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PURIFICATION OF RIBOSOMAL 30S PROTEINS FROM THE ARCHAEBACTERIUM *SULFOLOBUS ACIDOCALDARIUS* BY ION-EXCHANGE AND DISCONTINUOUS REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

JOACHIM R. GRÜN and RICHARD REINHARDT*
Max-Planck-Institut für Molekulare Genetik, Abt. Wittmann, Ihnestrasse 73, D-1000 Berlin 33 (Dahlem) (F.R.G.)

SUMMARY

Ribosomal total protein from the 30S particle of the archaebacterium *Sulfolobus acidocaldarius* was isolated by ion-exchange and reversed-phase high-performance liquid chromatographic methods. By using a variety of different buffers and columns, six purified proteins were obtained by ion-exchange and up to ten proteins by reversed-phase methods. The separated proteins were tested for homogeneity by sodium dodecyl sulphate or two-dimensional polyacrylamide gel electrophoresis. Efforts to determine the N-terminal amino acid sequence of the purified proteins were also initiated. The selectivity and load capacity of reversed-phase chromatography were increased by the development of a "discontinuous reversed-phase" method. Scale-up to semi-preparative column combinations and re-chromatography of selected fractions under appropriate conditions will increase the number and yield of purified proteins.

INTRODUCTION

It is now generally accepted that archaebacteria represent a third line of descent[1], although the evolutionary status of these microorganisms is not yet clear. They show characteristics which in some instances suggest a close relationship to the eubacterial class, while other traits appear to be closer to those of the eukaryotic kingdom.

Ribosomes, because they play a central role in protein biosynthesis and are therefore present in all organisms, represent an excellent model for comparative studies in evolutionary aspects. Both archae- and eubacterial ribosomes consist of a large 50S and a small 30S subunit. In the *Escherichia coli* ribosome, 21 proteins of the 30S subunit have been identified and their amino acid sequences have been determined, whereas for the 50S subunit 32 proteins have been sequenced[2]. Initial studies on the archaebacterial ribosomes from *Sulfolobus* suggest that the total number of different proteins is higher[3,4] than in *E. coli*.

The isolation of ribosomal proteins by open-column chromatography and de-

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naturing agents, such as 8 *M* urea, was described in ref. 5, and the use of high-performance liquid chromatographic (HPLC) systems was demonstrated in, *e.g.*, refs. 6–8. HPLC methods are now widely used for the purification of proteins[9], and reversed-phase HPLC is becoming the method of choice in protein separation[10]. We have utilized advanced HPLC methods to separate individual ribosomal proteins from the mixture of total protein (TP) in the 30S subunit of the archaebacterium *Sulfolobus acidocaldarius*. The N-terminal amino acid sequence determination of these proteins can be used as a basis for synthesizing oligonucleotide probes to locate and isolate the coding genes for these proteins. Once a ribosomal protein gene has been identified, the complete amino acid sequence of the protein can be obtained from the DNA sequence. The comparative analysis of sequence information of ribosomal proteins from different archaebacteria that will become available in the future will undoubtedly help to shed light on the relative evolutionary position of this class of organism.

In contrast to the procedure described for *E. coli* ribosomal proteins[6–8], the separation of ribosomal proteins from the thermoacidophile *S. acidocaldarius* is limited by the selectivity of a single reversed-phase (RP) column. Often more than one protein is eluted within one peak. Therefore, it is always necessary to verify the purity of the HPLC peaks by sodium dodecyl sulphate (SDS) or two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE).

Multi-column chromatography is used in different ways. Except when several columns are running in a parallel system[11], there are two main applications for the use of more than one column in one separation: first, the "column coupling"[12,13] technique combines identical columns to increase the plate number; and second, the non-homogeneous field of multi-dimensional or two-dimensional (*cf.* ref. 14) column chromatography "separates multi-component samples were a fraction (or fractions) of elutions from one column is selectively transferred to one or more secondary columns for additional separations"[15]. "The columns may be of the same type ... but with different phase ratios or they can be of different types ..."[16]. This technique can be carried out manually[17] (better called re-chromatography if there is a collection step between the two separations) or automated (called tandem[18] or better tandem column array[19] chromatography). The difference between two-dimensional and tandem column array chromatography is that the former uses more than one column of the same kind (*e.g.*, ion-exchange–ion-exchange chromatography[15]) and the latter uses two different kinds of chromatography in the first and second dimensions (*e.g.*, size exclusion–reversed-phase chromatography[16] or ion-exchange–reversed-phase chromatography[18]). Proper two- or three-dimensional chromatography[14], where the "column" is a flat bed, similar to thin-layer chromatography, or a cube, respectively, was described in ref. 20.

