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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

XLVII*. ANALYTICAL AND SEMI-PREPARATIVE SEPARATION OF SEVERAL PITUITARY PROTEINS BY HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The chromatographic separation of several pituitary proteins on a Mono-Q anion-exchange column is described. The effect of eluent pH and buffer composition on the resolution is demonstrated with several standard proteins. The experimental data indicate that good protein recoveries and resolution can be obtained on this essentially monodisperse microparticulate ion-exchange resin when the pH of the eluent is chosen *ca.* 0.5 pH units below the p*I* of the most basic component in the mixture. With this new column separations are *ca.* 30 times faster than with conventional cellulosic anion exchangers at similar sample loads.

INTRODUCTION

Because of its compatibility with fully aqueous buffers, ion-exchange chromatography has been widely used as an integral part of the isolation and characterisation of proteins for more than three decades. Conventional cellulosic ion exchangers often exhibit low rates of mass exchange with proteins. This generally necessitates low flow-rates, and a low rate of change of appropriate counterions or pH if acceptable levels of resolution are to be achieved. Further, the chromatographic swollen volume of these exchangers may also be affected by changes in flow-rate, ionic strength, pH, temperature and type of buffer used. Over the past several years, ion-exchange packing materials which are more mechanically stable, resistant to matrix deformation in the presence of various buffer ionic components or solvents and useable at high flow-rates have attracted increasing attention. Spherical, porous polystyrene (PS)–divinylbenzene (DVB) copolymers, bonded to appropriate ionic functional groups, having particle diameter of 6 to 12 μm and 8–12% cross-linking, have been available for some time and widely used for the separation of amino acids and small peptides. However, many PS–DVB ion-exchange resins suitable for polypeptide and protein

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separation are also deformed at high flow-rates and, due to the lower extent of cross-linkage, they are sensitive to eluent composition. To circumvent these disadvantages, a number of rigid organic resins, *e.g.* DEAE- and CM-Spheron, and a variety of fully porous inorganic ion exchangers, *e.g.* Toyosoda TSK-545 DEAE or SynChropak AX, have been investigated[1,2]. In this paper, we examined several of the chromatographic characteristics of the recently introduced[3] monodisperse Mono-Q packing material, a composite hydrophilic polyether anion exchanger. Its application to the purification of several pituitary proteins is now described.

MATERIALS AND METHODS

Equipment

A Pharmacia (Uppsala, Sweden) Fast Protein Liquid Chromatography (FPLC) system was used which consisted of two P-500 syringe pumps, a V-7 injector and a 278-nm fixed-wavelength single-path UV monitor, coupled to a two-channel REC 482 pen recorder. Gradient elution was controlled with a GP-250 programmer. Mono-Q prepacked columns (HR 5/5) 50 × 5 mm I.D., were used throughout. Column effluents were collected with a FRAC-100 fraction collector. Sample injections were made with a 250-μl Type A-RN liquid syringe obtained from SGE (Melbourne, Australia). All chromatographic separations were carried out at ambient temperatures (approx. 20°C). The flow-rate for the protein hormone isolations was 1.0 ml/min.

Buffers and samples

Piperazine, tris(hydroxymethyl)aminomethane (Tris) and bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris) were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium chloride (Univar grade) was from Ajax (Sydney, Australia). Distilled water was further purified on a Milli-Q system (Millipore, Bedford, MA, U.S.A.). All buffers were filtered through 0.45 μm cellulose acetate filters (HAWP 04700) from Millipore. The pH measurements were made with a combination glass electrode attached to a Radiometer pHM62 meter (Radiometer, Copenhagen, Denmark).

Sperm whale myoglobin, horse heart cytochrome *c*, human transferrin, hen egg ovalbumin, hen egg lysozyme and bovine thyroid stimulating hormone (bTSH, 1 IU/mg) were obtained from Sigma. Pentex bovine serum albumin (BSA) was obtained from Miles Biochemicals (Elkhart, IN, U.S.A.). Crude ovine prolactin and ovine thyroid stimulating hormone were isolated in this laboratory, essentially following the procedure of Reichert[4] as modified by Stanton *et al.*[5]. Human prolactin was isolated according to the method of Hwang *et al.*[6]. Immunoreactive prolactin in column fractions was determined in competitive binding radioimmunoassays using highly purified radio-iodinated prolactin tracer and radio-inert standards. A commercial radioimmunoassay kit from Diagnostic Products Corp. (Los Angeles, CA, U.S.A.) was used for the human prolactin determinations. Highly purified bTSH (30–40 IU/mg, kindly supplied by Dr. J. G. Pierce) was radio-iodinated by established methods[5,7] using lactoperoxidase to a specific activity of *ca.* 30 μCi/μg. Radioactivity in column fractions was quantitated with a Packard Auto-Gamma scintillation spectrometer Model 5360 (Packard Instruments, Downers Grove, IL, U.S.A.).

