A rapid and preparative method for the separation of yeast ribosomal proteins by using high-performance liquid chromatography

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Ribosomal proteins from the yeast *Saccharomyces cerevisiae* were separated, on a preparative scale, by ion-exchange h.p.l.c. Proteins from the small and large ribosomal subunits were resolved, respectively, into 33 and 23 peaks, and most of the proteins present in these peaks were identified by using one- and two-dimensional gel electrophoresis. Several of the peaks appeared to contain a single protein uncontaminated by other species. Ribosomal proteins were also separated by using reverse-phase h.p.l.c. Analysis of the peaks resolved indicated that the order of elution for the proteins of both ribosomal subunits is, in certain cases, different for each of the two h.p.l.c. techniques used. Thus a combination of the two chromatographic methods employed here has the potential to facilitate the rapid and preparative separation of each of the proteins present in yeast ribosomes.

INTRODUCTION

Ribosomal proteins (r-proteins) of the yeast *Saccharomyces cerevisiae* have, like those from other organisms, M_r values generally below 30000 and contain a predominance of basic amino acids (Warner, 1982). Although these properties have made separation and purification a problem, r-proteins from *Escherichia coli* have been resolved by using ion-exchange and size-exclusion chromatography (Wittmann, 1974). These methods have also been employed to separate eukaryotic r-proteins (Tsurugi *et al.*, 1977; Itoh *et al.*, 1979, 1980; Higo & Otaka, 1979; Otaka *et al.*, 1982, 1984), but have the disadvantages of being very laborious and of producing low yields for some proteins. More recently, reverse-phase h.p.l.c. has been used to separate both prokaryotic (Kerlavage *et al.*, 1982, 1983a,b; Kamp *et al.*, 1983; Kamp & Wittmann-Liebold, 1984) and eukaryotic (Ferris *et al.*, 1984; Kerlavage *et al.*, 1985) r-proteins. The use of h.p.l.c. in such experiments has resulted in an improved resolution of components, and a decrease in column run times, from days to hours.

The application of ion-exchange h.p.l.c. to the separation of r-proteins has to date been very limited. The basic nature of r-proteins indicated that a separation on the basis of charge might not be satisfactory (Kerlavage *et al.*, 1983b). Nevertheless, the potential for using ion-exchange h.p.l.c. to separate *E. coli* r-proteins under selected conditions was recognized (Kamp *et al.*, 1984) and such a separation has been achieved, on a preparative scale, in our laboratory (Capel *et al.*, 1986).

Although an attempt to resolve rat liver ribosomal proteins by ion-exchange h.p.l.c. was only partially successful (Dalrymple *et al.*, 1983), we have subsequently used a combination of ion-exchange and reverse-phase

h.p.l.c. to purify and characterize r-protein L3 from trichodermin-resistant yeast mutants (Threadgill *et al.*, 1986). We have now extended our new method to the separation of proteins from both 40 S and 60 S ribosomal subunits of *S. cerevisiae* and have identified, by gel electrophoresis, most of the components resolved.

EXPERIMENTAL

Yeast strains

S. cerevisiae strain Y166 (α , *trp-5*[−], *his-4*[−]) was grown in 150 l batches of YEPD medium (1% yeast extract, 2% Bacto-peptone, 2% glucose) to an A_{600} of 2.0 (Threadgill *et al.*, 1986). Cells were slow-cooled, harvested, washed with distilled water (Threadgill *et al.*, 1986) and then frozen and stored at −20 °C until required.

Breakage of yeast cells

In preparation for each column run, cells (50–100 g wet wt.) were resuspended in 300 ml of low-salt buffer (100 mM-Tris/HCl, pH 7.4, 12.5 mM-magnesium acetate, 80 mM-KCl, 5 mM-dithiothreitol). The cells were broken by four passages through a French Pressure Cell (Aminco) at 7000 lbf/in² (48.3 MPa). Debris was sedimented by centrifugation at 12000 g for 15 min, and the supernatant fraction was retained. The pellet was extracted with 100 ml of low-salt buffer, and centrifugation was again carried out as above. The supernatant fractions were combined and cleared by centrifugation at 20000 g for 30 min. All operations were carried out at 4 °C.