The method described in this paper was developed to increase the resolving power of reversed-phase HPLC without the need for a two-dimensional system. This technique, which we call discontinuous reversed-phase chromatography (disc.-RPC), combines different RP columns in a sequence of increasing retention times and is used in place of a single column. It is referred to as disc.-RPC because (1) all of the injected sample passes through a discontinuous bed of stationary phases and (2) we found an enrichment effect that minimizes peak broadening in analogy with discontinuous gel electrophoresis. Discontinuous chromatography simply substitutes single

columns, and therefore both two-dimensional and tandem column array chromatography can be carried out with this discontinuous technique.

EXPERIMENTAL

Apparatus

The fast protein liquid chromatographic (FPLC) system from Pharmacia (Uppsala, Sweden) consisted of a GP250 gradient programmer with two P-500 pumps, a UV-1 monitor (214 nm) and a FRAC-100 fraction collector.

The HPLC systems from LKB (Bromma, Sweden) consisted of (1) a 2152 HPLC controller with two 2150 pumps, a 2151 variable-wavelength monitor, a Superrac 2211 fraction collector, a Rheodyne (Cotati, CA, U.S.A.) 7125 injection valve, and (2) a single-pump system with controller, monitor, fraction collector and injection valve as above and an LKB 2040 mixing valve.

The HPLC system from Beckman (Berkeley, CA, U.S.A.) consisted of a Model 420 HPLC controller with two 114-M pumps and a 210 A injection valve, a BT3030 variable-wavelength monitor from Biotronik (Maintal, F.R.G.) and an LKB Superrac 2211 fraction collector.

Chemicals and materials

All organic solvents used as mobile phases in HPLC were of LiChrosolv grade from Merck (Darmstadt, F.R.G.). Trifluoroacetic acid (TFA) from Fluka (Neu-Ulm, F.R.G.) was distilled over calcium sulphate. All other chemicals were of analytical-reagent grade from Merck.

Deionized water, doubly distilled in a quartz system, was used for all aqueous solutions. All reversed-phase chromatographic eluents contained 0.1% (w/v) of sodium azide and 0.001% (v/v) of 2-mercaptoethanol and were degassed with helium.

The stainless-steel columns, glass columns and column support materials used for separations are summarized in Table I.

Methods

S. acidocaldarius (DSM 1616)[21] cells were grown in a 150-l glass fermenter and harvested by continuous-flow centrifugation. The cells were broken in a Gaulin–Manton press (General Electric, Fort Wayne, IN, U.S.A.) and ribosomes were purified by gel filtration as described in ref. 21. The separation into subunits was performed by zonal centrifugation, while the preparation of total protein from the 30S subunit (TP30) was achieved by acetic acid–urea extraction[22]. After extensive dialysis against water, the extracted TP30 solution was lyophilized and stored at −20°C. To separate the proteins by reversed-phase HPLC, 9.2 A_{230} units of the lyophilized TP30 were dissolved in 1 ml of 15% (v/v) formic acid, centrifuged for 5 min at 9000 *g* and filtered through a Millipore (Milford, MA, U.S.A.) 0.45-μm SJHV filter. The sample was then injected immediately into the HPLC system. For ion-exchange chromatography (IEC), the lyophilized TP30 was dissolved in 5 *M* urea or 5% (v/v) acetic acid. The separation of the protein mixture was tested by SDS-PAGE[23] or 2D-PAGE[24]. The N-terminal sequence determination of some ribosomal proteins of the 30S subunit was performed by the double-coupling method[25], the automated liquid-phase technique in the Berlin sequencer[26] or a modified solid-phase method[27].

