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REVERSED-PHASE CHROMATOGRAPHY OF PROTEINS WITH DIPHENYL-SILICA COLUMN AND HYDRO-ORGANIC ELUENTS CONTAINING TWO ORGANIC SOLVENTS

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SUMMARY

High-performance liquid chromatography of proteins with silica bonded diphenyl stationary phase and hydro-organic mobile phases with linear gradient elution has been carried out with binary organic modifiers. The use of a mixture of 2-propanol and 1-butanol facilitated elution at total organic modifier concentrations significantly lower than with the use of 2-propanol alone. Furthermore higher protein recovery and retention of biological activity was obtained with the binary organic modifier because the increase in eluent strength with binary organic modifier was greater than that of the denaturing strength of the eluent. The use of a short, 3 cm long, column, relatively high flow-rates and steep gradients was also advantageous in attenuating protein denaturation. The results were interpreted within the framework of a theoretical treatment of the combined effect of the retention process and denaturation reaction simultaneously occurring in the column.

INTRODUCTION

The separation of proteins on silica-bound hydrocarbonaceous stationary phases has been widely investigated during the past few years[1]. Many reports suggested that the best column materials for use in high-performance liquid chromatography (HPLC) of proteins have larger pore dimensions and smaller alkyl or aryl ligates than those employed in conventional reversed-phase chromatography. As a result diphenyl-silica of wide pore diameter has been widely employed as the stationary phase with gradient elution in the HPLC of proteins[2]. Nevertheless the use of such a stationary phase requires relatively high concentrations of organic solvents in the mobile phase with concomitant denaturation of the protein in the chromatographic process that is considered a serious drawback of conventional reversed-phase chromatography for protein separations. Short columns are frequently used for the rapid separation of proteins by gradient elution[3–6] since it is believed that the mag-

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nitude of denaturation can be reduced when retention times are short and mobile phase effects are mainly responsible for denaturation. In order to attenuate denaturation in the column we have explored the use of hydro-organic mobile phases containing more than one organic solvent and certain additives in protein separation with short diphenyl-silica columns. The rationale of this approach is that by manipulation of the organic moiety of the eluent its denaturing strength, which accounts for poor protein recovery and/or loss of biological activity, can be reduced without diminishing eluent strength. Indeed the use of butanol in conjunction with organic modifiers usually employed in reversed-phase chromatography facilitated a better recovery of the native protein and biological activity than possible under the usual conditions in reversed-phase chromatography. The results are interpreted within the framework of a simplified theory for the interplay of chromatographic retention and the denaturation process which takes place simultaneously in the column.

EXPERIMENTAL

Apparatus

The eluent delivery system consisted of two Model 100A pumps and Model 420 gradient controller and a magnetic mixer (Altex, Berkeley, CA, U.S.A.). Samples were introduced by using a Model 7105 sample injector (Rheodyne, Cotati, CA, U.S.A.). Columns (3 × 0.466 cm I.D.) were packed by using a slurry of 10-μm Protesil 300 diphenyl-silica (Whatman, Clifton, NJ, U.S.A.) in 2-propanol–methanol (3:1). Column effluent was monitored at 225 and 280 nm with a Model SF-770 Spectroflow detector and a Model BD-41 recorder (Kratos, Westwood, NJ, U.S.A.).A Model 25 spectrophotometer (Beckman, Fullerton, CA, U.S.A.) was used in the measurement of enzyme activity and protein concentration.

Chemicals

All organic solvents were HPLC-grade from Fisher (Fairlawn, NJ, U.S.A.). Other chemicals were of reagent grade. Zonyl FSN and Zonyl FSB fluorosurfactants were obtained from Du Pont (Wilmington, DE, U.S.A.). Brij 35 and Tween-20 were from ICI (Wilmington, DE, U.S.A.). Polyoxyethylene octadecylamine was purchased from Armak (Chicago, IL, U.S.A.). Water was purified by using a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Insulin (Ins), pepsin (Pep), ovalbumin (Oval), Hemoglobin (Hem) and γ-globulin (Glo) were purchased from Sigma (St. Louis, MO, U.S.A.). Trypsin (Trp) and α-chymotrypsin (Chy) were from Worthington Biochem. (Freehold, NJ, U.S.A.). Cytochrome *c* (Cyto) was obtained from United States Chem. (Cleveland, OH, U.S.A.). Bovine serum albumin (BSA) was from J. T. Baker (Phillipsburg, NJ, U.S.A.). Protein samples were dissolved in water and insulin in 1 m*M* H_3PO_4, to give a concentration of 10 mg/ml. Coomassie Brilliant Blue G 250 was purchased from Bio-Rad (Richmond, CA, U.S.A.).