RESULTS AND DISCUSSION

Because of their biological importance, much experimental effort has been expended over the past two decades on methods for the extraction and purification of protein hormones from human and animal pituitary glands. In order to permit optimal yields of these proteins, several mild isolation procedures[4,5,8,9] have been devised. Conventional open column gel permeation chromatography and ion-exchange chromatography have often formed an integral part of these isolation schemes, particularly during the latter stages of the purification. Although these chromatographic procedures permit good mass and biological recoveries under appropriate elution conditions they are time consuming and frequently lack sufficient resolving power. Over the past several years the attention of this laboratory has been directed to the use of high-performance liquid chromatographic (HPLC) techniques for the purification of polypeptides and proteins at the micropreparative (low μg) through prepar-

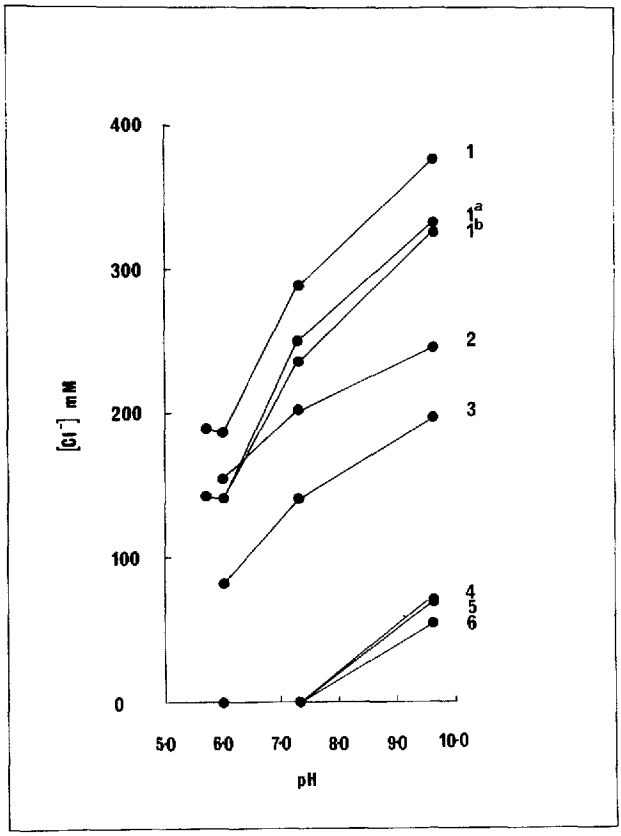

Fig. 1. Effect of eluent pH and buffer composition on resolution of several protein standards on a Mono-Q anion-exchange column. In each experiment, the rate of change of [Cl^-] was 17.5 mM/min and the flow-rate was 1.0 ml/min. The primary eluent buffers were 20 mM Bis-Tris–HCl (pH 5.70), 20 mM piperazine (pH 6.0), 20 mM Tris–HCl (pH 7.2) and 20 mM piperazine (pH 9.6). Proteins: 1 = BSA; 2 = ovalbumin; 3 = transferrin; 4 = sperm whale myoglobin; 5 = hen egg white lysozyme; 6 = cytochrome c, components 1a, 1b correspond to BSA aggregates.

ative (multigram) level. Because of its uniform particle size and distribution (particle diameter, d_p = 9.8 ± 2% μm) and high small-ion capacity (0.28–0.36 mmol/ml) the Mono-Q anion exchanger appeared well suited for the purification of individual pituitary proteins, particularly for relatively small amounts, *e.g.* 1–10 mg, of a specific component. Previously, Hearn and co-workers[10,11] demonstrated that a variety of basic non-atmospheric buffers such as piperazine and Bis-Tris exhibit a number of advantages for the separation of proteins, including pituitary proteins, in buffer electrofocusing experiments and on soft gel ion-exchange columns under chromatofocusing conditions. In preliminary experiments with several protein standards, the performance of the Mono-Q packing material was examined with several similar buffer combinations known to exhibit good buffering capacity at the operational pH of the eluent.