TABLE I

STAINLESS-STEEL AND GLASS COLUMNS USED FOR SEPARATIONS

Method	*Material**	*Length × I.D. (mm)*	*Bonded phase*	*Source***
Ion-exchange chromatography	TSK SP5 PW	75 × 7.5	Strong	LKB
	TSK IEX-530 CM	300 × 4.0	Weak	Bio-Rad Labs.
	Mono-S HR 5/5 (glass)	50 × 5.0	Strong	Pharmacia
Reversed-phase chromatography	(a) TSK TMS-250	75 × 4.6	C_1	LKB
	(b) Synchropack RP-P C_1	125 × 8.0	C_1	Bischoff***
	(c) Vydac-214 TP	250 × 4.6	C_4	Chrompak
	(d) Vydac-201 TP	250 × 4.0	C_{18}	Macherey, Nagel & Co.***
	(e) Vydac-201 TP	250 × 8.0	C_{18}	Macherey, Nagel & Co.***
	(f) TSK ODS 120 T	250 × 4.6	C_{18}	LKB
	(g) TSK ODS 120 T	300 × 7.8	C_{18}	LKB
	(h) Nova Pak C_{18}	125 × 3.9	C_{18}	Waters Assoc.
	(i) Nucleosil 300-7	250 × 4.0	C_6H_5	Macherey, Nagel & Co.***

* For discontinuous RPC, the following column combinations were used (details are given under Results and discussion): a + d, a + f, a + d + f, b + e, b + g, b + e + g, h + i. All RPC columns and combinations were used with guard columns.

** Columns were purchased from LKB, Pharmacia, Bio-Rad Labs. (Richmond, CA, U.S.A.), Chrompack (Middelburg, The Netherlands) and Waters Assoc. (Milford, MA, U.S.A.).

*** Columns packed in the laboratory with column support materials from Bischoff (Leonberg, F.R.G.) and Macherey, Nagel & Co. (Düren, F.R.G.); stainless-steel columns were obtained from Knauer (Bad Homburg, F.R.G.).

RESULTS AND DISCUSSION

Ion-exchange chromatography

From previous work on the separation of ribosomal proteins from *E. coli* and *B. stearothermophilus* for crystallization experiments, it was apparent that the isolation of these proteins under strictly non-denaturing conditions[28] was the predominant factor in succesful crystallization. Although it is unnecessary to obtain native proteins for sequence determination, we examined the separation of TP30 with non-denaturing IEC buffer. On the Mono-S column, eluted with 0.01 *M* phosphate buffer (pH 6.5) and a gradient of 0–1 *M* potassium chloride in 360 min, most of the proteins remained bound, and the yield of the eluted proteins was very low (data not shown). Under different conditions, on the Mono-S column, eluted with a gradient of 0–1 *M* potassium chloride in 6 *M* urea and 0.05 *M* 2-(N-morpholino)ethanesulphonic acid (MES) buffer, we achieved the complete separation of TP30 within 45 min at a flow-rate of 2 ml/min (Fig. 1). SDS-PAGE demonstrated that five proteins were sufficiently pure for sequence determination (Fig. 2). On the TSK SP5-PW column with an optimized gradient and MES–urea buffers, no more than five ribosomal proteins were purified.

A comparison of the peak fractions from the separations on the two columns by SDS-PAGE is shown in Fig. 2. This yielded ten reasonably pure different proteins: slots 22–26 in Fig. 2 for the Mono-S separation and slots 28–33 for the TSK SP5 PW column.