Mobile phases

In most cases the starting eluent was 0.1 *M* H_3PO_4 in water having pH 2.3 adjusted by potassium hydroxide, containing additionally enough gradient former to adjust the volume fraction of total organic modifier to 12%. In certain instances water containing 0.1% (v/v) trifluoroacetic acid was used. Gradient formers were

prepared from the starting eluent by adding the organic solvent components at the volume percents indicated: A, 60% 2-propanol; B, 22% 2-propanol and 15% 1-butanol; C, 16.5% acetonitrile and 16.5% 1-butanol; D, 20% methoxyethanol and 20% 1-butanol; E, 33% 2-propanol and 4% 2-methyl-1-butanol; F, 22% 2-propanol and 33% 1-butanol. The capital letters refer to the gradient former composition given in Fig. 1 and Table I.

Protein recovery

An amount of 100 μg of protein was injected into the diphenyl-silica column (3 × 0.46 cm I.D.) and chromatographed at a flow-rate of 2 ml/min and a 2%/min increase in the organic solvent concentration in the mobile phase. The protein was collected in polyethylene centrifuge tubes and thereafter lyophilized. The content of each tube was dissolved in 5 ml water, from each tube 1 ml aliquots were transferred to a second polyethylene tube and assayed for protein by the Coomassie Blue method of Bradford[7]. Protein recovery was expressed as percent of initially injected protein found in the tubes containing the eluted protein peak.

Enzyme recovery

Enzyme samples were prepared in the same manner as described above. Enzyme activities in the sample and in the effluent were determined by Worthington assay procedures[8] and percent activity recovered was calculated.

RESULTS AND DISCUSSION

Chromatograms of five proteins depected in Fig. 1 illustrate the effect of the organic solvent composition of the gradient former in linear gradient elution by using a short diphenyl-silica column. The results indicate that gradient elution with the organic solvent mixture as the modifier in the gradient former yields at least as efficient separations as the use of aq. 2-propanol alone shown in Fig. 1A. The broken lines on the chromatograms give the organic solvent concentration in the eluent as a function of time. It is seen that the proteins are eluted at much lower organic modifier concentrations when binary organic modifiers are used instead of 2-propanol alone. The enhancement of eluent strength is attributed to the presence of butanol in the gradient former. The reduction in organic modifier concentration required to elute a wide variety of proteins having molecular weights ranging from 11,000 to 160,000 dalton can be assessed more precisely from the data presented in Table I which lists the position of the eluites in terms of the total organic modifier concentration in the eluent at their elution time.

The governing idea for these experiments was to enhance elution strength by using organic modifiers which contain an organic solvent poorly miscible in water in addition to the solvent usually employed as the organic modifiers in reversed-phase chromatography. In this way a more lipophilic and concomitantly stronger eluent can be obtained at a given overall organic solvent concentration than with the use of a single organic modifier. A variety of solvents has been tested and 1-butanol has been found to be the most appropriate. Diethylether is too volatile for use in the eluent, whereas the solubility of 1-hexanol and 2-octanol were too low to yield useful eluents. Only 2-methyl-1-butanol proved to be suitable as the more lipophilic component of the binary organic modifier.

In fact the latter solvent was found to be an excellent "booster" for the eluent and by using it with 2-propanol together most proteins listed in Table I, with the exception of lysozyme and γ-globulin, could be eluted from the diphenyl-silica column. Moreover superior resolution was obtained with this mobile phase in the separation of the protein mixture as shown in Fig. 1E.

As far as the less lipophilic component is concerned 2-propanol was found to be preferable in combination with 1-butanol to methanol or acetonitrile. Results obtained with 2-methoxy-ethanol, which has also been used for protein separations by reversed-phase chromatography[9,10] were encouraging. this solvent is miscible with a variety of polar and non-polar solvents and has a low UV cut-off value. The mixture of 2-methoxy-ethanol and butanol resulted in relatively short retention times but invariably yielded a "solvent peak" in the course of gradient elution as shown in Fig. 1D.

