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Note

Ion-exchange columns in high-performance liquid chromatography of proteins; a simple loading technique that improves resolution

ALEXANDER VARDANIS
Research Centre, Agriculture Canada, University Sub Post Office, London, Ontario, N6A 5B7 (Canada)
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The introduction of new media in the past few years has made the separation of proteins by high-performance liquid chromatography (HPLC) a popular technique. One of the available methods for these separations is ion exchange, where the protein is adsorbed onto a column at a specific pH and ionic strength and subsequently eluted by altering one or both of these parameters. Ion-exchange columns used in HPLC separations are not characteristically of high resolution, particularly if one is trying to separate a single component out of a complex mixture of proteins. We have found that if the sample is applied onto the column with a decreasing gradient of ionic strength and eluted with an increasing gradient, in many cases one obtains more efficient separations.

MATERIALS AND METHODS

The HPLC apparatus was a Waters system consisting of two pumps, a manual injector, a Model 441 absorbance detector and an 840 system controller. The experiments were done with two different anion-exchange columns, the Waters Protein Pack DEAE-cellulose 5PW (7.5 cm × 7.5 mm I.D.) and the Pharmacia Mono Q HR 5/5 (50 × 5 mm I.D.). The sample was injected either in the absence or in the presence of various concentrations of sodium chloride up to 0.4 *M*. Thus, in the case of the sodium chloride-containing samples, as the running buffer carried the sample to and through the column, proteins were adsorbed in the presence of a decreasing sodium chloride gradient. Adsorbed proteins were eluted with an increasing gradient of sodium chloride in the usual manner.

With some experiments, the method is shown as it is used to separate cyclic nucleotide-dependent protein kinases from a crude mixture of proteins. The protein kinase assay used, measures incorporation of ^{32}P from [γ-^{32}P]ATP into histone. The 75-μl incubation mixture contained 10 m*M* potassium phosphate (pH 6.8), 0.24 m*M* [γ-^{32}P]ATP (8000 to 20 000 cpm/nmol), 3.24 m*M* magnesium acetate, 50 m*M* sodium fluoride, 6.6 mg/ml of histone II or IIA (Sigma), 1 μ*M* cyclic AMP and enzyme. Incubation was for 10 min at 30°C. The reaction was stopped by spotting an aliquot (50 μl) of the mixture on a phosphocellulose filter paper disk and immersing in 75 m*M* phosphoric acid according to the method of Roskoski[1].

The protein mixtures applied to the columns were high-speed (100 000 *g*) su-

pernatant solutions obtained from 20% (w/v) homogenates of mouse skeletal muscle or an insect preparation previously described[2], prepared in 10 m*M* potassium phosphate (pH 6.8). In one of the runs described the preparation was an enzyme peak fraction from a DEAE-cellulose open column fractionation of an insect protein kinase. Ferritin and soyabean trypsin inhibitor were obtained from Sigma.

RESULTS AND DISCUSSION

The original observation was made in a series of experiments where an enzyme preparation from an open column DEAE-cellulose separation was applied to an anion-exchange HPLC column. The starting sample was a pooled concentrated fraction that contained approximately 0.075 *M* sodium chloride. Following routine procedure we dialyzed away the salt before proceeding with the injection onto the HPLC column. The profile of this run was rather unimpressive in terms of resolution of the components in the protein mixture (Fig. 1A). When, however, we injected the same sample without bothering to eliminate the salt, several components of the mixture separated from each other with good resolution (Fig. 1B).

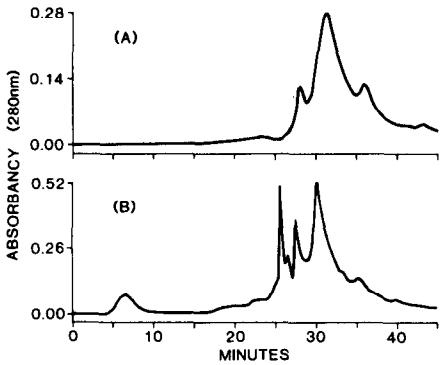

Fig. 1. Fractionation by HPLC of a preparation obtained by open column anion-exchange chromatography. In A the solution (200 μl) was injected after elimination of sodium chloride by dialysis. In B the solution (200 μl) was injected in the presence of approximately 0.075 *M* sodium chloride. Column: Waters Protein Pack DEAE-cellulose 5PW. Buffer A: 10 m*M* potassium phosphate (pH 6.8), buffer B: 10 m*M* potassium phosphate (pH 6.8) + 0.2 *M* sodium chloride. Elution by a linear gradient 0–100% buffer B at 5–35 min; held at 100% buffer B at 35–45 min. The column was washed with 1 ml of 1 *M* sodium chloride in buffer A and equilibrated with buffer A between injections. Flow-rate: 0.5 ml/min.