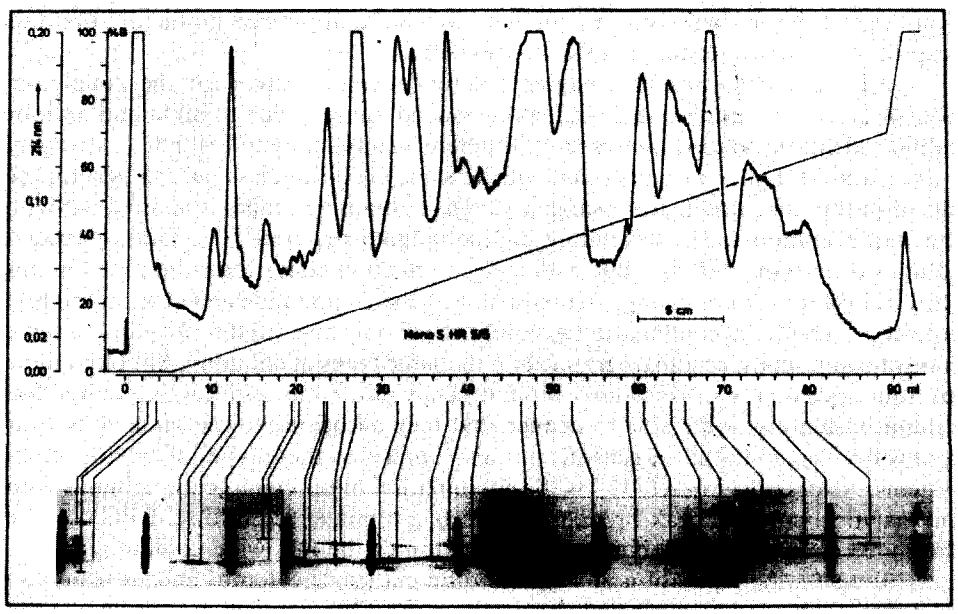

Fig. 1. Ion-exchange chromatography under denaturing conditions. Column, Mono-S HR 5/5; buffer A, 6 *M* urea in 0.05 *M* MES, pH 6.5; buffer B, 1 *M* potassium chloride in buffer A; gradient, 43 min from 0 to 70% B; flow-rate, 2.0 ml/min; FPLC system from Pharmacia; detector wavelength, 214 nm, range 0.2; 20 A_{230} units of TP30 injected. SDS-PAGE, 30 μl of each 500-μl fraction.

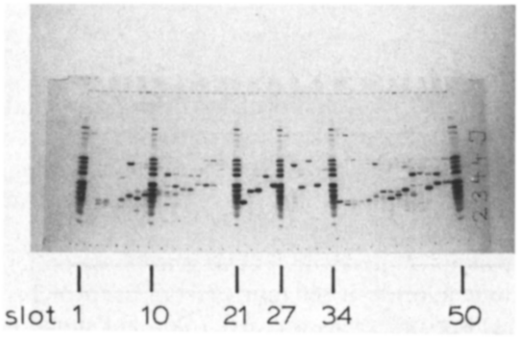

Fig. 2. Pooled fractions of two different ion-exchange chromatograms. TP30 as marker in slots 1, 10, 21, 27, 34 and 50. Slots 2–26, fractions of a Mono-S separation (conditions as in Fig. 1); slots 28–49, fractions of a TSK SP5 PW separation. Conditions: gradient, 130 min from 0 to 70% B; flow-rate, 1.0 ml/min; LKB two-pump HPLC system; detector wavelength, 230 nm, range 0.64; 25 A_{230} units of TP30 injected. Proteins sufficient pure for sequencing are in slots 22–26 (Mono-S) and 28–33 (TSK SP5 PW column).

Reversed-phase chromatography

Reversed-phase chromatography was tested on the same separation problem (TP30) on six different analytical columns, three semi-preparative columns and seven different disc.-RPC combinations with three buffer systems. Although in some instances the separation of proteins appeared to be good, as judged by the number of separated peaks, this was found to be misleading in this kind of protein separation. When the pooled fractions were examined by SDS-PAGE, only incomplete separations were achieved with single columns, even with optimized gradients. For this reason, we used the technique of disc.-RP-HPLC.

Different RPC columns, having different retention times for the same compounds, may contain chemically different bonded phases, such as alkyl and aryl, or contain identical bonded phases from different manufacturers[29] (for C_{18}, compare Figs. 6 and 7). Different compounds of the same chemical class will, in general, be eluted in the same order, from single columns containing different phases, but with different retention times, depending on the column material[29,30]. Thus, a second column of similar material, but with longer retention times than the first for this chemical class, will not change the sequence of the compounds eluted from the first column. Therefore, a combination of columns will only give valid results for the same class of compounds, chromatographed on the same types of columns. Although there are some differences in ribosomal protein behaviour, from initial work with single column systems we were able to deduce that they all belong to the same class. Our results here suggest that, in general, this also applies to these types of multi-column systems. As described in ref. 31 for the combination of different guard columns with an analytical column, peak-broadening effects are minimized in disc.-RPC because the eluted compounds will bind again to the top of the following column.

Initially, the separation of TP30 on different single columns and their protein retention times were investigated. From the TSK TMS-250 column, proteins were eluted at 30% of eluent B (0.1% TFA in 2-propanol; *cf.*, Fig. 3). However, this eluent concentration only eluted the first few proteins from the Vydac-201 TP column (Fig. 4). TSK ODS 120T showed the longest retention times for TP30 (Fig. 5).