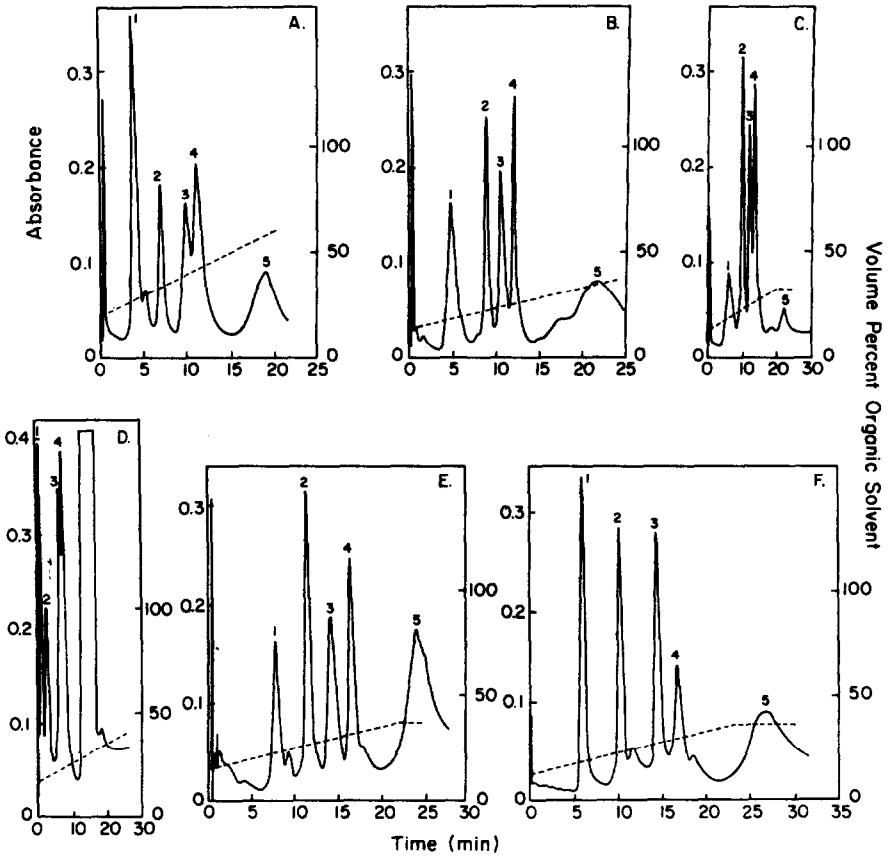

Fig. 1. Effect of organic solvent composition of gradient former on protein separation. Column, 3 × 0.46 cm, diphenyl-silica; flow-rate, 1.0 ml/min, except in (D) where it was 2 ml/min.; temperature, 24°C; detector setting, 225 nm; except in (E) where it was 280 nm. Starting eluent: 0.1 *M* phosphate buffer, pH 2.3; except in (F) it contained 0.1% (v/v) trifluoroacetic acid as well. Gradient former: expressed as volume percent of organic solvent in the starting buffer; (A) 60% 2-propanol; (B) 22% 2-propanol and 15% 1-butanol; (C) 16.5% acetonitrile and 16.5% 1-butanol; (D) 20% methoxy-ethanol and 20% 1-butanol; (E) 33% 2-propanol and 4% 2-methyl-1-butanol; (F) as in (B). Sample: 1, insulin; 2, cytochrome *c*; 3, bovine serum albumin; 4, myoglobin; 5, ovalbumin; 20 μg of each.

TABLE I

ELUTION POSITION OF PROTEINS IN LINEAR GRADIENT ELUTION WITH GRADIENT FORMERS OF DIFFERENT ORGANIC SOLVENT COMPOSITIONS

Column, 3 × 0.46 cm I.D., wide pore diphenyl-silica, 10 μm. Starting eluent was 0.1 *M* phosphate buffer, pH 2.3, except in F where it was water containing 0.1% (v/v) trifluoroacetic acid. For composition of gradient former see footnote. Flow-rate, 2 ml/min; gradient, 2% increase in organic solvent content per minute; temperature, 25°C.

