*Journal of Chromatography*, 473 (1989) 71–77
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 468

# MICROPREPARATIVE SEPARATION OF PEPTIDES DERIVED FROM SODIUM DODECYL SULPHATE-SOLUBILIZED PROTEINS

ARMIN BOSSERHOFF, JOCHEN WALLACH and RAINER W. FRANK*
*Biochemical Instrumentation Programme, European Molecular Biology Laboratory, Meyerhogstrasse 1, D-6900 Heidelberg (F.R.G.)*
(First received November 14th, 1988; revised manuscript received January 24th, 1989)

## SUMMARY

A systematic investigation of the influence of the detergent sodium dodecyl sulphate (SDS) on micropreparative peptide separations on microbore reversed-phase high-performance liquid chromatographic columns is reported. A tryptic digest of bovine serum albumin and a mixture of synthetic peptides were used to monitor the separation behaviour of a 1.6 mm I.D. Nucleosil $C_{18}$ column in the presence of various amounts of SDS. The data demonstrate that even traces of SDS in the sample reduce the separation efficiency and peptide recovery. An extraction method is presented which reduces the SDS content in peptide mixtures below the critical concentration without affecting significantly the recovery of individual peptides. After acidification of the sample, the detergent is extracted into heptane–isoamyl alcohol (4:1, v/v). In combination with chemical or enzymatic fragmentation techniques, this extraction method facilitates the sequence analysis of minute amounts of SDS-solubilized hydrophobic proteins. The applicability of the method is demonstrated on the example of the integral membrane protein bacteriorhodopsin.

## INTRODUCTION

Technological advances over the past few years now permit the sequence analysis of proteins and peptides to be performed routinely at low picomole levels. However, the micropreparative isolation of microgram amounts of proteins and peptides with suitable purity for microsequence analysis appears to be a persistent problem. In particular, large hydrophobic proteins, *e.g.*, integral membrane proteins and membrane-associated proteins, pose difficulties[1]. Hydrophobic proteins require the presence of detergents such as sodium dodecyl sulphate (SDS) during the purification procedure. SDS is also required to solubilize Coomassie blue-stained and fixed proteins before electroelution from the gel[2].

Several methods for the removal of SDS from protein preparations have been published which use inverse gradients on reversed-phase columns[3], methanol–chloroform precipitation[4] or ion-pair extraction[5]. Although these methods work effectively even with minute amounts of soluble proteins, they often cannot be applied to hydrophobic proteins. After efficient removal of SDS, many hydrophobic proteins

0021-9673/89/$03.50 

become insoluble in aqueous buffers and form precipitates which are inaccessible to endoproteases. The presence of small amounts of SDS is therefore required in order to keep the proteins in solution and to ensure efficient enzymatic fragmentation[6]. Accordingly, the enzymatic digests derived from these proteins and also proteins that were digested within SDS gels[7] contain various amounts of detergent. However, the data presented in this paper demonstrate that the SDS concentration in the sample is a critical parameter affecting the separation drastically when microbore reversed-phase high-performance liquid chromatography (HPLC) is used for peptide purification. Therefore, an efficient extraction procedure for removal of SDS from complex peptide mixtures was developed, which is generally applicable to the micropreparative isolation of peptides derived from SDS-solubilized proteins.

## EXPERIMENTAL

### *High-performance liquid chromatography*

Separation of the peptides was performed using a Hewlett-Packard 1090 liquid chromatograph equipped with a Kontron 430 UV detector. The peptides were detected at 215 nm. The column (250 × 1.6 mm I.D.) was Nucleosil $C_{18}$ (Macherey, Nagel & Co.) packed by MZ-Analysentechnik (Mainz, F.R.G.). The synthetic peptides were separated at a flow-rate of 100 μl/min using a 20-min linear gradient from 5 to 50% B followed by a 10-min isocratic elution at 50% B; buffer A was 0.1% trifluoroacetic acid (TFA) in water and buffer B was acetonitrile containing 0.07% TFA. The tryptic peptides obtained from bovine serum albumin (BSA) were separated under the same conditions, but using a 60-min linear gradient from 0 to 60% B. All gradient separations of SDS containing samples were followed by a 10-min isocratic elution at 80% B. A 90-min linear gradient from 10 to 90% B was used for separation of the peptides derived from tryptic digestion of bacteriorhodopsin.

### *Trypsin digestion*

BSA (Serva, Heidelberg, F.R.G.) was digestd in 100 m*M* ammonium hydrogencarbonate for 6 h at 37°C. The protein concentration was 2 μ*M* and the enzyme/substrate ratio was 1:5 (w/w). Bacteriorhodopsin (Serva) was solubilized by boiling in 1% SDS and was diluted with 100 m*M* ammonium hydrogencarbonate to yield a 2 μ*M* protein solution containing 0.08% (w/v) SDS. Digestion of 200-μl aliquots of this solution was performed for 12 h at 37°C and the enzyme/substrate ratio was 1:4 (w/w). Trypsin (sequencing grade) was obtained from Boehringer (Mannheim, F.R.G.).