Abbreviations used: r-protein, ribosomal protein; TP40, total protein from 40 S ribosomal subunits; TP60, total protein from 60 S ribosomal subunits.

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Preparation of ribosomes, ribosomal subunits and ribosomal proteins

Ribosomes were prepared from supernatant fractions and ribosomal subunits isolated exactly as described elsewhere (Threadgill *et al.*, 1986). Proteins were extracted from ribosomal subunits by the acetic acid extraction method (Hardy *et al.*, 1969), dialysed into 5% (v/v) acetic acid and freeze-dried. Proteins obtained from 60 S and 40 S ribosomal subunits are referred to, respectively, as TP60 and TP40.

Ion-exchange h.p.l.c.

For the separation of r-proteins, a preparative Bio-Rad

SP-5-PW cation-exchange column (150 mm $\times$ 21.5 mm) was utilized in conjunction with a KCl gradient and a low-pH buffer. The buffer solutions used were as follows. Buffer A: 8 M-urea, 0.02 M-KH₂PO₄, 0.5 mM-dithiothreitol. Buffer B: 8 M-urea, 0.02 M-KH₂PO₄, 0.5 mM-dithiothreitol, 1 M-KCl. The pH of both was adjusted to 5.6 with methylamine.

The freeze-dried proteins were dissolved in buffer A before their separation. Between 100 and 600 A_{230} units of protein (20–120 mg) could be loaded on to the column. The flow rate was 1.5 ml/min, and fractions were collected for 2 min each. The concentration of KCl in the eluting buffer is indicated on the relevant profiles. The A_{230} of each fraction was recorded.

Fig. 1. Separation of TP40 by ion-exchange h.p.l.c.

(a) Approx. 20 mg (100 A_{230} units) of protein, extracted from the small ribosomal subunit and dissolved in 3 ml of buffer A, was applied to a preparative SP-5-PW column as described in the Experimental section. The KCl concentration of the eluting buffer is indicated by the bold line. Peaks were pooled as shown and prepared for electrophoresis as described in the Experimental section (b). Samples (20 μ l) were run on a SDS/17.5% -acrylamide gel with TP40 and M_r markers. The M_r marker lane for the left half of the gel (peak nos. 5–22) did not include the M_r -17000 or -12300 markers and has an extra M_r -29000 marker.

Identification of r-proteins by gel electrophoresis

Peaks obtained from ion-exchange h.p.l.c. columns were pooled, dialysed against 5% acetic acid and freeze-dried. The freeze-dried samples were then dissolved in 200 μ l of 8 M-urea to allow subsequent analysis by gel electrophoresis.

One-dimensional SDS/polyacrylamide (17.5%) gels were run as described by Laemmli (1970). Two-dimensional gel electrophoresis was performed by the method of Kaltschmidt & Wittmann (1970). The nomenclature used to identify the separated proteins is that of Michel *et al.* (1983). All gels were stained with Coomassie Blue R250 unless otherwise noted.

Reverse-phase h.p.l.c.

For further purification of r-proteins (i.e. r-protein L2), the relevant pooled peak obtained from an

Table 1. Identification of proteins from 40 S ribosomal subunits

TP40 was separated by ion-exchange h.p.l.c. (Fig. 1a). Proteins were identified by a combination of one- (Fig. 1b) and two- (Fig. 2) dimensional gel electrophoresis.

Peak no.	Protein
1	—
2	—
3	—
4	—
5	S1a
11	S27a
12	S1, S7, S27
13	S11, S16
14	S20
15	S25
16	S6
17	S13
18	—
19	S17
20	S33
21	S12
22	S0, S21
23	S24
24	S3, S10
25	S22
26	S4
27	—
28	S36 or S37
29	S28
30	S14
31	S18
32	S31

Fig. 2. Identification, by two-dimensional gel electrophoresis, of proteins separated from TP40 by ion-exchange h.p.l.c.