Protein	*Molecular weight (10^{-3} dalton)*	*A**	*B*	*C*	*D*	*E*	*F*
Insulin	11	28.8	17.4	12.4	15.7	22.0	18.8
Cytochrome *c*	12	34.7	20.2	22.3	17.8	25.8	22.1
Ribonuclease *a*	13.7	27.8	14.9		19.1	20.4	20.0
Lysozyme	14	37.0	18.2	21.0			24.8
Myoglobin	17.5	43.1	23.0	24.7	21.2	31.0	27.6
Trypsin	23	37.0	22.4		27.8	24.3	30.4
α-Chymotrypsin	24	43.8	26.2		32.0	30.0	34.1
Pepsin	34	25.6	16.1	16.5	19.3	20.7	20.4
		27.2	17.3	17.5	20.8	22.1	21.7
Ovalbumin	40	58.0	34.9	33.0		37.0	41.4
Hemoglobin	64.5	49.1	24.9	24.6	30.0	29.6	31.2
		52.9	26.3	26.8	31.8		34.1
Bovine serum albumin	68	50.9	21.8	26.1	20.4	28.6	25.7
γ-Globulin	160	26.8			27.6		35.3

* The composition of the gradient former was as described in Fig. 1 and in the experimental section.

The starting eluent in some experiments was 0.1% aq. trifluoroacetic acid, a frequently used eluent component in reversed-phase chromatography of proteins, and no phosphate buffer was employed. No significant change in resolution was observed in these cases, *vide* Fig. 1F, but the proteins eluted at organic modifier concentrations in the eluent invariably higher than those with the use of phosphate buffer. If denaturation is to be minimized the use of trifluoroacetic acid, a known denaturing agent, is not recommended even at such low concentrations.

The results demonstrate that the use of binary organic modifier facilitates rapid elution of proteins from hydrocarbonaceous bonded phase columns and the organic solvent concentration necessary for the elution of a given protein is lower than that with a single organic modifier. This is consistent with the observation by Tarr and Crabb[11], which was published after completion of the present study, that ternary solvent systems are advantageous for the separation of hydrophobic proteins and peptides in reversed-phase chromatography.

Another possibility to enhance eluent strength without increasing the denaturing strength of the eluent arises from the suggestion by Barford *et al.*[12] to add surfactants to the eluent at very low concentrations. Therefore we examined the use of various surfactants at concentrations not exceeding 0.5% (w/v). The results on the retention time of five proteins are qualitatively given in Table II. It is seen that in the experiments with gradient elution both increase and decrease of retention times was observed. Addition of non-ionic surfactants such as Tween-20 and Zonyl FSN to the eluent caused the proteins to elute slightly faster whereas that of ionic and amphoteric

TABLE II

EFFECT OF THE ADDITIVES ON THE RETENTION OF THE PROTEINS

Column, 3 × 0.46 cm I.D. wide pore diphenyl column. Mobile phase, solvent A; 0.1 *M* phosphate buffer (pH 2.3); solvent B; 60% 2-propanol in A. Gradient, linear from 40–100% solvent B for 20 min. Flow-rate 1 ml/min. + increased < 10%; + + increased > 10%; + + + increased > 50%; − decreased < 10%; − − decreased > 10%; − − − decreased > 50%.

Additive	*Percentage in mobile phase*	*Retention time*				
		Ins	*Cyto*	*BSA*	*Myo*	*Oval*
Zonyl FSN	0.03	− −	− −	− −	− −	− −
Zonyl FSB	0.1	+	+	+	+	+
Tween-20	0.05	−	−	− −	− −	−
Sodium dodecyl sulfate	0.5	+ + +	+ + +	+ + +	+ + +	+ + +
tert.-Butylaminoethanol	0.3	−	−	+		
Glycerol	1	− −	−	−	−	
	5	− − −	− −	−	− −	
Leucine	0.5	+ +	+	+	+	
Polyoxyethylene octadecylamine	0.2	Peaks broadened				
Brij 35	0.5	Peaks broadened				

surfactants generally increased the retention time. Certain surfactants such as Brij 35 and polyoxyethylene octadecylamine gave rise to excessive band broadening which may be due to inhomogeneity of these products at the molecular level.

The effect of other additives which are not surfactants was also examined. Glycerol at concentrations of 1–5% in the eluent reduced retention but at the cost of increased viscosity of the mobile phase. *tert.*-Butylaminoethanol gave mixed results and addition of 0.5% leucine to the mobile phase gave rise to an increase in protein retention. The latter effect is likely due to binding of leucine to the protein and thereby enhancing the hydrophobic character of the protein surface.

