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Modification of the selectivity of a reversed-phase high-performance liquid chromatographic system by binding sodium dodecyl sulphate to peptides

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SUMMARY

Cytochrome *c* and myoglobin were subjected to sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis, electroeluted from the gel and fragmentated with cyanogen bromide. High-performance liquid chromatographic (HPLC) separation of the peptide mixtures obtained was improved when using a gradient of acetonitrile containing phosphates in comparison with a gradient containing trifluoroacetic acid. This finding may be useful for the HPLC analysis of peptides derived from SDS–protein complexes.

INTRODUCTION

Polyacrylamide gels are widely used media for the analytical separation of proteins and peptides. Although these procedures are generaly thought of as analytical techniques, they also provide a powerful preparative technique for small amounts of protein if the protein can be removed from the gel once the protein positions have been located[1]. It should be noted that for many proteins, particularly large hydrophobic proteins, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) is an easy method for the final stage of the purification procedure. Interestingly, the recovered protein is suitable for chemical and enzymatic fragmentations. The peptides derived can be separated and purified by reversed-phase liquid chromatography, in order to obtain information about the primary sequence of a given protein[2].

By reversed-phase chromatography and C_{18} column, we have analysed the cyanogen bromide fragments derived from proteins purified by SDS-PAGE and then electroeluted. Employing the most widely used mobile phase system, *i.e.*, a gradient of a water–acetonitrile mixture in the presence of low concentrations of trifluoroacetic acid (TFA), we observed that peptides displayed long retention times and were very poorly separated. In this paper we present a method that overcomes these difficulties, allowing a better resolution of the peaks.

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EXPERIMENTAL

Reagents

Water was distilled from glass and filtered through a 0.45-μm Millipore filter. Potassium phosphate buffer (analytical-reagent grade) was obtained from Merck (Darmstadt, F.R.G.), TFA from BDH (Poole, U.K.), acetonitrile from Carlo Erba (Milan, Italy) and cyanogen bromide from Fluka (Buchs, Switzerland).

Protein preparation and cyanogen bromide treatment

Myoglobin and cytochrome *c* (horse heart) were obtained from Sigma (St. Louis, MO, U.S.A.) and Boehringer (Mannheim, F.R.G.), respectively. About 10 mg of each protein were subjected to preparative SDS-PAGE on 15% polyacrylamide gel, as described previously[1]. After equilibration in electroelution buffer, the portion of the gel containing each protein was electroeluted in a ISCO (Lincoln, NE, U.S.A.), apparatus and lyophilized. In some experiments SDS was removed from proteins by ion-pair extraction[3]. The lyophilized proteins were dissolved in 70% formic acid and cleaved at methionyl residues with cyanogen bromide[2]. After recovery, the resulting mixtures of peptides were dried under a stream of nitrogen and dissolved in 70% formic acid.

Apparatus

A Perkin-Elmer (Norwalk, CT, U.S.A.) high-performance liquid chromatographic (HPLC) system was used as described previously[4]. A Bakerbond TM C_{18} (15 μm) wide-pore (330 Å) column (25 cm × 0.46 cm I.D.) was obtained from Baker (Phillisburg, NJ, U.S.A.). A guard column obtained from Upchruch (Oak Harbour, WA, U.S.A.) was always used to protect the main column.

HPLC method

All chromatografic runs were carried out at room temperature af a flow-rate of 1.0 ml/min. Peptide elution was accomplished by the application of a binary gradient from 0 to 50% acetonitrile containing either 0.1% (w/v) TFA or potassium phosphate buffer (pH 5.9) as indicated in the legends of the figures.

RESULTS AND DISCUSSION

The cyanogen bromide fragments derived from proteins electroeluted from polyacrylamide gels were analysed by reversed-phase HPLC on a C_{18} column using two different mobile phase modifiers, TFA and phosphate buffer. Significant differences in the peptide elution profiles are evident when different separation conditions are used, as illustrated in Fig. 1.

When the elution is performed with a mobile phase containing 0.1% TFA (Fig. 1a and b), the separations are poor and several peptides are strongly retained by the column. On replacing TFA by 20 m*M* phosphate buffer, not only a marked reduction in retention times of the peptides but also an improvement in their separation are obtained (Fig. 1c and d).