Samples of r-protein, prepared as described in the legend to Fig. 1, were identified by two-dimensional gel electrophoresis (Kaltschmidt & Wittmann, 1970). Approx. 4 μ g of each protein sample was applied to each of the two gels shown. Approx. 10 μ g of TP40 was applied additionally to the gels illustrated on the right-hand side of the photographs. Gels were silver-stained (Morrissey, 1981). Proteins were identified by the nomenclature of Michel *et al.* (1983). (a) Peak fraction 13 (S11 and S16); (b) peak fraction 17 (S13). Migration in the first dimension was from left to right and in the second from top to bottom.

ion-exchange h.p.l.c. column was loaded directly on to a reverse-phase h.p.l.c. column. For separation of TP40 and TP60 by reverse-phase h.p.l.c., freeze-dried samples were dissolved in buffer A before loading. The column used was a preparative SynChropak RP-P C₁₈-silica column (50 mm $\times$ 10 mm). A gradient of acetonitrile in 0.1% trifluoroacetic acid was used as described elsewhere (Kerlavage *et al.*, 1983a). The flow rate was 2.5 ml/min, and fractions were collected for 1 min each. The A_{230} or A_{214} was determined for each of the fractions collected, as indicated on individual Figures.

RESULTS AND DISCUSSION

Ion-exchange h.p.l.c. has gained only limited appeal as a method for separating r-proteins (Kamp *et al.*, 1984; Dalrymple *et al.*, 1983). This is surprising, as classical methods for their resolution involve ion-exchange chromatography as the main purification step. More recently, however, work in our laboratory has indicated that ion-exchange h.p.l.c. can be used very successfully to separate r-proteins from both *E. coli* (Capel *et al.*, 1986) and *S. cerevisiae* (Threadgill *et al.*, 1986). In the latter work we showed that ion-exchange h.p.l.c. could be used to purify large amounts of a single r-protein (L3). This allowed us to study structural changes in this protein isolated from various trichodermin-resistant mutants of *S. cerevisiae*. We have now extended our earlier studies to the separation of proteins from both 40 S and 60 S ribosomal subunits isolated from *S. cerevisiae*. We have succeeded in identifying most of the proteins present in

Fig. 3. Separation of TP60 by ion-exchange h.p.l.c.

(a) Approx. 20 mg (100 A_{230} units) of protein extracted from the large ribosomal subunit were dissolved in 3 ml of buffer A and analysed exactly as described in the legend to Fig. 1. (b) The SDS/polyacrylamide gel is pictured, with the peak numbers indicated.

the separated peaks, and further demonstrate the use of reverse-phase h.p.l.c. as an important final purification step in the preparative method.

A typical separation of TP40 from *S. cerevisiae* by ion-exchange h.p.l.c. is shown in Fig. 1(a). Analysis, by SDS/polyacrylamide-gel electrophoresis (Fig. 1b), of the proteins present in the 33 peaks resolved indicates that in many cases the peaks appear to contain a single protein: a vast improvement on previously published separations of eukaryotic r-proteins by ion-exchange chromatography. The identification of the major protein(s) present in each peak involved pooling of each peak, dialysis into 5% acetic acid, freeze-drying, resuspension in urea and separation by two-dimensional gel electrophoresis (Kaltschmidt & Wittmann, 1970). Only basic proteins were separated on these two-dimensional gels, although certain acidic proteins, also present in ribosomes, were identified by SDS/polyacrylamide-gel electrophoresis by reference to their known M_r values (Michel *et al.*, 1983). Fig. 2 illustrates the

identification of r-proteins S11 and S16 in peak 13 and of r-protein S13 in peak 17, by using two-dimensional gel electrophoresis. In each case, two gels were run in duplicate, one with only the sample of interest and the other with the sample of interest plus a background of TP40 as an aid to placement of the protein spots in the TP40 pattern (Fig. 2). Each peak with enough material present was subjected to this type of analysis. The identification of the basic proteins present in the separated peaks was made on the basis of both these two-dimensional gels and the position of the proteins on the SDS/polyacrylamide gels in cases of ambiguous placements. The results are presented in Table 1, with identifications indicated for the major protein species detected in each peak.