A comparison of the protein recoveries obtained from the chromatography of

TABLE III

PROTEIN AND ENZYMIC ACTIVITY RECOVERIES OBTAINED ON DIPHENYL-SILICA COLUMN WITH DIFFERENT ORGANIC SOLVENT SYSTEMS

Column, 3 × 0.46 cm I.D. column packed with diphenyl bonded silica (Protesil 300). Mobile phase, solvent A: 0.1 *M* phosphate buffer (pH 2.3); solvent B: (A) 60% 2-propanol in A; (B) 22% 2-propanol + 15% 1-butanol in A. Gradient, linear from 35 to 100% solvent B for 20 min (A); 24 min (B). Flow-rate, 2 ml/min.

Protein	*Protein (%)*		*Enzymic activity (%)*	
	A	*B*	*A*	*B*
Insulin	98	101		
Cytochrome *c*	96	102		
α-Chymotrypsin	84	99	40	72
Trypsin			32	56
Hemoglobin	86	100		
Bovine serum albumin	76	82		
γ-Globulin	67	89		

a number of proteins on the 3-cm diphenyl column using the mobile phases A and B is given in Table III. All the proteins chromatographed were eluted in high yields with the B system. One reason for this may be that proteins are less strongly absorbed on the stationary phase and be eluted more readily. In addition, the higher activity recovery of trypsin and α-chymotrypsin were also obtained using the B ternary solvent system.

An additional observation made during this study was that the biological activity of adenosine deaminase was almost completely lost in 60% 2-propanol–phosphate buffer (pH 6.5) after 40 min, while 50% of the activity was retained when using the 22% 2-propanol + 16% 1-butanol–phosphate buffer (pH 6.5) system after the same period.

In general, low flow-rates are used in reversed-phase HPLC of proteins to increase the efficiency by compensating for the lower diffusion rates of the larger protein molecules. As seen in Fig. 2, however, with the 3 × 0.46 cm I.D. column

