Journal of Chromatography, 397 (1987) 81–89
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1236

EVALUATION OF ADVANCED SILICA PACKINGS FOR THE SEPARATION OF BIOPOLYMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

IV. MOBILE PHASE AND SURFACE-MEDIATED EFFECTS ON RECOVERY OF NATIVE PROTEINS IN GRADIENT ELUTION ON NON-POROUS, MONODISPERSE 1.5-μm REVERSED-PHASE SILICAS

R. JANZEN, K. K. UNGER* and H. GIESCHE
Institut für Anorganische Chemie und Analytische Chemie, Johannes Gutenberg-Universität, 6500 Mainz (F.R.G.)
J. N. KINKEL
Chemical Reagents Division, R & D Chromatography, E. Merck, 6100 Darmstadt (F.R.G.)
and
M. T. W. HEARN
Department of Biochemistry, Monash University, Clayton, Victoria 3168 (Australia)

SUMMARY

The reversed-phase chromatography of proteins by gradient elution with acidic, low-ionic-strength aqueous–organic eluents is often associated with losses of the biological activity of the protein. In this study, the enzymatic activities of catalase, horseradish peroxidase and pepsin were examined under static and dynamic column conditions on non-porous, monodisperse 1.5-μm reversed-phase silicas with various *n*-alkyl ligands. Catalase readily lost its enzymatic activity under the influence of the acidic aqueous–organic eluents in the absence of the reversed-phase packing, whereas peroxidase was partially deactivated as a result of combined mobile phase and stationary phase effects but regained its activity on storage after elution. The enzymatic activity of pepsin was found to be very dependent on the column residence time and on the type of bonded *n*-alkyl ligand employed.

INTRODUCTION

The retention of polypeptides and proteins on reversed-phase (RP) silicas and with acidic, low ionic strength eluents is often associated with asymmetry or multiple peaks and losses of the biological activity. These phenomena, which result from the elution conditions and the strong hydrophobic solute surface interactions, are due in part to the dynamic effects of conformational equilibration of the solute, which occur either in the bulk mobile phase or at the stationary phase surface. These effects

0021-9673/87/$03.50

have been observed on a variety of proteins, *e.g.*, ribosomal proteins[1,2], papain[3], soybean trypsin inhibitor[3,4], collagen chains[5], lysozyme[3,6], ribonuclease[7] and multi-subunit protein hormones[8].

Studies of the kinetics of unfolding and refolding of proteins under high-performance liquid chromatographic (HPLC) conditions[9,10] are attracting increasing interest, in view of their importance to the purification of natural and biosynthetic recombinant DNA protein products. Hearn and co-workers[11–13] have developed multi-stage models for protein retention with *n*-alkylsilicas with particular emphasis on the kinetics. Experimental data have validated these models for a number of test cases[11–13]. Kinetic and mass-transfer effects due to pore diffusion of proteins into and out of porous *n*-alkylsilicas are eliminated when non-porous silicas are employed as packing materials. Further, the kinetics of adsorption and desorption of the solute at the hydrocarbon interface should be improved. All these factors together are expected to have a significant influence on the chromatographic behaviour of proteins and on the recovery of biological activity in particular.

In this work, we examined the enzymatic activity of three test proteins, catalase, horseradish peroxidase and pepsin, under static and dynamic column conditions on non-porous, monodisperse 1.5-μm reversed-phase silicas, chemically bonded with various *n*-alkyl ligands.

EXPERIMENTAL

Materials

The various *n*-alkyl stationary phases on non-porous silicas were prepared in our laboratory.

All chemicals were obtained from E. Merck (Darmstadt, F.R.G.). Solvents were of HPLC grade and also obtained from Merck. Column hardware was supplied by Bischoff (Leonberg, F.R.G.). Catalase was from Boehringer (Mannheim, F.R.G.), horseradish peroxidase from Sigma (St. Louis, MO, U.S.A.) and pepsin from Merck.

