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Note

Separation of acidic proteins from mineralized tissues by reversed-phase high-performance liquid chromatography

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Proteins of which approximately a quarter or more of their amino acids are aspartic acid (Asp) or glutamic acid (Glu) are important constituents of mineralized skeletons. They have been identified and partially characterized in vertebrate tooth dentine[1] and enamel[2], vertebrate bones[3,4], mollusk shells[5] and coral skeletons[6] and are inferred to be present in the mineralized tissues of a number of other different phyla. Their widespread distribution, their observed rapid migration after biosynthesis to the mineralization front in dentine[7] and their oft-demonstrated potential for binding calcium ions suggest that the acidic proteins perform important, but as yet poorly understood, functions in the process of crystal growth during mineralization. Part of the problem to date is due to the numerous technical difficulties encountered when analysing these unusually acidic molecules.

Since their initial identification in tooth dentine[1], further characterization of acidic matrix proteins from mineralized tissues has proven to be unusually difficult. This is in part due to their tendency to aggregate rapidly, necessitating, for example, the use of dissociative conditions during molecular sieve chromatography[2]. They are relatively labile under mild conditions and hence exposure to pH extremes has to be avoided. They cannot be assumed to adopt a globular conformation and therefore their chromatographic behaviour is often anomalous when compared to "standard" proteins. Furthermore, they do not bind many of the commonly used protein stains. One good method for isolating this class of proteins from other matrix constituents is ion-exchange chromatography[2,3,5]. As the method is usually not effective in separating the different acidic proteins themselves, I have developed a new technique using reversed-phase high-performance liquid chromatography (RP-HPLC). In this report I illustrate the technique using acidic proteins from a mollusk shell in which approximately a third of the amino acid residues are aspartic acid.

MATERIALS

The body chamber of a freshly collected *Nautilus repertus* (Palau) shell was used as a source of acidic proteins. Technical grade EDTA (Fluka, Buchs, Switzerland) was used for decalcification. All the solvents and chemical reagents used for preparing the HPLC buffers were spectra grade or analytical grade, respectively.

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METHODS

Preparation of the acidic protein fraction for liquid chromatography

The aragonite of the body chamber was dissolved in 10% (w/v) EDTA pH 7.0 and the fraction soluble after demineralization was purified according to the method of Weiner[5]. The total soluble fraction (about 150 mg including some non-dialyzable adsorbed EDTA) was fractionated on a DE 52 ion-exchange column (Whatman, Maidstone, Great Britain) under conditions described by Weiner[5]. Both a linear NaCl gradient and two step gradients (0.35 *M* NaCl, 0.55 *M* NaCl) were used. The fraction which eluted with the onset of the 0.55 *M* NaCl flush, was collected and concentrated from about 20 to 8 ml with a stream of nitrogen at room temperature. Prior to analysis, 0.5-ml aliquots were dialyzed overnight against 300 ml of buffer (A) (see below).

Chromatographic apparatus and conditions

A high-performance liquid chromatograph with a Waters UV detector (Model 440, wavelenght 254 nm), an Eldex pump (Model A-30-S), a Rheodyne injector (Model 7125) with a 100-μl sample loop and a simple low-cost programmer[8] was used. A reversed-phase column (200 × 4.6 mm I.D.) self-packed with C_{18} Spherisorb S5 ODS2 (5 μm) (Phase Separations, Hauppauge, NY, U.S.A.) was used. The column was equilibrated with buffer (A) comprising 0.05 *M* sodium acetate pH 6.50, 2.5% acetonitrile. Buffer (B) comprised 0.05 *M* sodium acetate pH 6.50, 50% acetonitrile. The pH of both buffers was readjusted to 6.50 with glacial acetic acid after addition of the acetonitrile and before the buffers were degassed under reduced pressure in an ultrasonic bath for a few minutes. When using the low pressure gradient maker, care must be taken to maintain both buffers under equal hydrostatic pressures. A linear 45-min gradient was initiated immediately after sample injection. The proteins were eluted at room temperature at a flow-rate of 0.6 ml/min. When all the peaks had been eluted, in this case 35 min after injection, 100 μl of dimethylsulphoxide (DMSO) were injected in order to release the remaining material from the column. After the DMSO had been eluted from the column, buffer (B) was pumped for an additional 10 min. Re-equilibration with buffer (A) was achieved in 15 min.