It is well known that salts in the mobile phase may be corrosive towards the steel of the pumps and of the chromatographic column. Moreover, low concentra-

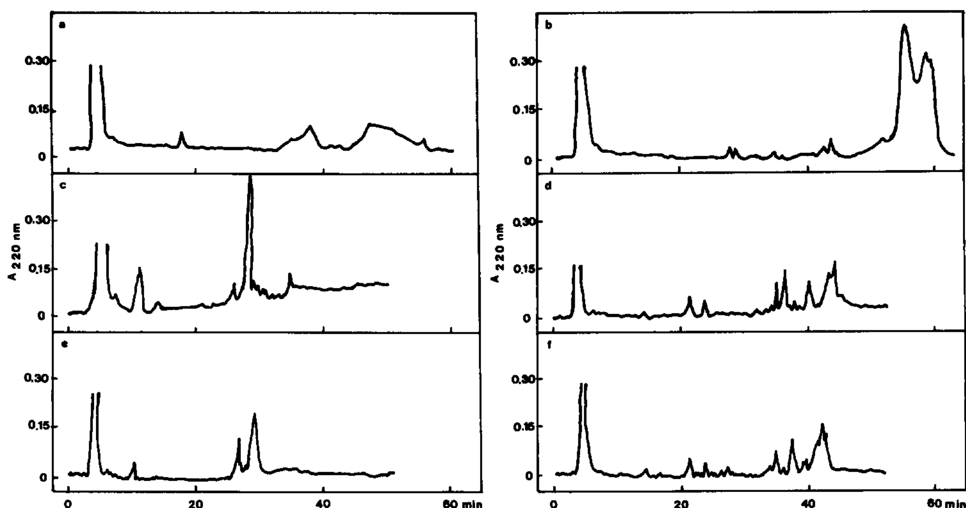

Fig. 1. Separation by HPLC of cyanogen bromide fragments of proteins electroeluted from acrylamide gels. The protein fragments were applied to a Bakerbond TM C_{18} wide-pore (330 Å) column (25 × 0.46 mm I.D.) and eluted with a 50-min linear gradient from 0 to 50% acetonitrile. Temperature, ambient; flow-rate, 1.0 ml/min. Mobile phase additives: (a), (b) 0.1% TFA (w/v); (c), (d) 20 m*M* potassium phosphate buffer (pH 5.9); (e), (f) 5 m*M* potassium phosphate (pH 5.9). (a), (c), (e) Cytochrome *c*; (b), (d), (f) myoglobin.

tions of salts in the chromatographic system make it easier to perform further analyses of the collected peptides, such as amino acid analysis. We therefore tested the quality of the separation obtained by the use of a mobile phase containing 5 m*M* phosphate buffer. These separations are very similar to but not identical with those resulting from 20 m*M* phosphate in that they are characterized by a lower quality (compare Fig. 1c and d with e and f). Nevertheless, it is worth stressing that the addition of such a small amount of buffer (5 m*M*) still produces separations that are better than those obtained with 0.1% TFA (compare Fig. 1a and b with e and f).

The profile obtained from an electroeluted protein is different form that typical of a non-electrophoresed protein, *i.e.*, in the absence of SDS. As an example, one can compare the cyanogen bromide digestion profile of the electroeluted form of cytochrome c (Fig. 1a) with that of the non-electrophoresed form of cytochrome c (Fig. 2a), obtained using a gradient of acetronitrile containing TFA in both instances.

The loss of separation observed in the elution pattern of fragments derived from electroeluted proteins could be related to the presence SDS bound to the peptides. To test this possibility, we performed the cyanogen bromide digestion of cytochrome *c* subjected to electroelution and then to removal of SDS by ion-pair extraction, as described elsewhere[3]. The chromatographic profile is shown in Fig. 2b. It is evident that the separation of fragments performed with TFA as mobile phase modifier is poorer than that of non-electroeluted cytochrome *c* (Fig. 2a); however the quality of the separation is better than that obtained for electroeluted protein which contains SDS (Fig. 1a).

Electrophoretic purification is a very powerful technique that allows the separation of small amounts of very pure proteins. It is also known that the SDS used in