We made the assumption that the probable reason for this result was that when the sample is injected in the presence of salt and as the running buffer carries the sample through the column, some salt dilution occurs and thus proteins adsorb in the presence of a decreasing gradient of ionic strength. Only the highly charged proteins are adsorbed at or near the head of the column, with other species adsorbing through the length of the column as the ionic strength decreases. Thus some separation occurs during loading and is superimposed on the separation obtained when elution is effected through the usual application of an increasing gradient of ionic strength. With the above in mind we set out to run a number of experiments with different protein mixtures in order to investigate the utility of this technique. Two

typical examples are shown here. In the series of runs shown in Fig. 2 a high-speed supernatant solution from a mouse skeletal muscle homogenate was applied to a Waters anion-exchange column. The injections (200 μl) were in the absence of added salt (A) and in the presence of 0.1, 0.2, 0.3 and 0.4 *M* sodium chloride (B, C, D and E, respectively). As can be seen the addition of salt prior to injection resulted in the spreading out of the elution profile of the adsorbed proteins with some species now being well resolved from the rest of the mixture. In order to show the extent of possible resolution we have enlarged the 20–25 min section of the chromatograms of Fig. 2 and present them in Fig. 3. As can be seen the species that elutes with a retention time of 20.8 min in chromatogram E was pulled out from the rest of the mixture and can be obtained in relatively pure form, by the simple addition of 0.4 *M* sodium chloride to the preparation before injection.

The possibility existed that when a single sample compound is loaded under the above conditions, it is eluted as more than one peak. If this was the case the improved resolution would be at least partly due to an artifact. In order to investigate

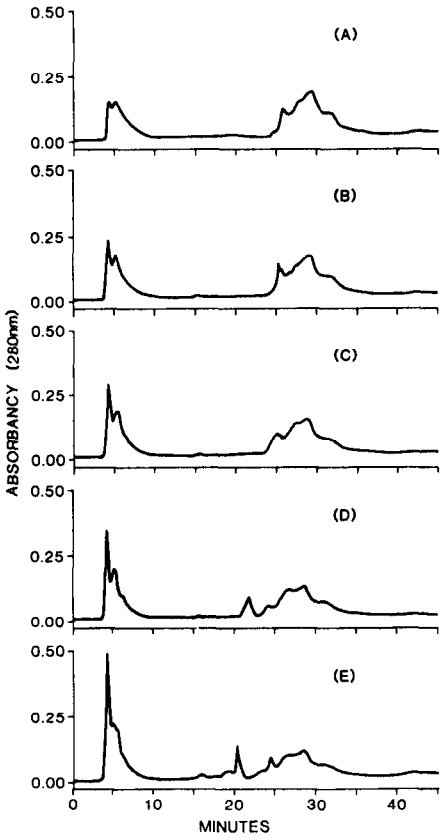

Fig. 2. Fractionation of a high-speed supernatant solution of a mouse skeletal muscle homogenate. Column: Waters Protein Pack DEAE-cellulose 5PW. Injections (200 μl) in the absence of added salt (A) or in the presence of 0.1, 0.2, 0.3 and 0.4 *M* sodium chloride (B, C, D and E, respectively). Elution and column equilibration and flow-rate as for Fig. 1.