Amino acid analysis

The fractions eluted were dried under vacuum and then hydrolyzed under reduced pressure in 0.5 ml 6 *N* HCl at 110°C for 20 h after flushing twice with nitrogen. The hydrolysates were analyzed on a Durrum D500 amino acid analyzer. The amount of protein in each fraction was calculated from the molar yields of amino acids obtained. The fraction which elutes with DMSO was diluted in 20 times its volume of double distilled water, dialyzed (Spectrapor 3 tubing) against two changes of 1 l of double distilled water, lyopholized and then hydrolyzed under the above conditions.

N-Terminus analysis

Corresponding peaks from three different runs were pooled, dried and then dansylated under denaturing conditions for proteins according to the method of Zanetta *et al.*[9]. After hydrolysis (110°C for 16 h in 6 *N* HCl) the dansylated amino

acids were fractionated by HPLC under conditions which the major amino acids commonly found in proteins are separated, according to the procedure of Weiner and Tishbee[10].

RESULTS

Ion-exchange chromatography using a linear sodium chloride gradient separates the more acidic proteins (which elute between 0.35 *M* and 0.55 *M* NaCl)[5] from the remaining matrix constituents. Only poor separation, however, is achieved between the different acidic proteins themselves (Fig. 1). In order to prepare a well defined fraction for HPLC analysis, a sodium chloride step-gradient is used. The bulk of the acidic proteins elute at the onset of the 0.55 *M* NaCl flush (fraction C, Fig. 2). Under the HPLC chromatographic conditions described, fraction C is separated into many sub-fractions (Fig. 3), almost all of which contain protein as determined by amino acid composition analysis (Table I).

Some of the peaks which elute prior to the onset of the gradient may be due to trace contaminants such as Tris which were not completely removed by dialysis. If dialysis against buffer (A) prior to analysis is omitted, these peaks are greatly enhanced. The broad shape of the major protein-containing peaks is similar to that observed in many protein separations using HPLC. Raising the column temperature to 40°C, reducing the quantity of material loaded onto the column or using a C_8 sorbent instead of a C_{18} sorbent does not significantly alter the shape of the peaks. The major protein-containing fractions elute at relatively low concentrations of acetonitrile (about 5–25%). Addition of isopropanol to the buffers causes the retention time to be reduced, whereas lowering the buffer pH to 3.5 prevents elution of the

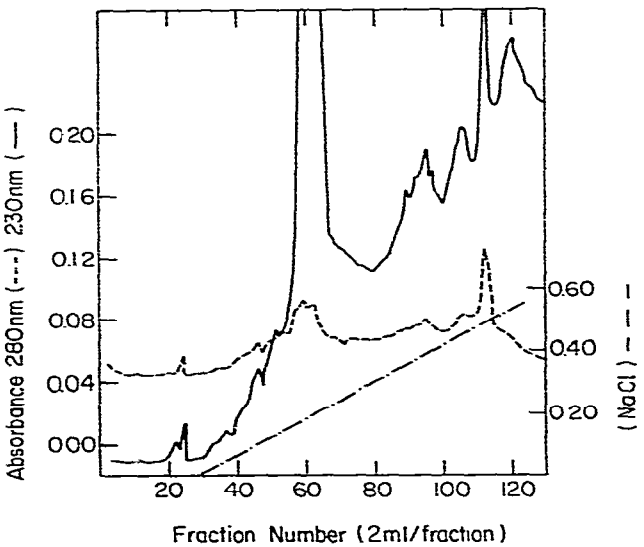

Fig. 1. DEAE-cellulose ion-exchange chromatography in 0.05 *M* Tris pH 8.20 buffer. About 10 mg of the soluble organic matrix fraction were eluted with a linear 0 to 0.6 *M* NaCl gradient. Contaminating EDTA elutes at about 0.2 *M* NaCl. The column was flushed at the end of the run with 6.0 *M* urea, 1.0 *M* NaCl.