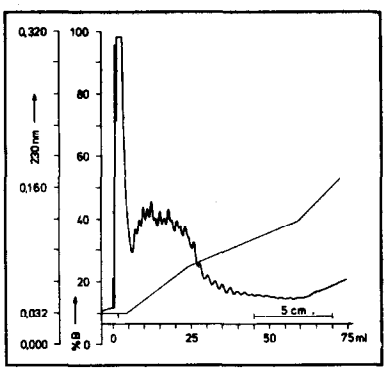

Fig. 3. Single-column reversed-phase chromatography with TSK TMS-250. Eluent A (here and in all other RP chromatograms), 0.1% TFA in water; eluent B, 0.1% TFA in 2-propanol; gradient, from 10 to 55% B in 145 min; flow-rate, 0.5 ml/min; detector wavelength, 230 nm, range 0.32; recorder, 5 ml/cm; two-pump HPLC system from LKB; 18 A_{230} units of TP30 injected.

Combinations of two or three of these columns in order of increasing retention times were then used, and the elution sequence was again checked by SDS-PAGE (Figs. 6 and 7). The combination of TSK TMS 250 (length 75 mm) and TSK ODS 120T (250 mm) resulted in a much better separation of TP30 (*cf.*, Fig. 6) than chromatography on TSK ODS 120T alone. The use of acetonitrile instead of 2-propanol in eluent B had only a negligible influence on the results of disc.-RPC for this column combination. For the combination of Vydac-201 TP and TSK ODS 120T columns,

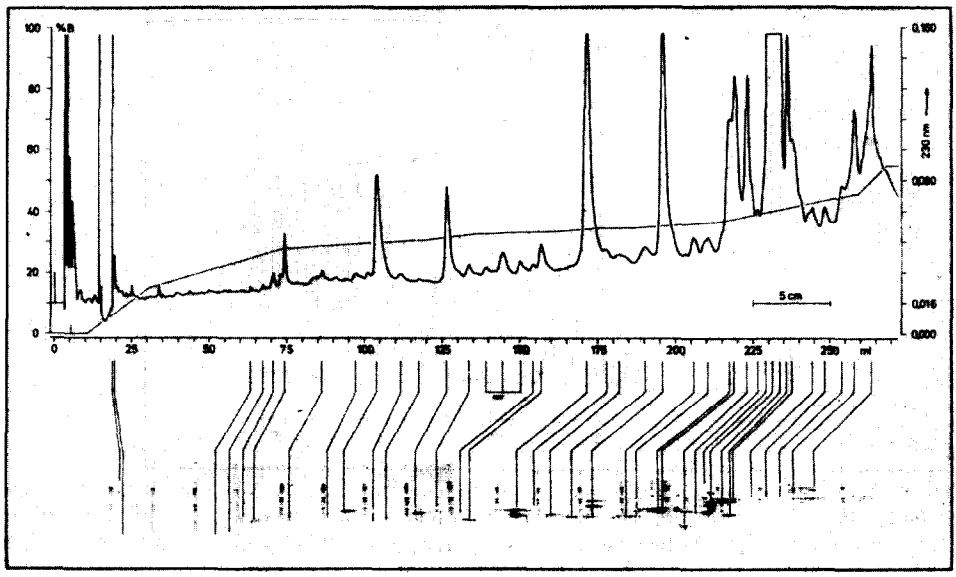

Fig. 4. Single-column reversed-phase chromatography with Vydac-201 TP Gradient, from 0 to 55% B in 526 min; detector, range 0.16; 5 A_{230} units of TP30 injected; all other conditions as in Fig. 3.

