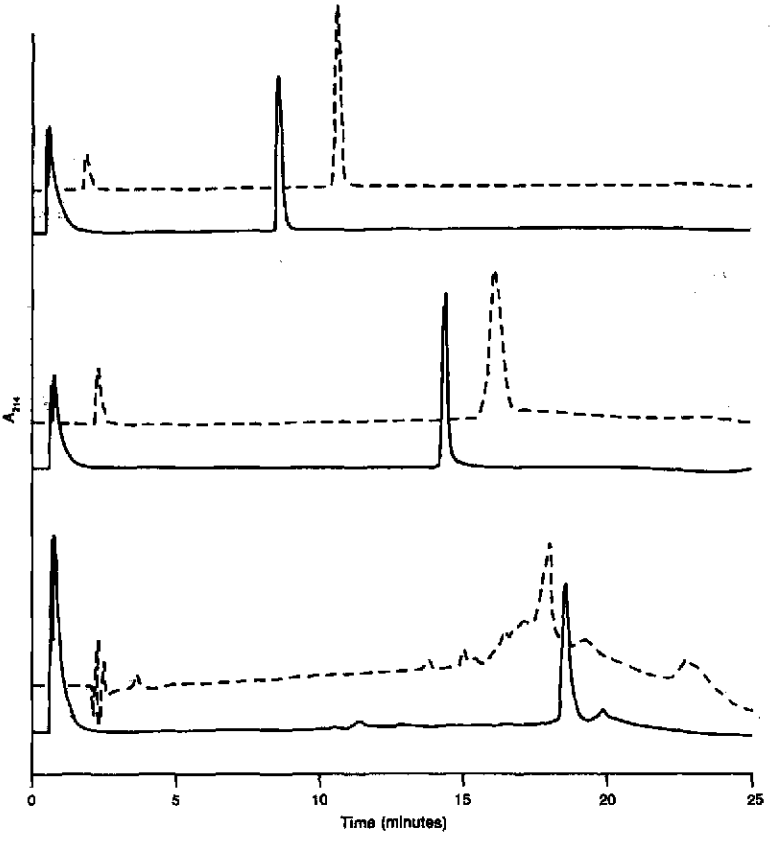

Fig. 5. Representative chromatograms showing the results obtained for (top) "good" (ribonuclease A), (middle) "bad" (alcohol dehydrogenase) and (bottom) "ugly" (β-galactosidase) proteins. The dashed traces show the results using "consensus" conditions, while the solid traces show the results using "optimized" conditions.

TABLE IV

SUMMARY OF THE IMPROVEMENTS IN CHROMATOGRAPHIC PERFORMANCE ACHIEVED FOR "CONSENSUS" *VS.* "OPTIMIZED" CONDITIONS

Performance	*Consensus*	*Optimized*
Peak capacity	10–20	50–100
Peak volumes (μl)	300–1200	15–60
Applicable mol. wt. range (kilodalton)	1–50	1–670
"Good" proteins (%)	*ca.* 30	*ca.* 90
Recoveries (%)	5–95	80–100
Ghosting (%)	0–50	0–5

The chromatophoresis process

The objective of the work described here and in ref. 1 was to improve the performance of RP-HPLC for protein separations in order for the chromatophoresis process to attain a comparable level of resolving power for complex protein samples as other two-dimensional techniques. Table IV summmarizes the progress we have made, as measured by the separation of the 33 proteins listed in Table I of ref. 1.

It should be noted that the peak capacity achievable by RP-HPLC (50–100) is now in the same range as the value of 70 for isoelectric focussing reported by O'Farrell[26]. This is illustrated in Fig. 6, which shows the RP-HPLC separation of the total proteins from *E. coli* using our optimized conditions. Although this separation is impressive in that 50–75 peaks can be seen in a single chromatogram, the