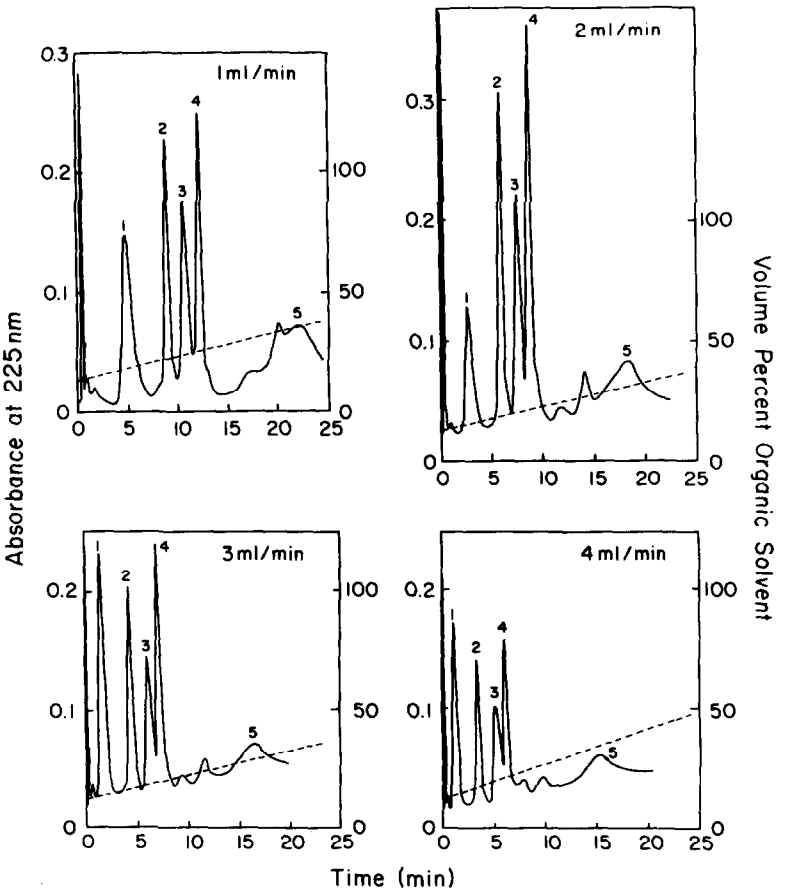

Fig. 2. Effect of flow-rate on protein separation by gradient elution. Column, 3 × 0.46 cm I.D. diphenyl-silica; mobile phase: starting eluent, 0.1 *M* phosphate buffer, pH 2.3; gradient former, 22% 2-propanol + 15% 1-butanol in linear gradient; temperature 24°; detector setting, 225 nm. Sample: 1, insulin; 2, cytochrome *c*; 3, bovine serum albumin; 4, myoglobin; 5, ovalbumin; 20 μg of each.

and gradient elution, high flow-rates can be employed in the protein separations without loss of the efficiency even at flow-rates of 4 ml/min. Furthermore, the use of 3-cm column also offers the advantage of rapid column re-equilibration and the employment of steep gradients in protein separation with concomitantly short elution times.

The results presented in Table III can be interpreted in terms of the theory previously developed for the interplay of the chromatographic retention process and first-order reaction which the eluite undergoes simultaneously in the column[13]. In the present case the reaction is the transformation of the native protein (N) into its denatured form (D) without accumulation of intermediates and denaturation may occur in both the mobile and the stationary phases. Conservation of mass in the absence of diffusional effects and with uniform flow profile in the column yields

$$(1 + k_N)\frac{L}{u_0}\frac{\partial C_{N,m}}{\partial t} + L\frac{\partial C_{N,m}}{\partial z} = -KDaC_{N,m}/(1 + K) + DaC_{D,m}/(1 + K) \quad (1)$$

where k_N is the retention factor of the native protein, L is the column length, u_0 is the superficial velocity of the eluent and K is the equilibrium constant for denaturation. The respective concentrations of the native and denatured protein in the mobile phase are given by $C_{N,m}$ and $C_{D,m}$, and t is time and x is axial position in the column. The dimensionless Damköhler number is given by

$$Da = L\,k_2\,(1 + 1/K)/u_0 \quad (2)$$

where the overall rate constant k_2 is defined by

$$k_2 = k_N k_{f,s} + k_{f,m} \quad (3)$$

as a function of $k_{f,s}$ and $k_{f,m}$, the first-order rate constants of denaturation in the stationary and mobile phases, respectively. The Damköhler number is a measure of the relative magnitude of the rate of eluent movement through the column and the rate of reaction.

Model studies for isocratic systems have shown that if $0.1 < Da < 1$ the chromatogram obtained with injection of a pure sample[13] will show two peaks due to on-column inter conversion. When Da is larger than unity the peak contains only the reaction product, *i.e.* denatured protein, and if Da is less than 0.1 the peak will contain only the native protein injected.

No corresponding model studies have been done yet for gradient elution such as is used in macromolecular chromatography and the analysis is complicated by change in value of Da during gradient development. Nevertheless an estimate of Da offers a means to interpret the effect of organic solvent on the retention of protein and loss of their activity. Denaturation in the mobile phase is assumed to occur by thermal denaturation with rate constant $k_{f,t}$ and by other processes which are dependent on the nature and concentration of each organic co-solvent and for simplicity are lumped together here. The overall apparent first-order rate constant in hydro-organic systems containing one organic solvent can be expressed by

$$k_{f,m} = k_{f,t} + k_{f,A}\,(1000\varphi_A/V_A)^{n_A} \quad (4)$$

where $k_{f,A}$ is the n_Ath order rate constant for denaturation by the organic modifier and V_A and φ_A are the molar volume and volume fraction of the organic solvent. For mixtures containing two organic solvents, A and B, the rate constant is

$$k_{f,m} = k_{f,t} + k_{f,A}\,(1000\varphi_A/V_A)^{n_A} + k_{f,B}\,(1000\varphi_B/V_B)^{n_B} \tag{5}$$

where subscripts A and B refer to quantities particular to solvents A and B.

The eqns. 4 and 5 can be extended to consider further effects leading to denaturation as well as protecting agents and synergy. Nevertheless, they suffice for the present discussion in which we consider the apparent first-order rate constant for denaturation on the surface to be independent of mobile phase composition.