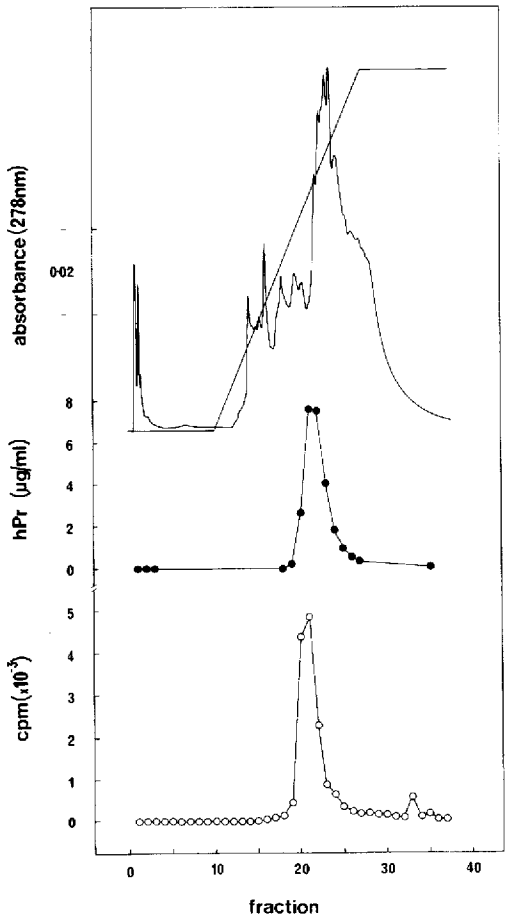

Fig. 2. Top: separation of a crude human pituitary fraction containing human prolactin (hPr) on a Mono-Q anion-exchange column with an elution gradient from 20 m*M* piperazine (pH 9.6) to 20 m*M* piperazine–300 m*M* sodium chloride (pH 9.6), flow-rate 1.0 ml/min. A 17-min linear gradient started 10 min after sample injection. Middle: hPr immunoreactivity profile of the recovered chromatographic fractions. Bottom: elution profile of [^{125}I]hPr chromatographed under the same conditions.

Fig. 1 shows typical data for the change in resolution of these standard proteins as a function of pH, where their effective elution volumes (expressed as the ionic strength values at the column inlet for a standard 0–400 m*M* linear sodium chloride gradient) are plotted against eluent pH. Although the elution order of these proteins effectively followed their respective p*I* values, resolution was clearly pH-dependent, the optimal separation for these standard proteins occuring when the pH of the eluent was *ca.* 0.5–1.0 pH units below the p*I* of the most basic component and within 0.5 pH unit of the pK_a of the buffer. Further, resolution remained essentially unchanged at flow-rates from 0.5–2.5 ml/min provided the gradient time was proportionately adjusted to ensure that the $\frac{d\,[Cl]}{dt} \cdot v^{-1}$ factor[2] was constant. However, at a constant flow-rate an apparent increase in resolution was evident with longer gradient times due to the larger gradient elution volumes involved (see also Fig. 4).

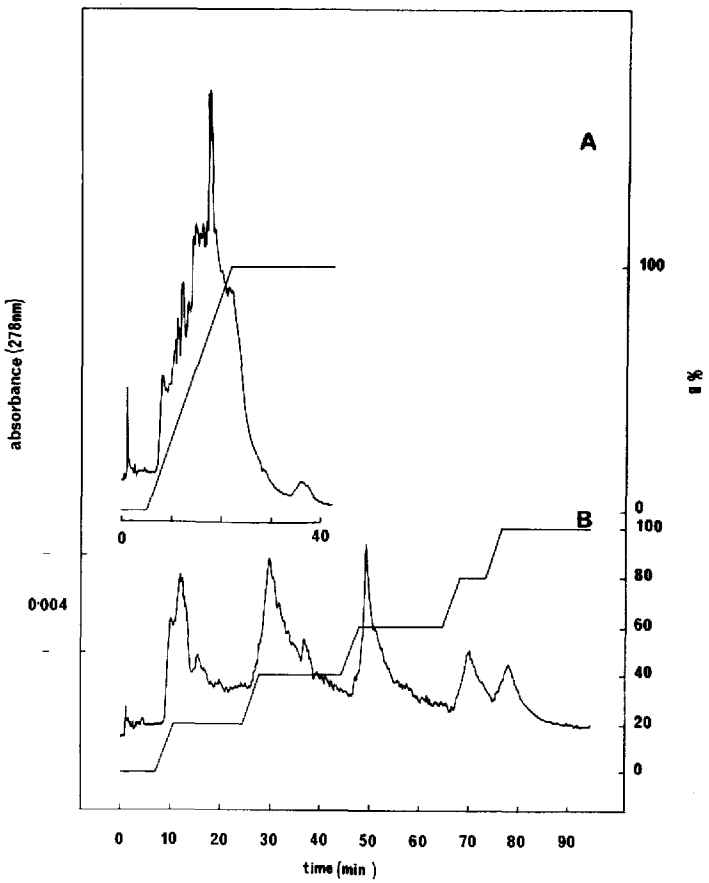

Fig. 3. (A) Separation of oPr preparation on a Mono-Q anion-exchange column with an elution gradient from 20 m*M* piperazine (pH 9.6) to 20 m*M* piperazine–300 m*M* sodium chloride (pH 9.6). A linear 17-min gradient started 5 min after sample injection. (B) Separation of the oPr preparation with the same buffer system but a segmented gradient: 0–5 min, hold, 5–8.4 min (0–20%) linear, 8.4–25 min hold, 25–28.4 min (20–40%) linear, etc. The sample load in each case was 250 μg in 100 μl. Flow-rate, 1.0 ml/min.