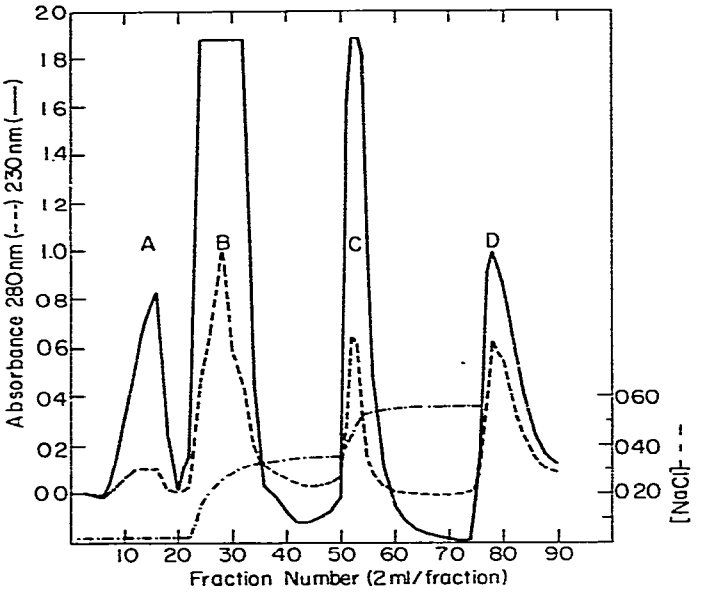

Fig. 2. DEAE-cellulose ion-exchange chromatography in 0.05 *M* Tris pH 8.20 buffer. About 150 mg of the soluble organic matrix fraction were eluted with two sodium chloride step gradients (0.35 *M* and 0.55 *M* NaCl) and a flush of 6.0 *M* urea, 1.0 *M* NaCl at the end of the run. Contaminating EDTA elutes in fraction B. Fraction C contains the bulk of the acidic proteins.

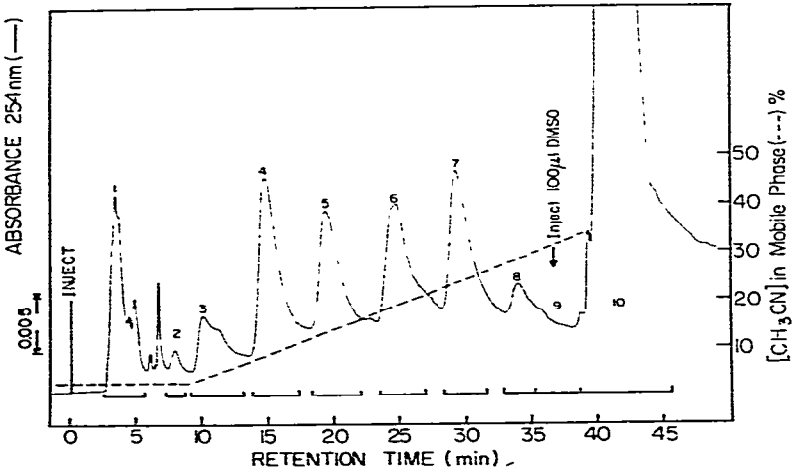

Fig. 3. The elution profile (original chromatogram) obtained for about 110 µg of fraction C (see Fig. 2) when analyzed on the C_{18} column. The peaks were collected for amino acid composition analysis as designated. The UV absorption of fraction 10 is primarily due to DMSO. The gradient shown corrects for the time-lag between the onset of the gradient program which is concurrent with sample injection and the appearance of the first elevated concentrations of acetonitrile in the detector.

TABLE I

AMINO ACID COMPOSITIONS (mole %) OF THE FRACTIONS ELUTED BY HPLC AFTER INJECTION OF 0.11 mg OF *N. REPERTUS* FRACTION C OBTAINED FROM THE ION-EXCHANGE COLUMN

Cys is absent or present in very small amounts in all samples; tr = trace amounts.

	Fraction no. (see Fig. 2.										
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Fraction C prior to injection*
Asp + Asn*	29.57	32.54	43.37	43.84	42.65	37.23	33.00	29.78	17.37	10.74	34.84
Thr	2.01	1.94	0.33	0.27	0.47	1.14	1.47	1.96	4.29	5.16	1.24
Ser	10.66	7.97	3.11	2.65	2.31	1.93	2.37	4.29	7.73	18.02	3.67
Glu + Gln*	8.54	6.25	1.31	1.24	1.77	4.01	6.69	8.70	10.31	16.09	4.14
Pro	1.64	tr	tr	0.13	0.59	1.97	2.67	3.68	3.15	3.74	1.66
Gly	31.83	31.46	41.62	38.98	38.80	33.60	31.00	29.17	20.99	19.02	35.04
Ala	6.83	2.16	0.10	0.29	1.51	3.24	2.89	2.57	11.93	2.24	3.12
Val	2.19	2.80	0.35	0.30	0.49	0.78	0.86	0.61	5.63	5.01	1.28
Met	1.09	tr	0.18	tr	tr	0.11	0.15	0.98	1.72	0.79	0.09
Ile	1.30	1.72	0.63	0.76	0.57	0.63	1.24	1.84	3.34	2.86	1.11
Leu	0.89	1.29	0.61	0.44	0.69	0.74	0.83	0.74	3.24	5.01	0.56
Tyr	tr	2.16	1.49	2.52	2.59	2.86	4.43	3.43	1.05	1.65	1.66
Phe	tr	2.80	0.68	0.38	0.47	0.72	1.58	1.96	2.58	1.76	0.94
His	1.50	2.80	3.41	4.30	3.65	4.12	4.58	4.04	1.91	2.54	3.93
Lys	1.84	2.80	2.78	2.69	2.61	3.78	6.28	6.62	4.77	4.22	4.18
Arg	tr	1.29	tr	1.24	0.87	3.11	tr	tr	tr	1.13	2.56
Proportion (%) of protein in each fraction	3.6	1.1	9.9	18.7	15.5	14.4	6.6	2.0	2.6	25.6	