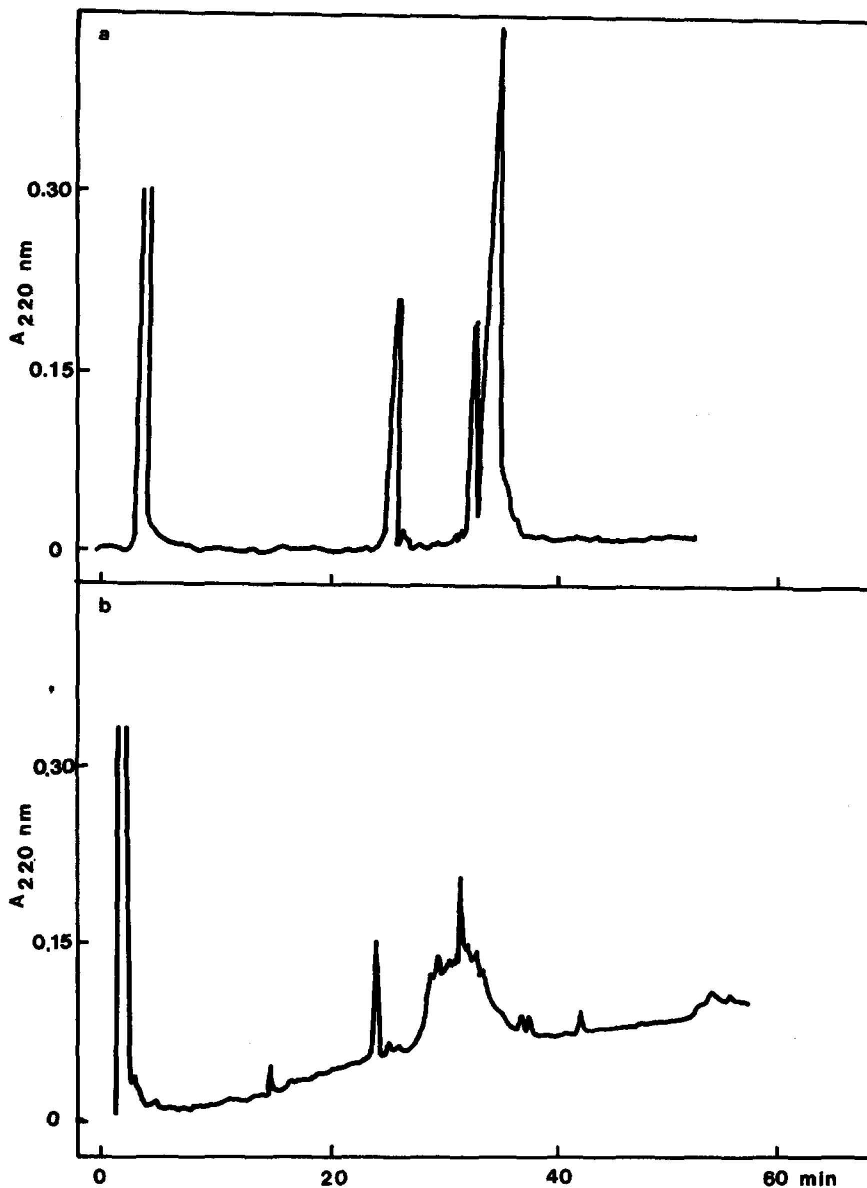

Fig. 2. Separation by HPLC of cyanogen bromide fragments of cytochrome *c*. Column and gradient as in Fig. 1, with acetonitrile containing 0.1% (w/v) TFA. (a) Cytochrome *c* not subjected to SDS-PAGE and electroelution; (b) cytochrome *c* subjected to SDS-PAGE electroelution and then removal of SDS.

the electrophoretic run binds tightly to most proteins. Several procedures have been described for the removal of SDS from electroeluted proteins, but have the disadvantage that losses are frequently encountered when only small amounts of samples are used.

We have observed that a mobile phase containing the most popular modifier, TFA[5], does not produce satisfactory separations of the fragments derived from cyanogen bromide cleavage, probably owing to the presence of SDS bound to the peptides. However, for the same peptides, we have found that the addition of millimolar amounts of phosphate buffer to the mobile phase reduces the retention times and improves the separation. This is not surprising, bearing in mind that phosphate in a variety of forms has found wide application as an additive in mobile phases used in the analysis of amino acids, peptides and proteins by HPLC (see ref. 6 for a review). Interestingly, the peptides so obtained are in a form suitable for gas-phase sequence analysis[2].

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REFERENCES

1 L. Dalla Libera, R. Betto, R. Lodolo and U. Carraro, *J. Muscle Res. Cell Motil.*, 5 (1984) 411–426.
2 J. H. Collins, J. L. Theibert and L. Dalla Libera, *Biosci. Rep.*, 6 (1986) 655–661.
3 W. A. Konigsberg and L. Henderson, *Methods Enzymol.*, 91 (1983) 254–259.
4 L. Dalla Libera, R. Betto and D. Biral, *J. Chromatogr.*, 264 (1983) 164–169.
5 F. E. Regnier, *Science*, 222 (1983) 245–252.
6 C. A. Bishop, in W. S. Hancock (Editor), *Handbook of HPLC for the Separation of Amino acids, Peptides and Proteins*, Vol. I, CRC Press, Boca Raton, FL, 1984, 153–159.