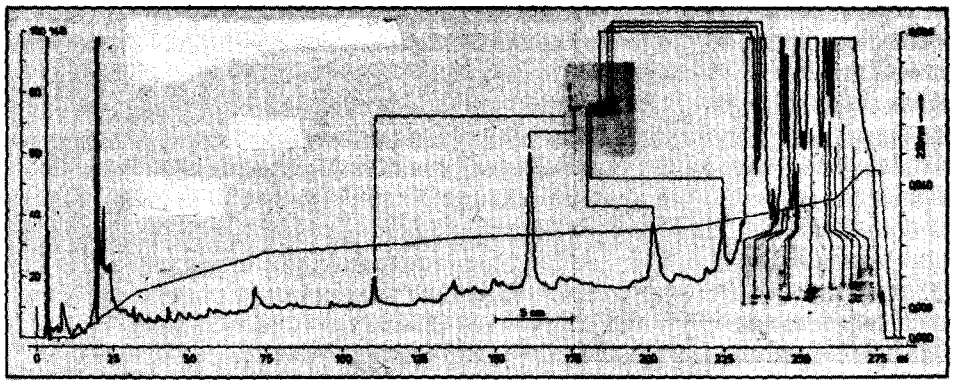

Fig. 5. Non-optimized single-column reversed-phase chromatography with TSK ODS 120T. Detector range, 0.08; 2.5 A_{230} units TP30 injected; all other conditions as in Fig. 4. The gradient used is not optimized but is the same as shown in Fig. 4 to illustrate the differences in retention times with "different" phases. Both Vydac-201 TP and TSK ODS 120T are of octadecylsilyl (ODS) material.

2-propanol as eluent B gave a slightly better separation (data not shown).

Finally, a three-column combination with an additional pre-column was tested with three different buffer systems. The separation of TP30 on TSK TMS 250, Vydac-201 TP and TSK ODS 120T eluted with 2-propanol as eluent B was not significantly better than that with the two-column system on Vydac-201 TP and TSK ODS 120T. To optimize the elution conditions, acetonitrile–2-propanol (9:1, v/v) was sub-

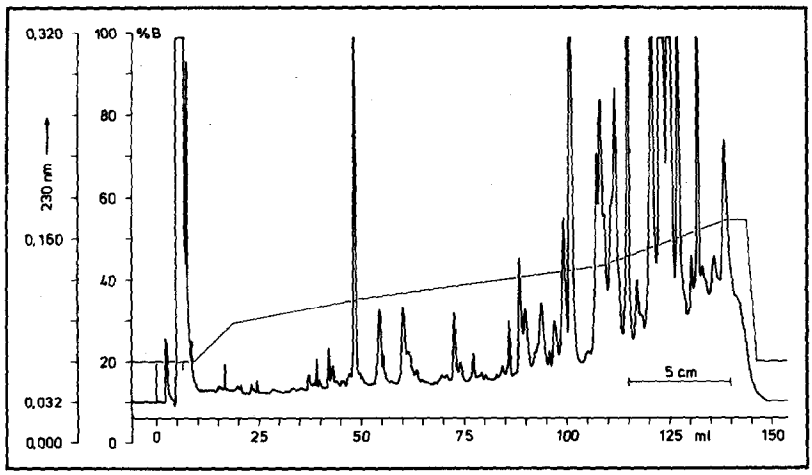

Fig. 6. Discontinuous reversed-phase chromatography with TSK TMS 250 and TSK ODS 120T. Eluent B, 0.1% TFA in acetonitrile; gradient, from 20 to 55% B in 265 min; flow-rate, 0.5 ml/min; temperature, 20°C; detector wavelength, 230 nm, range 0.32; recorder, 5 ml/cm; HPLC system, two-pump system from LKB; 36 A_{230} units of TP30 injected. Compared with the TSK ODS 120T column alone there is not much increase in separation length (250 to 325 mm) but an increase in selectivity and a decrease in peak width are observed.