Certain peaks seen in Fig. 1(a) (peaks 6–10) did not contain enough protein to allow detection by gel electrophoresis. As our separations on ion-exchange h.p.l.c. were carried out on a preparative scale, with a total loading in each experiment of 20 mg of protein, it

Fig. 4. Identification, by two-dimensional gel electrophoresis, of proteins separated from TP60 by ion-exchange h.p.l.c.

Samples of r-protein, prepared as described in the legend to Fig. 3, were identified by two-dimensional gel electrophoresis (Kaltschmidt & Wittmann, 1970). A 50 μ l portion of each pooled peak sample was applied to each of the two gels shown. Approx. 30 μ g of TP60 was applied additionally to the gels illustrated on the right-hand side of the photographs. Proteins were identified by using the nomenclature of Michel *et al.* (1983). (a) Peak fraction 20 (L35); (b) peak fraction 21 (L13). Migration in the first dimension was from left to right and in the second from top to bottom.

is possible that those proteins present in peaks 6–10, and barely detectable by their A_{230} , are not true r-proteins, since they are present in such low amounts. However, since we have recently shown (M. Cannon, G. J. Threadgill & R. C. Conrad, unpublished work) that ethanol precipitation of yeast ribosomal subunits can cause selective losses of certain proteins from these subparticles, peaks 6–10 may indeed contain low amounts of r-proteins that were largely lost during preparation. Further analyses will be required in order to identify these proteins correctly. Other peaks which remain unidentified include those eluted early (peaks 1–4). These peaks possibly contain one or more of the acidic proteins (S19, S26, S26a) that we have not identified, as indicated by our failure to resolve them by two-dimensional gel electrophoresis under the experimental conditions used in our studies. Furthermore, since r-proteins S26 and S26a have M_r values lower than 10000, we did not

Table 2. Identification of proteins from 60 S ribosomal subunits

TP60 was separated by ion-exchange h.p.l.c. (Fig. 3a). Proteins were identified using a combination of one- (Fig. 3b) and two- (Fig. 4) dimensional gel electrophoresis.

Peak no.	Protein
1	—
2	—
3	L15
4	L8
5	L32
6	L26
7	L16, L33
8	L6 and/or L7, L33
9	L9
10	L1a, L40
11	L4, L4a, L22
12	L34
13	L12, L20, L25, L31, L36
14	L2, L39
15	L5, L10, L19
16	L23
17	L3, L38
18	L21, L30
19	L23
20	L35
21	L13
22	—
23	L29

attempt to analyse the four early-eluted peaks by SDS/polyacrylamide-gel electrophoresis. Some of the peaks illustrated in Fig. 1(a) are less well resolved than others. As a result, there is evidence of spill-over in certain of the fractions that we analysed. An example of such spill-over is typified by peaks 23, 24 and 25 in Fig. 1(a). These peaks are incompletely resolved by ion-exchange h.p.l.c. under our experimental conditions, and a doublet protein-banding pattern spills over from peak 24 into peaks 23 and 25 (Fig. 1b). We have not attempted in this work to improve the resolution of such closely eluted proteins. We suggest, however, that controlled changes in the salt concentration gradient of the eluting buffer for the ion-exchange column could well be a fruitful approach in solving this problem.

Previously (Threadgill *et al.*, 1986) we showed the separation of TP60 using ion-exchange h.p.l.c. Here we identify many of the proteins resolved by this method.

A typical separation of TP60 is shown in Fig. 3(a). In this case the separation is not as clear-cut as for the TP40 preparation. To a degree, this is to be expected, since there are more proteins present in the 60 S ribosomal subunit, but the problem is compounded by the fact that fewer peaks are resolved (cf. Figs. 1a and 3a). Proteins in these peaks were again identified by using one-dimensional (Fig. 3b) and two-dimensional polyacrylamide-gel electrophoresis. Examples of these identifications by using the latter system are shown in Fig. 4, and a summary of the identifications is given in Table 2. The acidic protein L1a was identified by SDS/polyacrylamide-gel electrophoresis by reference to its known M_r (Michel *et al.*, 1983), but no attempt was made to identify the low- M_r acidic protein L44. It is clear that, although many of the peaks separated by the ion-exchange column (Fig. 3a) appear to contain single

Fig. 5. Purification of r-protein L2 by reverse-phase h.p.l.c.

