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Note

Ion-exchange high-performance liquid chromatography of mouse epidermal growth factor and its congeners: mobile phase optimization with ion-pairing additives

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Ion-pairing agents have been used previously to extend the resolving power of reversed-phase high-performance liquid chromatography (HPLC) by imparting a degree of dynamic ion-exchange function into the system [1]. As applied to commercial preparations of mouse epidermal growth factor (EGF), they have been shown to enhance markedly the separation of the closely related multiple components of these preparations [2]. The model system which these preparations represent has been used here to examine whether hydrophobic ion-pairing agents can also be used to impart a degree of dynamic hydrophobic interaction into an ion-exchange chromatographic system, thereby enhancing its resolving power.

EXPERIMENTAL

Acetonitrile (S grade) was obtained from Rathburn Chemicals (Walkerburn, U.K.). Single glass-distilled water was prepared from Milli Q low-conductivity feedstock. The following ion-pairing agents were used: heptafluorobutyric acid (HFBA, sequencer grade, Pierce, Rockford, IL, U.S.A.), pentadecafluoro-octanoic acid (PDFOA, Aldrich, Milwaukee, WI, U.S.A.), sodium dodecyl sulphate (SDS, specially pure, Sigma, U.K.), triethylamine (TEA, SLR grade, Fisons, Loughborough, U.K.), tetrabutylammonium chloride (TBAC, Aldrich), octylamine (OA, Aldrich), dodecylamine (DDA, Aldrich). Mouse EGF preparations were culture-grade material from Sigma.

Reversed-phase HPLC of EGF was carried out using an Altex Ultrapore

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RPSC (C_3-bonded phase, 5 μm particle size) 75 mm × 4.6 mm I.D. column. Ion-exchange HPLC of EGF was carried out using a TSK IEX 545K (silica-based, DEAE-bonded phase, 10 μm particle size) 150 mm × 7.5 mm I.D. column obtained from Anachem (Luton, U.K.). Operating conditions were controlled using an Altex 340-40 chromatograph. Eluted proteins were monitored by sequential UV absorbance (280 nm, Kratos Spectroflow 773) and endogenous tryptophan fluorescence (254/340 nm, Schoeffel FS 970).

RESULTS

Composition of EGF preparation

To examine the degree of heterogeneity of the EGF preparations, reversed-phase HPLC was carried out in the presence of the hydrophobic anionic ion-pairing agent PDFOA as described previously [2]. The elution profile was seen to be complex, consisting of two major and several minor peaks (Fig. 1A). A similar profile was obtained if SDS was used as an ion-pairing agent (Fig. 1B). The major peaks have been shown to consist of $_{1\text{-}53}$EGF and $_{2\text{-}53}$EGF, respectively [2]; the minor peaks consist of bioactive EGF-like moieties, probably including oxidised and deamidated forms of $_{1\text{-}53}$EGF and $_{2\text{-}53}$EGF, being artefacts of the conditions encountered during preparation [2].

Elimination of mixed-mode chromatography

In order to study the effects of modifying the mode of chromatography

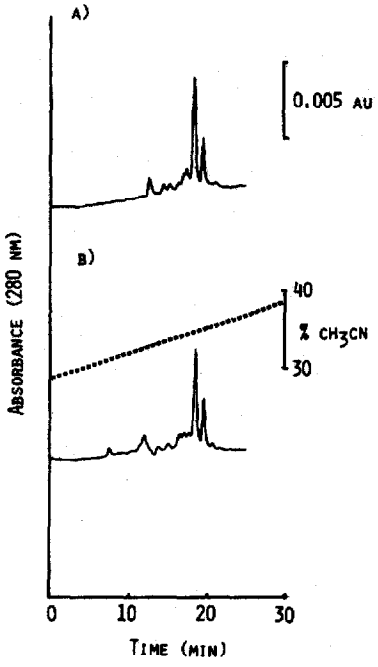

Fig. 1. Reversed-phase HPLC profiles of mouse EGF on an RPSC (C_3) column. Solvent A: 0.155 *M* sodium chloride, 10 m*M* hydrochloric acid, pH 2.1; solvent B: acetonitrile; each made 5 m*M* with PDFOA (A) or 3.5 m*M* with SDS (B). Flow-rate: 1 ml/min; temperature: 45° C; gradient of acetonitrile: 0.33% B per min as indicated; loading: 5 μg EGF.