Instrumentation

For chromatographic tests, a Merck–Hitachi high-pressure gradient system, consisting of two 655 A pumps, a gradient controller and a dynamic mixer, fitted with a Merck–Hitachi F 1000 fluorescence detector, was used. Enzymatic essays were monitored with a Zeiss (Oberkochen, F.R.G.) DM 4 spectrophotometer.

Enzymatic assays

Catalase was assayed by adding an aliquot, typically containing 80 ng of the enzyme, to a mixture of 2 ml of 0.1 *M* phosphate buffer (pH 7) and 1 ml of 0.06 *M* hydrogen peroxide. Decay of the hydrogen peroxide was monitored at 230 nm over the first 5 min of the reaction.

Peroxidase activity was determined by addition of an aliquot containing 200 ng of the protein to a mixture of 2.8 ml of 0.1 *M* phosphate buffer (pH 7.0), 0.1 ml of 3.7 m*M* hydrogen peroxide and 0.05 ml of 0.02 *M* guaiacol. The increase in optical absorbance at 436 nm was measured over an interval of 4 min.

A protease assay according to Anson[14] served to determine pepsin activity. In brief, 100 mg of haemoglobin in 5 ml of 0.06 *M* hydrochloric acid were digested by

incubation of 25 μg of pepsin for 10 min. The reaction was then stopped by addition of trichloroacetic acid. After filtration and addition of sodium hydroxide and Folin–Ciocalteu phenol reagent, the optical absorbance was measured at 546 nm against a control sample.

RESULTS AND DISCUSSION

Catalase

Catalase (E.C. 1.11.1.6) is a 240-kilodalton protein composed of four subunits, having a p*I* value of 5.8[15]. The pH optimum for the enzymatic activity is around pH 7[16]. At pH 3, dissociation into dimer subunits occurs, whereas under more severe denaturing conditions the monomers are obtained[17]. To assess the effect of the mobile phase composition on the enzymatic activity of catalase, static measurements were carried out using incubation times of 2–10 min, with various aqueous–organic solvents (Table I).

TABLE I

RESIDUAL ACTIVITY OF CATALASE AFTER INCUBATION WITH VARIOUS VOLUME FRACTIONS OF AQUEOUS–ORGANIC SOLVENTS

Organic solvent	*Incubation time (min)*	*Catalase activity (%)*			
		Organic solvent fraction			
		15%	*30%*	*45%*	*60%*
Acetonitrile	2	17	11	5.5	5.5
Acetonitrile	10	12	10	4	2.5
Methanol	10	92	75	34	1
Ethanol	10	94	67	35	0
1-Propanol	10	100	53	2	0
2-Propanol	10	100	97	87.5	85
Dioxane	10	86	80	70	53
Tetrahydrofuran	10	78	21	12.5	11

The results clearly indicate that the common organic solvents, *e.g.*, methanol, acetonitrile and 1-propanol, employed in the reversed-phase gradient elution of proteins lead to almost complete loss of the catalase activity when high molar fractions of the organic solvent are employed. There are fine gradations in the loss of activity as a function of the percentage of organic solvent in the aqueous–organic solution. Acetonitrile caused dectivation at 15%. With methanol the activity declined progressively in the range 15–60%. With 1-propanol the loss of activity occurred sharply above 30%. The most favourable solvent was 2-propanol, followed by dioxane. These results parallel the finding of Herskowitz *et al.*[18] on the solvent-induced denaturation of proteins in low ionic strength buffers. Similar observations have been made by Sadler *et al.*[19] and Hearn[20]. It also became evident that a reduction of the incubation time from 10 to 2 min gave a slight improvement in the biological recovery. However, these deactivation effects due to variations in incubation times are negligible compared with the differences observed between different solvents.

In addition to the effects of organic solvents, resulting in destabilization of the protein hierarchy, another cause of denaturation is expected to arise as a consequence of the application of an acidic pH. To examine the effect of the type of acid and the pH on enzymatic activity, various acidic solutions were first used and then combined with the most favourable solvents from the previous experiments (Table II).