Peak 14 from the ion-exchange h.p.l.c. separation illustrated in Fig. 3(a) was pooled as shown and run on a reverse-phase h.p.l.c. column as described in the Experimental section. The A_{214} of each fraction was measured. Peak fractions were pooled as shown, freeze-dried and total samples analysed on a SDS/17.5% acrylamide gel (insert) as described in the Experimental section. Lanes 1 and 8, M_r standards as in Fig. 1(b) (right-hand half of gel); lanes 2 and 7, TP60; lanes 3–6, peaks 1–4, as shown on the profile.

proteins, some contain multiple components (Table 2). In addition, there is again evidence of spill-over in certain fractions. Earlier work from our laboratory (Threadgill *et al.*, 1986) has indicated that this problem of co-elution might be overcome by using a combination of ion-exchange and reverse-phase h.p.l.c. Indeed, such an experimental approach enabled us to purify r-protein L3 to apparent homogeneity (Threadgill *et al.*, 1986). We therefore wished to illustrate the applicability of the combined approach to the purification of r-proteins other than L3.

Fig. 5 shows the further purification, by this technique, of peak 14 (see Fig. 3a), known to contain r-proteins L2 and L39 (see Table 2) and other minor contaminating species. Reverse-phase h.p.l.c. resolves the proteins present in this fraction into four clearly separated peaks. SDS/polyacrylamide-gel electrophoresis of these four peaks shows that peak 4 consists almost exclusively of r-protein L2 (see track 6 on the SDS gel illustrated in Fig. 5). A similar approach to ours has, in fact, been used by Dettmer *et al.* (1984), who employed a combination of CM-cellulose chromatography and reverse-phase h.p.l.c. in order to purify 32 proteins from the small subunit of rat liver ribosomes.

In order to test the feasibility of purifying each individual yeast r-protein by using the combination of the two h.p.l.c. techniques, we separated TP40 and TP60 preparations on reverse-phase h.p.l.c. columns (see Figs. 6a and 7a) and analysed the peaks by using one-dimensional SDS/polyacrylamide gels (see Figs. 6b and 7b). Although no identifications for all the proteins

present in these peaks are claimed, it appears that the order of elution for the proteins of both ribosomal subunits is, in some cases at least, different for each of the different h.p.l.c. techniques used. This general conclusion is given support by considering the resolution of the r-proteins S1, S7 and S27 and of the r-proteins L1a, L2, L10, L39 and L40. Thus peak 12 in Fig. 1(a) (illustrating the use of ion-exchange h.p.l.c.) contains three major protein bands (Fig. 1b), identified as S1, S7 and S27 (see Table 1). This combination of proteins is not detected in any of the peaks separated by reverse-phase h.p.l.c. Indeed, r-protein S1 is eluted from the reverse-phase h.p.l.c. column (Fig. 6a) as virtually a single protein, and there are no traces of either r-protein S7 or r-protein S27 in this fraction (see peak 10, Fig. 6b). Ion-exchange h.p.l.c. of TP60 (Fig. 3a) results in the co-elution of two major protein species (see peak 14, Fig. 3b), identified as r-proteins L2 and L39 (see Table 2). In contrast, reverse-phase h.p.l.c. of TP60 (Fig. 7a) resolves these components with L2 being eluted instead as a virtually pure protein (see peak 12, Fig. 7b). There is no trace of r-protein L39 in this fraction. A further example is provided by the elution behaviour of r-protein L1a. Ion-exchange h.p.l.c. of TP60 (Fig. 3a) results in the co-elution of this protein with L40 [see peak 10 (Fig. 3b), with r-protein identifications recorded in Table 2]. In contrast, reverse-phase h.p.l.c. of TP60 (Fig. 7a) results in the co-elution of r-protein L1a not with r-protein L40 but with a component tentatively identified as r-protein L10 (see peak 14, Fig. 7b). To allow easy comparison without recourse to detailed scrutiny of several Figures,

(b)

Fig. 6. Separation of TP40 by reverse-phase h.p.l.c.