* Weiner[5] reports that more than about 80% of the aspartate and glutamate is in the form of aspartic and glutamic acid respectively.

proteins under the described conditions. The addition of EGTA (Sigma, St. Louis, MO, U.S.A.) to the sample (to form a 20 m*M* solution) prior to injection does not fundamentally change the chromatogram except slightly to enhance the shoulder which elutes after fraction 6. The addition of EGTA to the buffers causes spurious peaks to form, presumably because of the relative insolubility of EGTA in acetonitrile.

The amino acid compositions of the peaks in Fig. 3 vary considerably (Table I) indicating that different proteins or assemblages of proteins are being separated. Significantly, peaks 3, 4 and 5 (Table I) have almost identical amino acid compositions, suggesting that these peaks represent different aggregation states of a single protein or that the proteins separated are very similar. There is no obvious trend in amino acid compositional changes of the proteins which corresponds to increasing retention time on the reversed-phase column. A more direct assessment of peak homogeneity was attempted by determining the N-termini of the different fractions. Despite the fact that large excesses of protein were used and the released Dns- (5-dimethylaminonaphthalene-1-sulphonyl) amino acids were examined using a very sensitive and quantitative HPLC separation procedure[10], no N-termini were identi-

fied. Presumably, the N-termini are blocked, and thus no information on peak homogeneity could be obtained using this approach.

The total yield of amino acids obtained from the fractions analyzed (Fig. 3) accounts directly for about 70% of the protein loaded on the column. As the mobile phase between peaks was not analyzed it is difficult reliably to estimate the recovery of protein from the column in this case. In another experiment, under the same conditions, a recovery in excess of 90% was obtained. It thus appears that almost all the protein is recovered by this procedure. Flushing the column with DMSO at the end of the run is, however, essential completely to recover the sample injected (Table I).

DISCUSSION

The technique described offers certain advantages over some other reversed-phase HPLC methods used for protein separation. The close-to-neutral pH of the sodium acetate buffers most likely preserves the *in vivo* conformations of the proteins, thus facilitating the separation of closely related proteins. These conditions do not, however, prevent aggregation. Low-pH phosphate buffers have been used successfully for protein separations[11]. Besides the danger of denaturation or spurious peptide bond cleavage, the presence of phosphate may interfere with the detection of phosphorylated amino acids, often present in unusually acidic proteins[1,4]. Pyridine–formate pH 4.0 buffers have also been used for protein separations[12]. These obviate the use of a fluorescence detection system and hence pre-column or post-column labelling of the proteins with an appropriate fluorescent reagent. The sodium acetate–acetonitrile system is transparent to relatively low wavelengths where the proteins absorb strongly. Some advantages of this method over standard protein separation procedures, including ion-exchange chromatography, are the greatly improved resolution of the proteins under examination, the short elution times and the high sensitivity of the detection system. For the study of mineralized tissue matrix constituents, the ability to detect and recover microgram quantitities of protein is of particular importance as in many cases, *e.g.*, forming enamel, only very small amounts of material are available.

This method thus provides a means of separating proteins which have proved to be unusually difficult to isolate and characterize. The technique is particularly effective when used after the acidic proteins have been separated from other matrix constituents by ion-exchange chromatography. Furthermore, it may well be applicable to acidic proteins not found in mineralized tissues. Preliminary results indicate, for example, that α-lactalbumin and parvalbumin are separated from each other under the conditions described.

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