TABLE II

RESIDUAL ACTIVITY OF CATALASE AFTER INCUBATION WITH ACIDIC AQUEOUS–ORGANIC SOLVENTS

Acidic medium	*Organic solvent fraction*	*Residual activity (%)*
TFA (pH 2)	–	0.5
H_3PO_4 (pH 2)	–	0.5
TFA (pH 3)	–	40
H_3PO_4 (pH 3)	–	55
TFA (pH 3)	45% 2-Propanol	0
H_3PO_4 (pH 3)	45% 2-Propanol	0
CH_3COOH (pH 3)	45% 2-Propanol	11
CH_3COOH (pH 3.5)	45% 2-Propanol	49
0.1 *M* Phosphate buffer (pH 4.8)	45% 2-Propanol	69
CH_3COOH (pH 3)	45% Dioxane	23
CH_3COOH (pH 3.5)	45% Dioxane	55
0.1 *M* Phosphate buffer (pH 4.8)	45% Dioxane	69

A nearly complete loss of activity was measured for trifluoroacetic acid (TFA) and phosphoric acid at pH 2, with a notable improvement in the recovery of enzymatic activity when the pH was increased by one unit, from 2 to 3. Addition of 2-propanol or dioxane at a level of 45% to the acidic solution of pH 3 resulted in a further reduction in the activity. Substitution of TFA or phosphoric acid by acetic acid or 0.1 *M* phosphate buffer of pH 3–4.8 led to conditions under which more than half of the enzymatic activity was preserved.

Based on the above data from static measurements, dynamic column experiments were carried out on *n*-octadecyl columns with TFA, phosphoric acid and acetic acid solutions of pH 3 (solvent A) and 67.5% 2-propanol in A (solvent B), applying a linear gradient from 0 to 100% B. The enzymatic activity of catalase recovered was less than 2% with TFA and phosphoric acid and about 7% for the acetic acid containing eluent. The total mass recovery for this protein (as subunits) was 92%. Hence catalase cannot be chromatographed under these conditions by employing acidic mobile phases without a loss of the enzymatic activity.

Peroxidase

Horseradish peroxidase (E.C. 1.11.1.7) is a 40-kilodalton protein with an isoelectric point of pH 7.2 and a pH optimum between 6 and 6.5[21]. Several chromatographic conditions were examined, based on the following mobile phase compositions: eluent A = 0.1% TFA in water; eluent B = 0.1% TFA in 2-propanol–water (4:1) or 0.1% TFA in acetonitrile–water (4:1) with linear gradients from 0 to 100% B.

The chromatographic fractions were dissolved in phosphate buffer, diluted to 50 ml and tested for their activity. The peroxidase activity following RP-HPLC was found to be dependent on the incubation time with the phosphate buffer. Such renaturation effects have previously been observed with lysozyme, chymotrypsinogen and ribonuclease[3,18,22].

The kinetics of renaturation were therefore studied without performing a chromatographic separation. Horseradish peroxidase was mixed with 2 ml of eluents containing various amounts of the organic solvent, 2 min later the phosphate buffer was added and the enzymatic activity was monitored over a period of 1–2 h. The same procedure was applied to fractions collected after chromatography on an *n*-octadecyl column with acetonitrile as the organic solvent.

The results are illustrated in Fig. 1. The static experiments reveal that the deactivation of peroxidase, induced by the acidic aqueous–organic solvent, was not completely reversible, even after an extended incubation time. Similar results were obtained from the column measurements. In order to estimate the extent to which the irreversible loss was dependent on the type of *n*-alkylsilica and the residence time