### *SDS extraction*

The extraction was performed on 100-μl samples containing 200 pmol of peptide mixture in 0.1% TFA–water and up to 0.05% (w/v) SDS. The sample was acidified with 5 μl of TFA and extracted with 100 μl of heptane–isoamyl alcohol (4:1, v/v) by briefly vortexing. After centrifugation in an Eppendorf centrifuge, the heptane phase was removed with a pipette and residual heptane was evaporated in a gentle stream of nitrogen. Samples containing more than 0.05% of SDS were extracted twice.

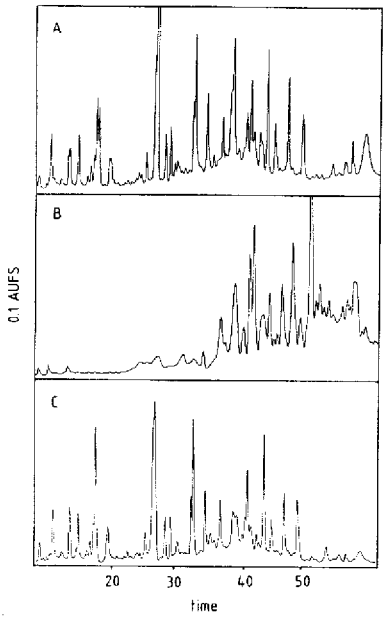


Fig. 1. High-performance liquid chromatography of tryptic peptides derived from bovine serum albumin. The peptides (200 pmol) were separated on a 250 × 1.6 mm I.D. Nucleosil $C_{18}$ column using a 60-min linear gradient from 0 to 60% B at a flow-rate of 100 $\mu$l/min. Sample A contained no SDS, samples B and C contained 50 $\mu$g of SDS. Before injection, sample C was extracted with heptane–isoamyl alcohol (4:1, v/v). Time in min.

## RESULTS AND DISCUSSION

Fig. 1A shows the elution profile of a tryptic digest of 200 pmol of BSA. The peptides were separated on the Nucleosil $C_{18}$ microbore column. The profile in Fig. 1B shows the same separation under identical chromatographic conditions, but with 50 $\mu$g (0.05%) of SDS in the sample. In the presence of the detergent the peptides were shifted to higher retention volumes and the number of separated peaks was markedly reduced.

TABLE I

AMINO ACID SEQUENCES OF THE SYNTHETIC PEPTIDES USED IN THE SEPARATION EXPERIMENTS

| *Peptide* | *Sequence* |
|---|---|
| | 1 5 10 |
| 1 | Lys-Val-Glu-Gly-Glu-Glu-Gly-Glu-Glu-Glu-Gly-Glu |
| | 1 5 10 |
| 2 | Lys-Cys-Arg-Asn-Arg-Arg-Arg-Glu-Leu-Thr-Asp-Thr-Leu-Gln |
| | 1 5 10 |
| 3 | Pro-Thr-Val-Thr-Ala-Ile-Ser-Thr-Ser-Pro-Asp-Leu-Gln |
| | 1 5 10 |
| 4 | Pro-Ala-Cys-Lys-Ile-Pro-Asp-Asp-Leu-Gly-Glu-Phe-Pro-Glu |
| | 1 5 10 |
| 5 | Ser-Glu-Glu-Pro-Ile-Tyr-Ile-Val-Thr-Glu-Tyr-Met-Ser-Lys-Gly-Ser-Leu |

The injection of 100 μg (0.1%) of SDS led to the complete breakdown of the separation and the peptides eluted as a single broad peak at 50–60% acetonitrile (data not shown).

To investigate the nature of the separation mechanism in the presence of various SDS concentrations, the elution behaviour of five synthetic peptides of similar size and of graded polarity (Table I) was monitored. The separations were performed under identical chromatographic conditions, but with the addition of increasing amounts of SDS (1–50 μg) to the sample. The results are shown in Fig. 2. In the absence of SDS, the most polar peptide (peptide 1) eluted first and peptide 5, containing the highest number of apolar residues, eluted last from the reversed-phase column. In Fig. 2b–e the effect of increasing concentrations of SDS in the sample is demonstrated. Whereas the retention time and the peak area of peptide 3 remained stable up to 50 μg (0.05%) of SDS, all other peptides were affected. The peaks were shifted to longer retention times and the peak areas were reduced. Even at 5 μg (0.005%) of SDS the recovery of the basic peptide 2 was reduced to 10% and at 50 μg (0.05%) of SDS the peptide failed to elute from the column. The effect of SDS on the retention volumes of the peptides can therefore be related to the number of Lys and Arg residues present in the individual sequences (Table I). This indicates an increasing interaction of the positively charged residues with the separation matrix due either to the binding of the anionic detergent to the peptide, thus rendering them more hydrophobic, or to the binding of SDS to the reversed-phase matrix. The latter would convert the hydrophobic $C_{18}$ phase into a negatively charged cation-exchange matrix. Therefore, as the amount of SDS applied to the column increases, the ion-exchange mechanism becomes predominant and, owing to the low ionic strength of the elution buffer, the basic peptides are retarded.