With eqns. 4 and 5 the Damköhler number can be written as

$$Da = L/u_0\,[K/(1 + K)]\,[k_N k_{f,s} + k_{f,t} + k_{f,A}\,(1000\varphi_A/V_A)^{n_A} + k_{f,B}\,(1000\varphi_B/V_B)^{n_B}] \tag{6}$$

Since the mobile phase composition change at any point, z in the column is related to time and rate of gradient development ϕ as

$$\varphi = \varphi_0 + \dot{\varphi}(t - z/u_0) \tag{7}$$

we may define a local Damköhler number, Da^*, for conditions that prevail in gradient elution as

$$Da^* = L/u_0\,[K/(1 + K)]\,\{k_{f,s} + k_{f,t} + k_{f,A}[\varphi_{A,0} + \dot{\varphi}_A\,(t - z/u_0)]^{n_A} \cdot (100/V_A)^{n_B} + k_{f,B}[\varphi_{B,0} + \dot{\varphi}_B(t - z/u_0)]^{n_B}(1000/V_B)^{n_B}\} \tag{8}$$

where $\dot{\varphi}_B$ is zero with hydro-organic systems containing a single organic modifier.

Inspection of eqn. 8 with $\dot{\varphi}_B$ set equal to zero suggests that one way to reduce the degree of denaturation and thereby to reduce band spreading due to the denaturation reaction is to minimize the retention time by using very steep gradient of the organic solvent. This can be illustrated as follows. Suppose elution occurs when the organic solvent concentration reaches a critical level so that the retention time is given as

$$t_R = (\varphi_c - \varphi_0)/\dot{\varphi} \tag{9}$$

On the other hand, the extent of denaturation as measured by the fraction of protein denatured in the column can be expressed in the case of one organic solvent present by

$$\ln [D]/[N + D] = \int_0^{t_R} k_D(\varphi)^n\,dt = (k_D\varphi_c^{n+1})/[(n + 1)\,\dot{\varphi}] + k_{f,s}(\varphi_c - \varphi_0)/\dot{\varphi} \tag{10}$$

The corresponding analysis for systems containing two organic solvents yields the extent of denaturation as

$$\ln[D]/[N + D] = k_{f,s}(\varphi_{c^*,A} - \varphi_{A,0})/\phi_A + k_{D,A}\varphi_{c^*,A}^{n_A+1}/(n_A + 1)\,\phi_A + \\ + k_{D,B}\varphi_{Bc,B}^{n_B+1}/(n_B + 1)\phi_B \tag{11}$$

where $\varphi_{c^*,A}$ and $\varphi_{c^*,B}$ are the critical concentrations of solvents A and B at which the protein elutes. The eqns 10 and 11 demonstrate that increase in ϕ minimizes extent and significance of denaturation on chromatographic profile.

Assume that retention with eluents containing binary organic modifier can be approximated as

$$\ln k = \alpha - \beta_A\varphi_A - \beta_B\varphi_B \tag{12}$$

where k is the retention factor and α, β_A and β_B are system parameters. We obtain for the critical organic solvent concentrations at which elution occurs

$$\varphi_{c^*,A} = \alpha/\beta_A - \beta_B\varphi_B/\beta_A \tag{13a}$$

or

$$\varphi_{c^*,B} = \alpha'/\beta_B - \beta_A\varphi_A/\beta_B \tag{13b}$$

Substitution of eqn. 13a or 13b into eqn. 11 reveals that the extent of denaturation caused by each of the organic solvents in eluents containing binary modifier must be less than that which would be obtained with each alone at the concentration required for elution. Furthermore, the strong power dependence of the second and third terms on the right-hand side of eqn. 8 suggests that the extent of denaturation can be manipulated by variation in $\varphi_{c^*,A}/\varphi_{c^*,B}$ ratio so that the sum of the last two terms on the right-hand side is minimized.

Indeed the results presented in Table III conform with the results of this analysis and less denaturation, consequently higher recovery of the protein and enzymic activity is obtained with eluents containing two organic modifiers than with use of a single organic modifier. It should be noted, however, that the favorable results obtained in chromatographic practice are restricted to organic solvents which have suitable values of the parameters involved as well as appropriate solubility characteristics. Nevertheless the prediction of the above theory that the employment of relatively short columns, high flow-rates and steep gradients favors the preservation of the integrity of the native form of the biopolymer appears to be quite general beyond the scope of the supporting data presented here.

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