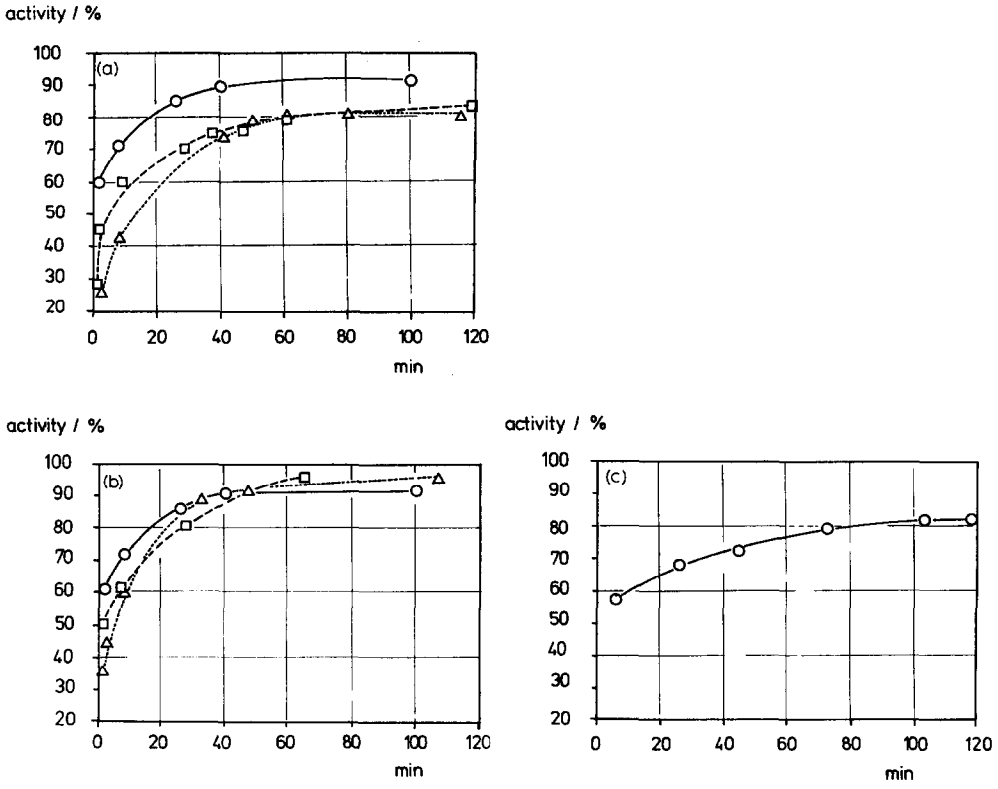

Fig. 1. Reactivation of peroxidase in phosphate buffer as a function of time: (a) after incubation with 0.1% TFA in (○) 0, (□) 32 and (△) 64% 2-propanol for 2 min; (b) after incubation with 0.1% TFA in (○) 0, (□) 32 and (△) 64% acetonitrile for 2 min; (c) after chromatographic separation with a 10-min gradient from 0 to 80% acetonitrile in 0.1% TFA; flow-rate, 0.7 ml/min.

TABLE III

INFLUENCE OF THE LIGAND CHAIN LENGTH OF THE REVERSED-PHASE PACKING ON THE IRREVERSIBLE DENATURATION OF HORSERADISH PEROXIDASE

Eluent A, 0.1% TFA: eluent B, 0.1% TFA in 2-propanol–water (4:1); gradient, 0–100% B in 10 min; flow-rate, 0.7 ml/min.

Stationary phase	*Residual activity after reactivation for 1 h in phosphate buffer (%)*
C_{18}	78
C_8	73
C_4	75
C_2	91
Phenyl	87

of a solute on a given reversed-phase column, the experiments illustrated in Fig. 1c were repeated under the conditions given in Tables III and IV.

These studies revealed that *n*-alkylsilicas with *n*-butyl, *n*-octyl and *n*-octadecyl ligands had similar effects with regard to irreversible denaturation. These effects were less pronounced with the phenyl- and ethylsilica columns. Increasing the residence time of the protein at the stationary phase surface up to 30 min in a given column (*e.g.*, *n*-octadecyl) had no significant effect on the remaining activity (Table IV). The data in Tables III and IV differed by less than 3% in repeated runs.

The results can be summarized as follows:

(i) At pH 2 and in the presence of organic solvents, horseradish peroxidase is largely inactivated but it can be reactivated by reincubation with a suitable buffer, *e.g.*, phosphate.

(ii) Irreversible losses of the enzymatic activity of horseradish peroxidase of *ca.* 5–10% were caused by the acidic medium (trifluoroacetic acid).