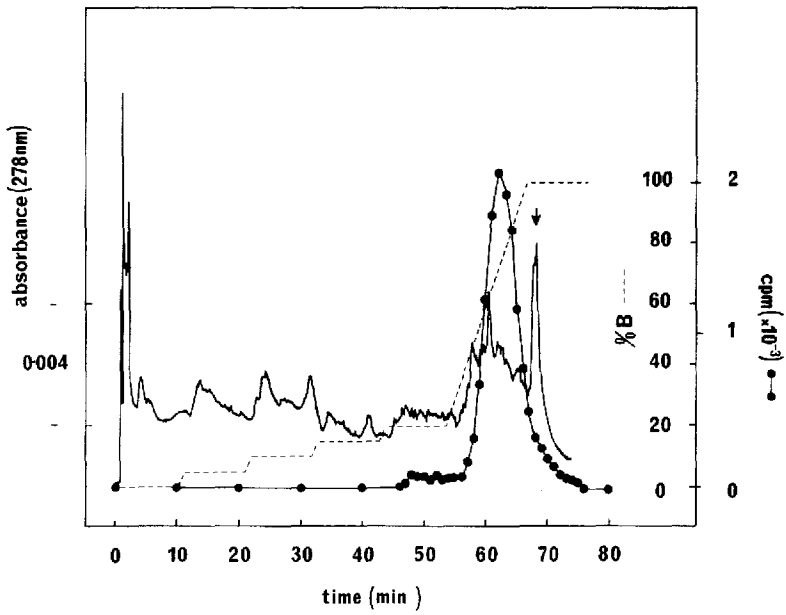

Fig. 4. Separation of a crude bTSH preparation on a Mono-Q anion-exchange column by elution with a segmented 100-min gradient from 20 m*M* piperazine (pH 9.6) to 20 m*M* piperazine–300 m*M* sodium chloride. The elution profile of [^{125}I]bTSH chromatographed under identical conditions is also shown. The elution position of serum albumin is indicated by the arrow.

Fig. 2 shows the chromatograms obtained with a crude human pituitary extract containing human prolactin (hPr) separated on a Mono-Q column with a linear gradient from 20 m*M* piperazine (pH 9.6) to 20 m*M* piperazine–300 m*M* sodium chloride (pH 9.6). Also shown in Figs. 2 are the immunoreactivity profile for this hPr preparation in the recovered chromatographic fractions and the radioactivity profile for [^{125}I]hPr, respectively. The total recovery of hPr under these conditions was 85%. Fig. 3 shows the separation of a crude ovine prolactin (oPr) preparation under similar chromatographic conditions. These and similar experiments confirm that the Mono-Q columns permit fast, convenient purification of prolactin preparations with sample capacities in the range of *ca.* 50 μg–20 mg protein.

Fig. 4 demonstrates the applicability of this new anion exchanger and step-gradient elution to the resolution of a crude bTSH preparation. Also shown in Fig. 4 is the elution profile for ^{125}I-BTSH under similar conditions. The total recovery of radioactivity was 84%. Compared to the separation of bTSH preparations on conventional soft gel anion exchangers[5], the new method is approximately 30 times faster. Recoveries at similar loads are comparable, while zones are *ca.* 10–20 times less diluted, because smaller gradient volumes are used. In view of the known structural microheterogeneity of the thyrotropins (and other glycoprotein hormones) the improvement in resolution and speed of separation indicated above enables the separation of structurally related glycoprotein hormone components exhibiting different biological potencies. Results of these studies will be reported elsewhere[5].

In summary, the results obtained with the separation of a variety of protein hormone preparations on Mono-Q anion exchanger show that this packing material

is a versatile addition to the currently available materials for their purification and analysis. The speed of separation, in combination with manipulation of pH and ionic strength of the eluent, allow complex mixtures of these proteins to be rapidly separated. Load capacities extend from the range required for microsequence determination, *i.e. ca.* 1 nmole, to the semipreparative level. Our data also indicate that linear gradients, where the rate of change of chloride ions (or an alternative counter ion) with time is held constant, will not necessarily produce optimal resolution of complex mixtures of the pituitary proteins. Better resolution can however be achieved with non-linear or stepwise elution gradients.

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