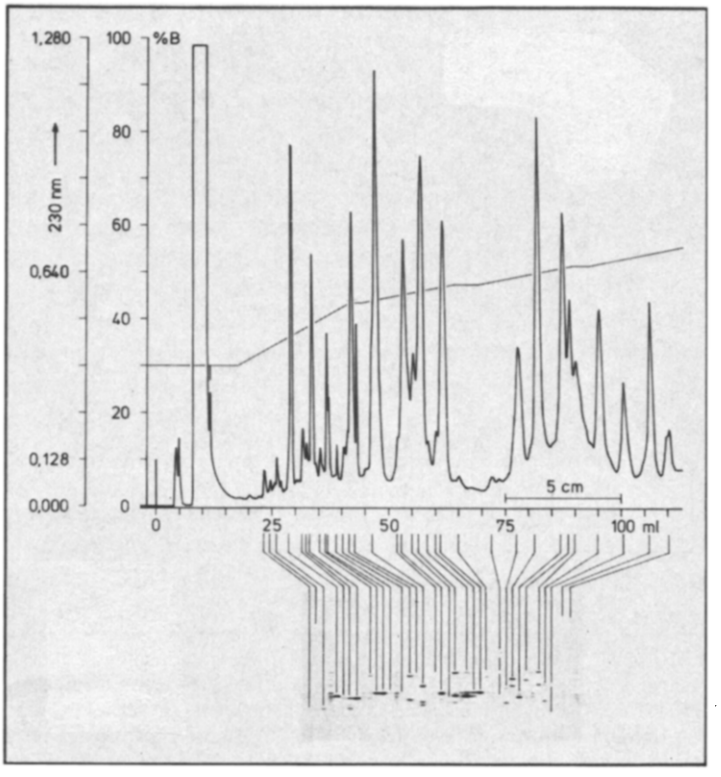

Fig. 7. Discontinuous reversed-phase chromatography with TSK TMS 250, Vydac-201 TP, TSK ODS 120T and acetonitrile with 2-propanol. Eluent B, 0.1% TFA in acetonitrile–2-propanol (9:1); gradient, from 30 to 56% B in 212 min (+26 min delay time); detector wavelength, 230 nm, range 1.28; 72 A_{230} units TP30 injected; other conditions as in Fig. 6. SDS-PAGE: aliquots of the peak fractions, collected in the peak mode by the Superrac fraction collector, were lyophilized, redisolved in 25 μl of sample buffer[23] and applied to the gel.

stituted for eluent B. Under these conditions, it was possible to separate TP30 into approximately 20 peaks, nearly all baseline-separated, within 240 min (Fig. 7). With disc.-RPC we were not only able to obtain better separations of TP30 proteins, but it was also possible to load about 70 A_{230} units of TP30 on to an analytical column combination in a single run.

There is no change in column cross-sectional area[32,33] when combining single RP columns, and therefore the increased load capacity may be an effect of the increasing selectivity, as Wehrli *et al.*[34] showed for isocratic separations, or may be caused by the increasing column length[35].

2D PAGE in Fig. 8 illustrates that 25 proteins could be isolated from 27 distinguishable ribosomal proteins of TP30, by a judicious column combination. At least ten of these proteins are sufficiently pure to be candidates for N-terminal sequence determination.

Using Synchropak RP-P C1 instead of TSK TMS-250, we investigated a semi-preparative combination (I.D. = 8 mm) of C_1–C_{18}–C'_{18}-type columns for the prefractionation of TP30 proteins. All fractions from this separation were subjected to

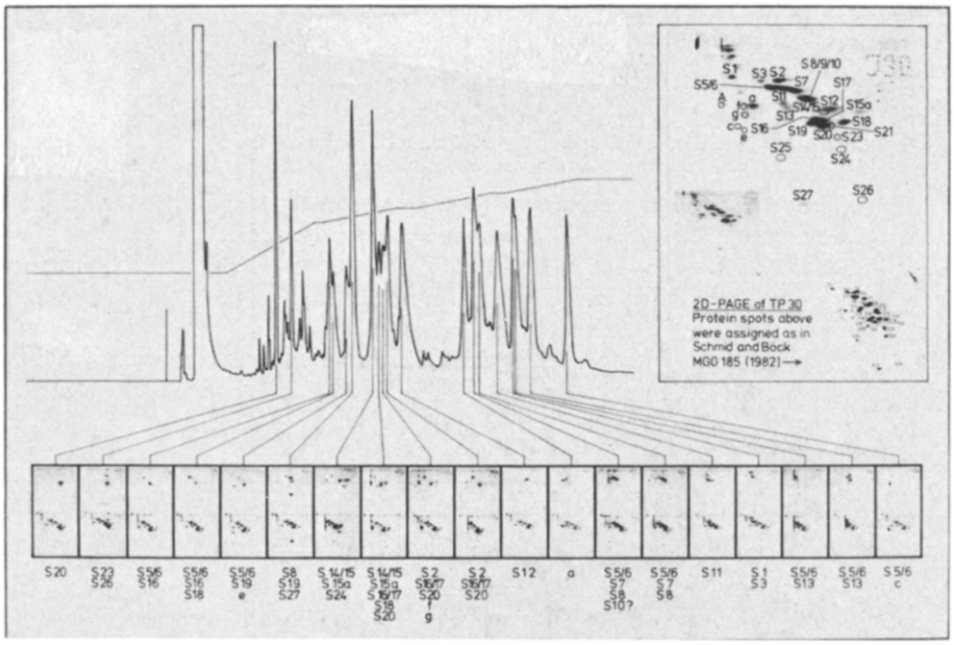

Fig. 8. Identification of 30S ribosomal proteins by discontinuous reversed-phase chromatography and two-dimensional polyacrylamide gel electrophoresis. HPLC conditions as in Fig. 7. Nineteen fractions of this repeated separation were tested by 2D PAGE[24] and assigned according to ref. 4 (2D gel in the lower right corner); 25 of 27 different ribosomal proteins distinguishable by 2D PAGE can be isolated. Upper 2D PAGE line, half the volume of the collected peaks; lower line, half the volume of the peaks plus TP30 as background marker.