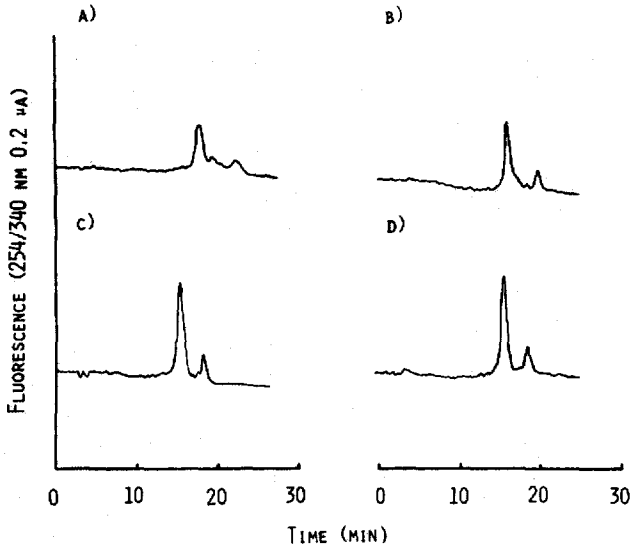

Fig. 2. Effect of acetonitrile on ion-exchange HPLC of mouse EGF on an IEX 545K (DEAE) column. Solvent A: 50 m*M* Tris—HCl pH 8; solvent B: 50 m*M* Tris—HCl, 0.5 *M* sodium chloride; each diluted with acetonitrile to a concentration of (A) 0%, (B) 10%, (C) 20%, (D) 40% (v/v). Gradient: 0—100% B in 30 min; flow-rate: 1 ml/min; temperature: ambient; loading: 2 μg EGF.

when using ion-exchange columns, it was first necessary to establish conditions under which gratuitous involvement of hydrophobic interactions was minimal. Acetonitrile was therefore added to the mobile phase at various concentrations to reduce hydrophobic interactions when EGF was chromatographed. Two major peaks of protein were observed, whose behaviour was altered in the presence of organic modifier (Fig. 2) producing a sharper peak shape. It was found that no significant change in profile was achieved by adding more than 20% acetonitrile.

TABLE I

EFFECTS OF ION-PAIRING AGENTS

Column: IEX 545K (DEAE). Solvents A and B as in Fig. 2, diluted with acetonitrile to 20%, and containing ion-pairing agents as indicated. Flow-rate: 0.5 ml/min; temperature: ambient; gradient: 0—100% B in 30 min. All results are taken from the second sequential run under conditions indicated, to allow stable chromatographic conditions to be attained.

Ion-pairing agent	Concentration (m*M*)	Time of first peak (min)	Separation time of main peaks (min)
None		25.4	3.8
Heptafluorobutyric acid	15	23.6	3.8
Sodium dodecyl sulphate	3.5	25.0*	2.3*
Dodecylamine	10.8	29.0*	6.1*
Octylamine	25	18.9	6.1
Tetrabutylammonium chloride	25	21.8**	5.5**
Triethylamine	40	19.9	5.7

*Equilibrium conditions were not attained; chromatographic efficiency deteriorated in subsequent runs.

**Results from fluorescence trace as TBAC contained UV-absorbing impurities.

Effects of ion-pairing agents

Ion-exchange chromatography was carried out in the presence of a number of ion-pairing agents, with the results shown in Table I. Only the cationic agents yielded an improved separation in this system. The limit of the effect on separation is indicated in Fig. 3. The distance between the major peaks was increased by 60%, and a third major peak was partially resolved with the addition of 25 m*M* octylamine to each solvent. It was not possible to increase the concentration of octylamine further because of its limited solubility in these solvents. Solubility constraints were even more limiting for some of the more hydrophobic ion-pairing agents.