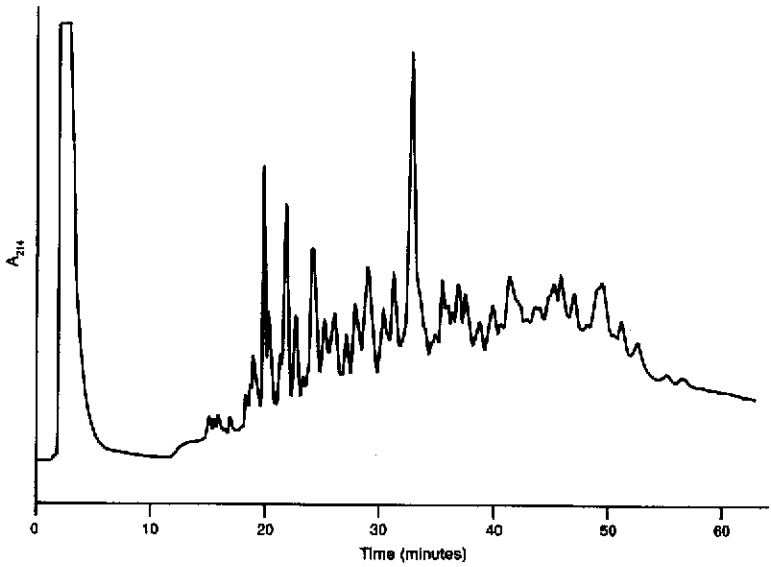

Fig. 6. The first-dimension (RP-HPLC) separation of an *E. coli* sample using optimized conditions on the prototype chromatophoresis system.

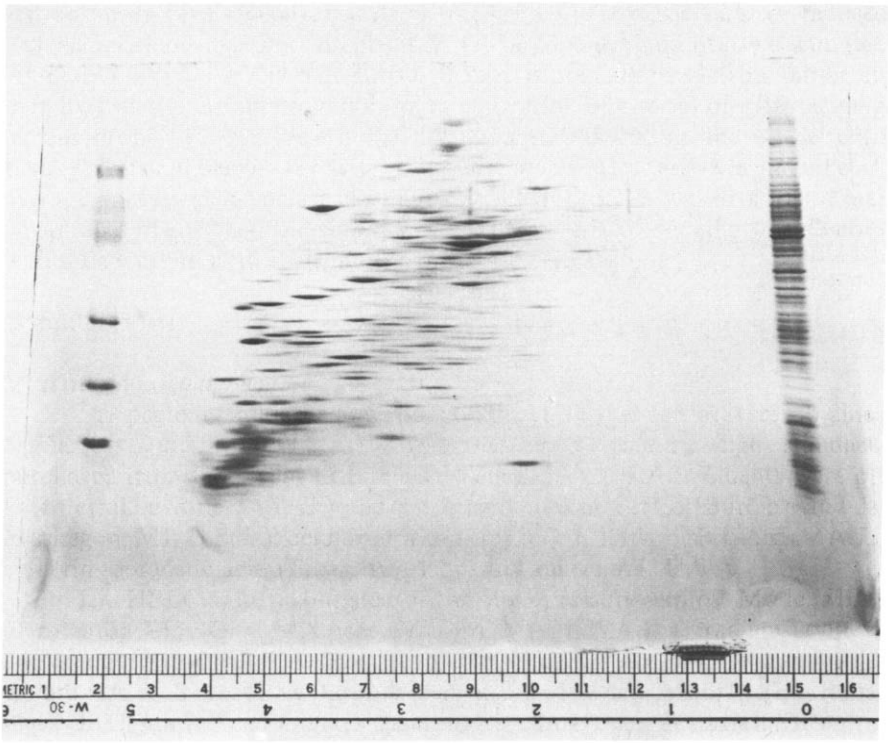

Fig. 7. A photograph of the second dimension output (SDS–polyacrylamide gel stained with Coomassie Blue) from the same *E. coli* sample shown in Fig. 6.

separation of this sample by chromatophoresis brings out the true power of this technique. Fig. 7 shows a chromatophoresis gel with over 250 distinct bands for a total protein load of 120 μg and stained with Coomassie blue dye.

CONCLUSIONS

The present paper completes our investigation of optimized conditions for the RP-HPLC separation of protein samples. From the results shown in Table IV and Figs. 5–7, one can see that we were able to achieve our initial goals for improving the resolution of the first dimension (RP-HPLC) of our new analytical method (the crhomatophoresis process).

It appears that the choice of both sample-pretreatment and separation conditions are of comparable inportance, if RP-HPLC is to be applicable to a wide range of protein samples. Generally these conditions should be selected so as to favor unraveling of the protein molecule into a random-coil structure, *i.e.* complete denaturation of the sample. The present separation conditions *in toto* constitute a good starting point for further work in this direction. In this sense our "optimum" conditions may be regarded as a "standard reference state", where a single variable at a time can be further changed and its effect on the chromatogram determined for any

protein sample. Some proteins show improved chromatographic separation for such minor variations in separation conditions.

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