The fact that the detergent binds to the column and not to the peptides is illustrated in Fig. 3. In this experiment the detergent was applied separately to the column 5 min (B) and 15 min (C) before injecting the peptide mixture. The change in the elution profile was almost identical with the profile obtained with simultaneous

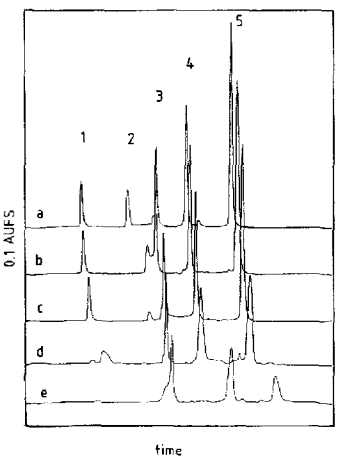


Fig. 2. Effect of SDS concentration on gradient elution of a mixture of five synthetic peptides. The peptides were separated on a 250 × 1.6 mm I.D. Nucleosil $C_{18}$ column using a 20-min linear gradient from 0 to 50% B followed by a 10-min isocratic elution at 50% B. The flow-rate was 100 μl/min. The peptides are designated according to their numbering in Table I. In each experiment 200 pmol of peptide mixture were injected in 100 μl of 0.1% TFA. SDS content in the sample: (a) 0; (b) 1; (c) 5; (d) 20; (e) 50 μg.

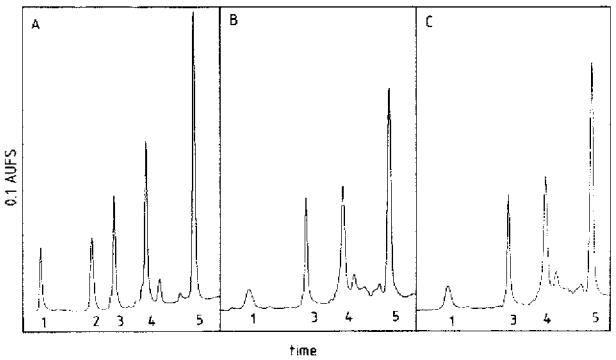


Fig. 3. Effect of SDS injection on gradient elution of peptides. In all instances (A–C) the peptides were injected in 100 ml of 0.1% TFA and eluted under the chromatographic conditions given in Fig. 2. In B and C the column was loaded with 20 mg of SDS and washed for (B) 5 min or (C) 15 min with starting buffer before sample injection.

injection of peptide and detergent. Owing to the strong binding of the detergent even after a 15-min wash with buffer A, no change in the retention behaviour was observed. To restore the separation, the detergent had to be eluted from the column with 80% acetonitrile.

From these results, it was obvious that even traces of SDS have to be removed from peptide mixtures before applying them to reversed-phase columns. As none of the cited methods for removal of SDS from proteins gave satisfactory results with peptide mixtures, we tried an extraction method which was proposed by Heukeshoven[8]. In this

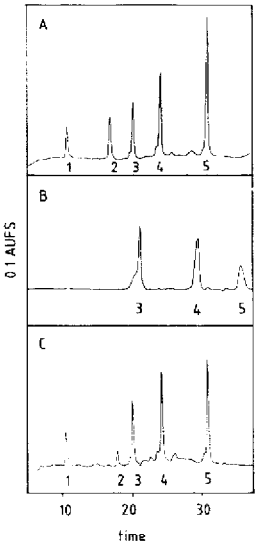


Fig. 4. Efficiency of removal of SDS from a peptide mixture (200 pmol in 100 μl of 0.1% TFA) by extraction with heptane–isoamyl alcohol (4:1, v/v). The chromatographic conditions were the same as in Fig. 2. The peptide numbering corresponds to the numbering in Table I. The peptides were separated (A) without SDS, (B) after addition of 50 μg SDS and (C) after a single extraction of B with heptane–isoamyl alcohol (4:1, v/v). Time in min.