(iii) A small amount (about 5%) of the original activity appears to be lost by solute–stationary phase interactions with residence times up to 30 min. This figure

TABLE IV

INFLUENCE OF COLUMN RESIDENCE TIME ON THE IRREVERSIBLE DENATURATION OF HORSERADISH PEROXIDASE

Gradient and flow stopped 2 min after injection for 0–30 min. C_{18} column; for other conditions, see Table III.

Duration of stopped flow (min)	*Residual activity after overnight reactivation in phosphate buffer (%)*
0	83
10	84
20	85
30	79

is decreased when short, *n*-alkyl-ligand silicas are used and remains unaffected by the residence time of solute in the column.

(iv) With acetonitrile as organic solvent, about 90% of the original activity was regained. In contrast, 2-propanol caused higher losses with the same volume fraction.

Pepsin

The other two proteins demonstrated extreme sensitivity towards the reversed-phase gradient elution conditions as a consequence of their high molecular weight, their quaternary structures and their sensitivity to low pH conditions.

In order to evaluate stationary phase effects on the denaturation of a more acid-stable protein, pepsin (E.C. 3.4.23.1) was chosen, as it is not or only slowly denatured as a result of mobile phase compositions similar to those used above. Pepsin is a 35-kilodalton protein, composed of a single polypeptide chain, having an isoelectric point below pH 3[23] and a pH optimum of about 1[24].

After chromatographic experiments similar to those described above for catalase and horseradish peroxidase, the enzymatic activity of pepsin was measured for the collected fractions. Eluent A was 0.1% TFA and B was 0.1% TFA in 2-propanol–water (4:1). A linear gradient of 0 to 100% B was applied at a flow-rate of 0.8 ml/min. The enzymatic activity of the collected pepsin was monitored as a function of the gradient time (between 5 and 60 min) on an *n*-octyl reversed-phase column (Table V) and as a function of the type of reversed-phase column at constant gradient time (Table VI).

TABLE V

ENZYMATIC ACTIVITY OF PEPSIN AS A FUNCTION OF GRADIENT TIME

C_8 column. Linear gradient from 0 to 80% 2-propanol in 0.1% TFA; flow-rate, 0.8 ml/min.

Gradient time (min)	*Retention time of main component (min)*	*Activity (%)*
5	6.34	81
10	7.73	73
20	9.96	59
60	17.35	53

Table V indicates that the loss of enzymatic activity of pepsin increases with increasing gradient time. Such behaviour is consistent with the increased loss of enzymatic activity being caused by surface-induced effects. Benedek *et al.*[3] examined the kinetics of the denaturation of papain on *n*-alkyl stationary phases and observed similar time-dependent effects. It is probable that these phenomena involve a rapid first step when the protein solute initially makes contact with the stationary phase, followed by a second, slow process while the protein remains in the adsorbed state. It should be noted that only a slight increase in denaturation (as a percentage of the total protein) occurred when a gradient time of 20 min was exceeded. The decreasing content of organic solvent with increasing gradient time might compensate for the denaturation arising from the column residence time of the solute.

TABLE VI

PEPSIN ACTIVITY AS A FUNCTION OF REVERSED-PHASE LIGAND CHAIN LENGTH

Gradient time, 10 min; for other conditions, see Table V.

Ligand	*Retention time (min)*	*Activity (%)*
C_{18}	7.75	48 ± 1
C_8	7.73	77 ± 4
C_4	7.59	81 ± 2
C_2	6.87	85 ± 2
Phenyl	8.06	73 ± 6

To assess the effect of the hydrophobic character of the reversed phase on the enzymatic activity, the same elution conditions were examined with the various reversed-phase silicas at a constant gradient time. As shown in Table VI, the pepsin activity declined as the chain length of the *n*-alkyl ligands of the reversed phase silicas increased. The highest recoveries were obtained on silica with ethyl and *n*-butyl ligands.