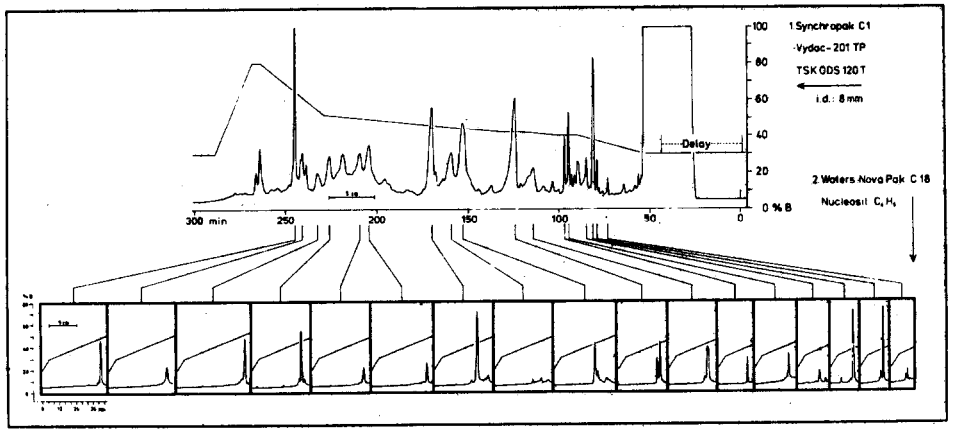

Fig. 9. Pre-fractionation by semi-preparative discontinuous reversed-phase chromatography followed by re-chromatography. Pre-fractionation: columns, Synchropak RP-P C1, Vydac-201 TP and TSK ODS 120T, all 8 mm I.D.; eluents and gradient, as in Fig. 8; flow-rate, 0.5 ml/min (linear velocity 1 ml/cm instead of 3 ml/cm with the analytical columns); recorder, 2.5 ml/cm; detector (1 mm cell path length) wavelength, 230 nm, 0.4 a.u.f.s.; HPLC system from Beckman; 92 A_{230} units TP30, diluted in 10 ml 15% formic acid, were injected. Re-chromatography: columns, Nova Pak C_{18} and Nucleosil 300-7 C_6H_5; eluent B, 0.1% TFA in 2-propanol; gradient, from 20 to 100% B in 70 min; flow-rate, 0.25 ml/min; recorder, 1.0 ml/cm; detector wavelength, 230 nm, range 0.64–2.56; HPLC system, LKB single-pump system. Up to 1 ml of the pre-fractionated fractions were injected without concentrating the sample to reduce the volume.

a disc.-RPC of a Nova Pak C_{18} and a laboratory-packed Nucleosil 300-7 C_6H_5 column. Some of the re-chromatographed fractions could be further subdivided as shown in Fig. 9.

CONCLUSION

We have demonstrated that both IEC and disc.-RPC are suitable methods for the separation of TP30 from *S. acidocaldarius*. It was possible to isolate six nearly purified proteins in one run by IEC. However, proteins purified by IEC must be desalted and concentrated before they can be re-chromatographed or sequenced. Desalting could be accomplished by traditional methods such as dialysis or by RPC on short guard columns. A major reason for using IEC for the separation of proteins is to avoid denaturing conditions during protein purification. Unfortunately, because of the low yields of proteins under the non-denaturing conditions that we used, it does not seem feasible for us to isolate proteins for crystallization.

In contrast, using disc.-RPC methods, up to ten proteins could be purified for sequence determination. Although none of the methods examined could separate all proteins in the TP30 mixture, the advantages of disc.-RPC are the use of volatile buffer systems and the high load capacity, which appears to be even higher than with IEC. We are now testing short-column disc.-RPC for the separation of TP30 and TP50 of *S. acidocaldarius* in a similar manner to the use of short single columns[35].

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