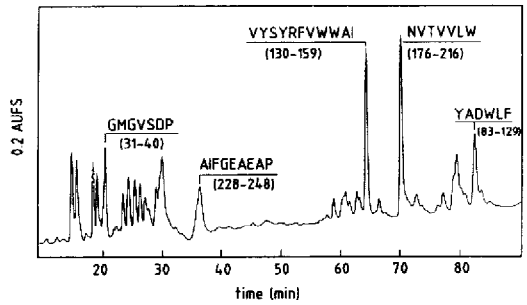


Fig. 5. Separation of tryptic peptides derived from SDS-solubilized bacteriorhodopsin (500 pmol). The protein was digested as described under Experimental and the fragments were extracted with heptane–isoamyl alcohol (4:1, v/v). The separation was performed on a 250 × 1.6 mm I.D. Nucleosil $C_{18}$ column using a linear gradient from 0 to 90% B at a flow-rate of 100 μl/min. The amino acid sequence data obtained from individual peaks are given as one-letter codes on top of the peaks. The numbers in parentheses give the positions of the corresponding peptides in the sequence of the mature protein.

method, SDS is extracted into heptane–isoamyl alcohol (4:1, v/v) after acidification of the sample with TFA. The efficiency of the method is illustrated in Fig. 4. After a single extraction of the peptide mixture containing 50 μg of SDS per 100 μl, the elution profile which was obtained from the SDS-free sample (A) was almost completely restored (C).

As determined by the methylene blue method[9], the SDS concentration was below 0.001%. The recovery of peptides 1, 3 and 4 was near 100%. Peptide 3 was still slightly retarded and only peptide 5 was recovered with reduced yield (90%) owing to extraction losses. A similar result was obtained when a tryptic digest of 200 pmol of BSA containing 50 μg of SDS (Fig. 1B) was extracted. After extraction, the elution pattern of the SDS-free sample was restored and the recovery of only a few peptides was reduced (Fig. 1C).

The applicability of the method to hydrophobic membrane proteins is demonstrated in Fig. 5, which shows the micropreparative separation of tryptic peptides obtained from 500 pmol of SDS-solubilized bacteriorhodopsin. The protein was digested in the presence of 0.1% of SDS and the digest was extracted twice with heptane–isoamyl alcohol (4:1, v/v). Corresponding to the distribution of the lysine and arginine residues in the primary structure of bacteriorhodopsin[10], several small hydrophilic peptides (eluting at 30–45% B) and a number of large hydrophobic fragments (eluting at 75–90% B) were obtained. The identities of some of the peptides were established by microsequence analysis in a gas-phase sequencer[11]. The sequences are given in Fig. 5.

In conclusion, the data presented demonstrate the extreme sensitivity of microbore reversed-phase HPLC columns to the presence of SDS in the sample. The detergent binds to the column and converts the reversed-phase matrix into a cation-exchange matrix. The separation capacity of the column and the peptide recovery are reduced. Therefore, control of the amount of detergent present in the sample is important for efficient micropreparative isolation of peptides. As many proteases, such as trypsin and V8-protease, are not inhibited in the presence of up to 0.1% of SDS, the proposed extraction method allows the enzymatic fragmentation of SDS-solubilized proteins to be performed without prior removal of the detergent. The

extraction reduces the amount of SDS in the resulting peptide mixture to below the concentration critical for micropreparative HPLC, without affecting the recovery of peptides. This strategy significantly improves the microsequence analysis of hydrophobic membrane proteins.

## REFERENCES


1 P. Tempst, D. D.-L. Woo, D. B. Teplow, R. Aebersold, L. E. Hood and S. B. H. Kent, *J. Chromatogr.*, 359 (1986) 403.
2 M. W. Hunkapiller, E. Lujan, F. Ostrander and L. E. Hood, *Methods Enzymol.*, 91 (1983) 227.
3 R. I. Simpson, R. L. Moritz, E. C. Nice and B. Grego, *Eur. J. Biochem.*, 165 (1987) 21.
4 D. Wessel and U. I. Flügge, *Anal. Biochem.*, 138 (1984) 141.
5 W. H. Konigsberg and L. Henderson, *Methods Enzymol.*, 91 (1983) 254.
6 H. Suzuki and T. Terada, *Anal. Biochem.*, 172 (1988) 259.
7 U. M. Benedum, P. A. Baeuerle, D. S. Konecki, R. Frank, J. Powell, J. Mallet and W. B. Huttner, *EMBO J.*, 5 (1986) 1495.
8 J. Heukeshoven, paper presented at the *6th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Baden-Baden, October 20–22, 1986.*
9 K. Hyashi, *Anal. Biochem.*, 67 (1975) 503.
10 H. G. Khorana, E. G. Gerber, W. C. Herlihy, C. P. Gray, R. J. Anderegg, K. Nihei and K. Biemann, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 5046.
11 H. Gausepohl, M. Trosin and R. Frank, in B. Wittmann-Liebold (Editor), *Advanced Methods in Protein Microsequence Analysis*, Springer, Berlin Heidelberg, p. 150.