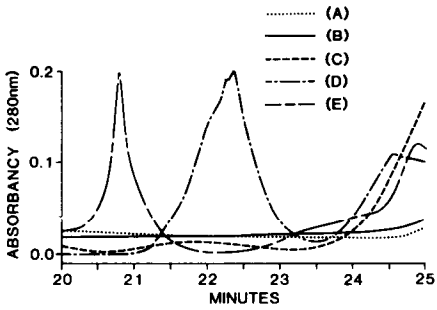

Fig. 3. Enlargement of the 20–25 min segment of the chromatograms of Fig. 2. The letter legends correspond to those of Fig. 2.

this possibility we subjected two purified proteins to the loading technique under the conditions outlined in the legend to Fig. 2. Soyabean trypsin inhibitor (100 μg) was eluted as a single peak with elution times of 31.7, 30.1 and 29.9 min when injected in 200 μl of buffer alone, buffer + 0.2 *M* sodium chloride and buffer + 0.4 *M* sodium chloride, respectively. Ferritin (100 μg), on the other hand, retained its elution time (32.5 min) in all of the above three methods of loading. We did not observe any peak splitting with these two purified proteins.

In another series of experiments we used a preparation from insect tissue that we already knew contained at least three species of cyclic nucleotide-dependent protein kinase. The enzymes in this mixture have been previously resolved in open column chromatography[2]. The preparation was applied to a Pharmacia anion-exchange column (Mono Q HR 5/5) in the absence of any added salt (Fig. 4A) and in the presence of 0.1 or 0.2 *M* sodium chloride (Fig. 4B and C, respectively). It can be seen that when the material was injected in the absence of salt the three enzymes were not resolved. The simple addition of 0.2 *M* sodium chloride before injection resolved at least three peaks of enzyme activity.

We have found that the effects of salt addition are not always predictable in terms of making a specific protein elute earlier. Some proteins will retain their elution time even at relatively high concentrations of salt at injection. In fact, with one of our enzyme preparations (results not shown here) we were able to obtain enzyme of much higher specific activity by this technique, by causing earlier elution of the majority of contaminating proteins while our enzyme retained its elution time. An example along these lines can be seen by comparing chromatograms A and B of Fig. 4.

In general this technique resulted in earlier elution of the protein of interest, a fact that was of considerable advantage especially in the case of enzymes that were as a result minimally exposed to room temperature. Another possible advantage in terms of enzyme stability is the fact that salt, at the concentrations used, often stabilizes some enzymes.

It should be pointed out that the size of the injected sample is an important parameter in this technique. Too large a sample would not allow enough dilution during application to make the technique effective. We have found that with the standard HPLC ion-exchange columns (*e.g.* 7.5 cm × 7.5 mm I.D.) the injection volume should be kept below 500 μl.

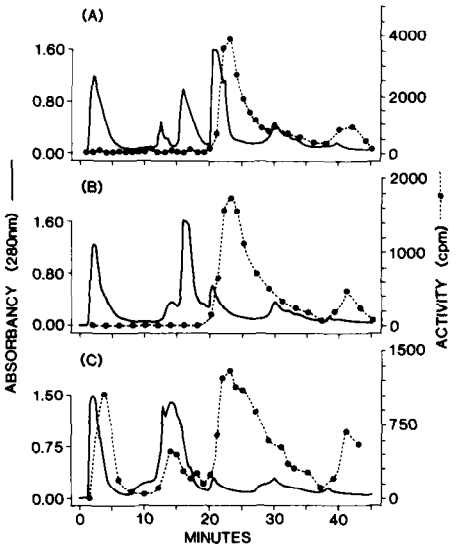

Fig. 4. Fractionation of a high-speed supernatant solution of an insect thorax homogenate[2]. Column: Pharmacia Mono Q HR 5/5. Injections (500 μl) in the absence of added salt (A) or in the presence of 0.1 and 0.2 *M* sodium chloride (B and C, respectively). Elution, column equilibration and flow-rate as for Fig. 1, except that the concentration of sodium chloride in buffer B was 0.5 *M*. Activity represents incorporation of ^{32}P from [γ-^{32}P] ATP into histone. For particulars of the protein kinase assay see Materials and methods.

Although we have not tried it, the technique should also work if pH rather than ionic strength is used for application and elution.

We feel that the addition of the right concentration of salt to a protein mixture prior to its application onto an HPLC ion-exchange column is a simple technique that could make some of the HPLC separations much more effective.

ACKNOWLEDGEMENT

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REFERENCES

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2 A. Vardanis, *J. Biol. Chem.*, 255 (1980) 7238–7243.