CONCLUSION

These studies have dealt with three test cases for protein behaviour in RP-HPLC systems. The first, typified by catalase, represents a mobile phase-induced loss of biological activity independent of any stationary phase effect. The second, typified by horseradish peroxidase, involves both stationary phase and mobile phase phenomena, which lead to an apparent loss of activity if the sample is assayed immediately after chromatography. However, if there is adequate time for re-equilibration in suitable buffers, the activity is regained (*i.e.*, the protein refolds to a "near-natural" structure). The third case, typified by pepsin, involves stability in the mobile phase but a pronounced column residence time effect. Clearly, in the latter two cases, appropriate choices of column configuration, packing material, etc., mean that there is a chance of successfully carrying out RP-HPLC with both oligomeric and monomeric proteins of low to medium molecular weight.

REFERENCES

1 A. R. Kerlavage, C. J. Weitzmann, T. Hasan and B. S. Cooperman, *J. Chromatogr.*, 266 (1983) 225.
2 H. P. Nick, R. E. H. Wettenhall, F. J. Morgan and M. T. W. Hearn, *Anal. Biochem.*, 148 (1985) 93.
3 K. Benedek, S. Dong and B. L. Karger, *J. Chromatogr.*, 317 (1984) 227.
4 S. A. Cohen, S. Dong, K. P. Benedek and B. L. Karger, in I. M. Chaiken, M. Wilchek and I. Parikh (Editors), *Affinity Chromatography and Biological Recognition*, Academic Press, New York, 1983, pp. 479–487.
5 M. T. W. Hearn, B. Grego and M.-I. Aguilar, submitted for publication.
6 K. A. Cohen, K. Schellenberg, K. Benedek, B. L. Karger, B. Grego and M. T. W. Hearn, *Anal. Biochem.*, 140 (1984) 223.
7 S. A. Cohen, Y. Tapuhi, J. C. Ford and B. L. Karger, *Anal. Chem.*, 56 (1984) 217.
8 P. G. Stanton and M. T. W. Hearn, submitted for publication.
9 S. A. Cohen, K. Benedek, Y. Tapuhi, J. C. Ford and B. L. Karger, *Anal. Biochem.*, 144 (1985) 275.

10 X. M. Lu, K. Benedek and B. L. Karger, *J. Chromatogr.*, 359 1986) 19.
11 M. T. W. Hearn, A. N. Hodder and M.-I. Aguilar, *J. Chromatogr.*, 327 (1985) 47.
12 M. T. W. Hearn, in C. J. King and J. Navratil (Editors), *Chemical Separations*, Vol. 1, Litarvan Press, Denver, CO, 1986.
13 M. T. W. Hearn and M.-I. Aguilar, *J. Chromatogr.*, in press.
14 M. L. Anson, *J. Gen. Physiol.*, 22 (1938/39) 79.
15 G. R. Schonbaum and B. Chance, in P. D. Boyer (Editor), *The Enzymes*, Vol. 8, Academic Press, New York, 2nd ed., 1963, p. 147.
16 A. C. Maehly and B. Chance, *Methods Biochem. Anal.*, 1 (1963) 147.
17 H. Sand, K. Weber and E. Molbert, *Eur. J. Biochem.*, 1 (1967) 400.
18 T. T. Herskowitz, B. Gadegbehn, and H. Jaillet, *J. Biol. Chem.*, 245 (1970) 2588.
19 A. J. Sadler, R. Micanovic, G. E. Katzenstein, R. V. Lewis and C. R. Middaugh, *J. Chromatogr.*, 317 (1984) 93.
20 M. T. W. Hearn, in preparation.
21 K. G. Paul, in P. D. Boyer (Editor), *The Enzymes*, Vol. 8, Academic Press, New York, 2nd ed., 1963, p. 227.
22 D. B. Wetlaufer, E. R. Johnson and L. M. Clauss, in E. F. Osserman, R. E. Canfield and S. Beychok (Editors), *Lysozyme*, Academic Press, New York, 1963, p. 269.
23 P. G. Righetti and T. Caravaggio, *J. Chromatogr.*, 127 (1976) 1.
24 A. J. Cornish-Bowden and J. R. Knowles, *Biochem. J.*, 113 (1969) 353.