(a) Approx. 7 mg of TP40 was dissolved in buffer A and applied to a preparative SynChropak C₁₈-silica column as described in the Experimental section. Proteins were eluted with acetonitrile, whose concentration is indicated by the bold line. (b) Pooled peaks were freeze-dried and resuspended in 100 μ l of 8 M-urea. Then 20 μ l of each sample was run on a SDS/17.5%-acrylamide gel as described in the Experimental section. M_r standards were as in Fig. 3(b).

the above data are presented in the form of a composite diagram (see Fig. 8). Potentially, therefore, this provides a most favourable situation, although, despite these very promising separations, we accept that a certain number of r-proteins that co-purify from the ion-exchange column may also co-purify under the conditions used by us for reverse-phase h.p.l.c.

In the separations presented here we have applied 20 mg of total protein to the ion-exchange column. In other experiments we have been able to increase this to 120 mg with no apparent effect on the separation achieved (results not shown). This is in marked contrast with previously published separations using CM-cellulose chromatography, where increasing the amount of

Fig. 7. Separation of TP60 by reverse-phase h.p.l.c.

Approx. 7 mg of TP60 was treated and analysed under conditions described in the legend to Fig. 6.

protein loaded on to the column caused a decrease in the resolution of the components (Itoh *et al.*, 1979). On the other hand, it is possible to achieve an analytical-scale separation of yeast r-proteins by using ion-exchange h.p.l.c., comparable with the preparative-scale separations presented here, in under 2 h (G. J. Threadgill, unpublished work). Thus the ion-exchange h.p.l.c. separation method described by us here can easily be adapted for either analytical or preparative purposes.

Our results demonstrate conclusively that ion-exchange h.p.l.c. can be used very successfully as an initial purification step for certain yeast r-proteins by using the high-urea low-pH system described in the present paper. The SP-5-PW column employed in our experiments has two main advantages over reverse-phase C_{18} -silica columns, certainly in our hands: losses are much less (10%, compared with 25%) and a larger amount of material can be applied. It was not our

Fig. 8. Resolution of selected r-proteins by using ion-exchange and reverse-phase h.p.l.c.

This composite diagram illustrates that certain r-proteins are co-eluted from ion-exchange h.p.l.c. columns but not from reverse-phase h.p.l.c. columns. The data shown are abstracted from the detailed data presented in Figs. 1, 3, 6 and 7, and indicate r-proteins identified by SDS/polyacrylamide-gel electrophoresis after elution from h.p.l.c. columns. Tracks *b*, *e* and *g* are samples resolved by ion-exchange h.p.l.c. Tracks *c*, *f* and *h* are samples resolved by reverse-phase h.p.l.c. M_r values for the r-proteins S1, S7, S27, L1a, L2, L10, L39 and L40 are respectively ($\times 10^{-3}$) 33.5, 27.0, 13.5, 38.5, 42.0, 27.5, 10.5 and 10.0 (see Michel *et al.*, 1983).

intention in this work to present an h.p.l.c. purification scheme for each individual r-protein. Instead, we have developed a new method that provides an excellent starting point for an investigator who wishes to obtain a particular r-protein from yeast. We suggest that the acetonitrile concentration gradient used in our present work (see also Threadgill *et al.*, 1986) might well be used successfully and without modification for the purification of many r-proteins (certainly including L2 and L3). Nevertheless, the acetonitrile concentration gradient that we have used can, if necessary, be manipulated to maximize the purification of the protein of interest from contaminating species. In addition, as mentioned above, the salt-concentration gradient used here for ion-exchange h.p.l.c. might also be modified to improve the separation of proteins at selected places in the elution profile. The availability of this important, rapid and reproducible technique should greatly assist in structural and functional studies of the yeast ribosome and yeast r-proteins and may facilitate the development of a method for the complete reconstitution of the eukaryotic ribosome from its RNA and protein components.

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