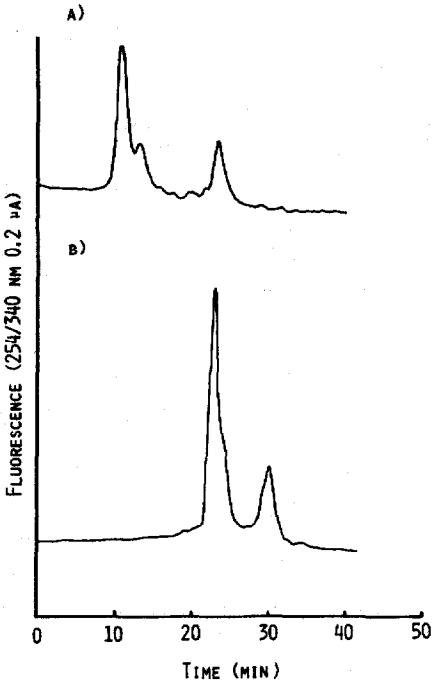

Fig. 3. Effect of octylamine on ion-exchange HPLC of mouse EGF on an IEX 545K (DEAE) column. Solvent A: 50 m*M* Tris—HCl pH 8; solvent B: 50 m*M* Tris—HCl, 0.5 *M* sodium chloride; each diluted with acetonitrile to 20%, with 25 m*M* OA (A) or without it (B). Gradient: 0—50% B in 60 min; flow-rate: 0.5 ml/min; temperature: ambient; loading: 5 μg EGF.

DISCUSSION

It has been generally recognised that the coverage of silicas by bonded stationary phases is less than perfect, with the result that HPLC is rarely performed using a single mechanism. Thus Schmidt et al. [3] observed both ionic and hydrophobic interactions when attempting size exclusion of proteins using a diol-bonded phase. Mixed-mode chromatography has been used with poorly covered reversed-phase columns [4] although this is normally associated with loss of efficiency and reproducibility with peptides and proteins [2]. Investigations of mixed hydrophobic/ion-exchange processes in the chromatography of proteins were made by Hofstee [5] using a series of alkylamine-substituted agarose matrices, and Kopaciewicz et al. [6] have used derivatised

polyethyleneimine coatings to produce HPLC ion-exchange stationary phases of varying hydrophobicities. Unwanted ionic or hydrophobic interactions may be suppressed by the use of a salt or an organic modifier, respectively, in the mobile phase [2, 3]. However, the mobile phase has not previously been constructed as a means of deliberately introducing hydrophobic interactions. That silica-based ion exchangers are not free from hydrophobic interactions is shown by the change in elution profile of EGF observed when acetonitrile is added to the mobile phase (see Fig. 2).

Once conditions are established in which these hydrophobic interactions are minimised, the effects of hydrophobic ion-pairing agents are detectable, and it is clear that hydrophobic interactions can be introduced into ion-exchange chromatography as readily as ion-exchange effects can into reversed-phase chromatography [2]. The similarity in elution profiles of EGF-related materials on separation in reversed-phase mode using quite different ion-pairing agents (PDFOA and SDS) suggests that for an ion-pairing agent of a particular charge only one effect is obtained, although its extent is different for different agents. The limiting factor for most of the ion-pairing agents is their solubility, which is low for some of the more hydrophobic agents. In this study, optimal separation was achieved with octylamine. However, the number of detectable components in these EGF preparations is less using the ion-exchange method than by reversed phase (cf. Figs. 1 and 3), mainly because the latter seems to give intrinsically more efficient chromatography. Also, in the former, anionic agents did not give useful additional separations. However, since the primary mode of separation is different, the separations achieved are also likely to be different, and ion-exchange chromatography on the anion exchanger Mono Q appears to separate deamidated forms of EGF [7] from the parent forms. This is likely to be the separation achieved in this study on the DEAE-bonded phase in the absence of ion-pairing agents. The separation of the $_{2\text{-}53}$EGF from $_{1\text{-}53}$EGF, which is the primary separation achieved by reversed-phase chromatography [2], would then account for the additional major peak observed in the presence of octylamine (Fig. 3A). Consequently this modification of ion-exchange chromatography offers the possibility of increasing the ability to achieve separations when used co-ordinately with reversed-phase chromatography. In addition such methods may make possible a type of chromatography using hydrophobic interaction for proteins which are not soluble in (e.g. ovalbumin [8]) or are dentured by (e.g. fibroblast growth factor [9]) the more extreme conditions employed in reversed-phase HPLC.

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