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## Note

# Purification of low-molecular-weight RNAs by high-performance gel permeation chromatography

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Interest in the purification of small RNAs[1] steadily increased in recent years when it was recognized that these RNAs as well as the corresponding ribonucleoprotein particles (RNPs) can be causative agents of important diseases. In humans several connective tissue disorders, *e.g.*, *lupus erythematosus*, are accompanied by autoantibody production against small nuclear RNPs[2] (snRNPs). Different classes of snRNPs contain different small nuclear RNAs (snRNAs) which have highly conserved sequences of approximately 100–220 nucleotides in length[1–3]. RNA viroids are the smallest known agents of several economically important diseases of higher plants[4].

The purification of individual RNA species still remains rather difficult, although methods for the separation of complex RNA mixtures are available. One-dimensional polyacrylamide gel electrophoresis under denaturing conditions gives rise to satisfactory separations of RNAs on an analytical scale[5]. An even better resolution of low-molecular-weight RNA mixtures can be obtained by two-dimensional gel electrophoresis[6,7]. For preparative purposes, electrophoretic procedures have the drawback of a partial loss of the precious material during the reextraction of the RNAs from the gel matrix. We have chosen high-performance liquid chromatography (HPLC) to fractionate RNA mixtures according to their molecular weights. Previously, we succeeded in separating cytoplasmic RNA mixtures by HPLC gel filtration, achieving a complete recovery of biologically active mRNA molecules[8]. The fractionation range of this procedure appeared from our experimental data to be particularly attractive for the separation of relatively small RNA molecules. In this report we describe the purification of low-molecular-weight RNA molecules consisting of 70–400 nucleotides by high-performance gel permeation chromatography.

## EXPERIMENTAL

### *Materials*

Procaryotic ribosomal RNAs and 4S RNAs (mixture of tRNAs) were obtained from Boehringer (Mannheim, F.R.G.). [$^3$H]uridine (specific activity 43 Ci/mmol) was purchased from Amersham Buchler (Braunschweig, F.R.G.). All other chemicals were analytical grade and came from E. Merck (Darmstadt, F.R.G.).

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*Extraction of small nuclear RNAs*

A [$^3$H]uridine-labelled nuclear RNA mixture containing snRNAs was isolated as described by Ireland *et al.*[9] with minor modifications. Briefly, HeLa cells growing in suspension were labelled with [$^3$H]uridine (0.7 $\mu$Ci/ml) for 24 h. Cell nuclei were prepared, disrupted by ultrasonication and total nuclear RNAs were extracted after proteinase K treatment with phenol–chloroform–isoamyl alcohol (50:50:1, v/v/v). The bulk of high-molecular-weight RNAs was removed by centrifugation. Total nuclear RNA was loaded on a 10–30 % sucrose gradient containing 100 m*M* NaCl, 1 m*M* EDTA, 10 m*M* Tris–HCl, pH 7.5 and 0.2 % sodium dodecyl sulphate (SDS) and centrifuged for 6 h at 200,000 *g* and 23°C in a Beckman SW41 rotor. Gradient fractions containing RNAs smaller than 18S were combined and the RNA was recovered by precipitation in ethanol.

*Separation of RNA mixtures*

HPLC gel filtration of RNAs was performed at 21°C with a LKB chromatography system consisting of the UltroPac columns (60 × 0.75 cm) TSK G 4000 SW, 3000 SW and 2000 SW, an UltroPac TSK precolumn GSWP (7.5 × 0.75 cm), a HPLC pump, an Uvicord S II monitor and a SuperRac fraction collector. The following buffers were used for chromatography: A, 100 m*M* sodium acetate, pH 7.0, 750 m*M* NaCl, 0.1 % velcorin and 1 % methanol; B, 200 m*M* NaCl, 5 m*M* $MgCl_2$, 10 m*M* sodium acetate, pH 5.5, 0.2 % SDS; C, 6 *M* urea, 1 m*M* EDTA, 75 m*M* Tris–HCl, pH 7.5, 0.1 % SDS.

The RNA samples were dissolved in 50–100 $\mu$l of elution buffer, loaded onto the column through a Rheodyne injector and eluted with a constant flow-rate of 6–12 ml/h. Fractions of 500 $\mu$l were collected. The RNA was recovered from the eluate by precipitation with 2.5 volumes of ethanol.

The RNA composition of the column fractions was analysed on 10 % polyacrylamide gels in 7 *M* urea, 100 m*M* Tris–borate, pH 8.3 and 20 m*M* EDTA[10]. $^3$H-labelled ribosomal RNA from HeLa cells and non-radioactive snRNAs from Ehrlich Ascites Tumor cells which contained U2, U4 and U6 were used as markers. Electrophoresis was carried out at 250 V for 11 h. The gels were prepared for fluorography according to the procedure of Laskey[11] and exposed to Kodak XAR film.

## RESULTS AND DISCUSSION

We have previously shown that RNAs of high molecular weight, *e.g.*, 23S and 16S rRNAs as well as 9S globin mRNA, can be highly purified by high-performance gel permeation chromatography[8] using a TSK G 4000 SW column which has a gel matrix of relatively large pore size[12]. The other two columns TSK G 3000 SW and 2000 SW which are commercially available are characterized by matrices of lower pore sizes as has been demonstrated by chromatography of protein mixtures[12]. The fractionation range of the TSK G 3000 SW column was determined for RNAs using a mixture of procaryotic ribosomal RNAs and 4S RNAs (Fig. 1). Both high-molecular-weight rRNAs, 23S and 16S, were eluted together in the exclusion volume of the TSK G 3000 SW column (Fig. 1). The low-molecular-weight RNAs, 5S and 4S, having chain lengths of 120 and about 80 nucleotides, respectively, gave rise to two peaks in the chromatographic profile which were very well separated from each other (Fig. 1).

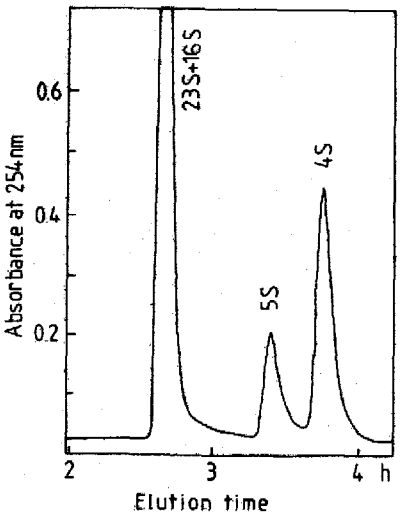


Fig. 1. RNA fractionation on a TSK G 3000 SW column. A mixture containing 23S, 16S, 5S and 4S RNA (75 μg) was dissolved in 100 μl of buffer A and loaded onto a TSK G 3000 SW column (60 × 0.75 cm) which was eluted at 21°C with the same buffer at a flow-rate of 100 μl/min.

As expected, the 4S RNA which was a mixture of different tRNAs could not be resolved into individual tRNA species by HPLC gel filtration. The difference in molecular weight between the various tRNAs was not great enough for a separation of the mixture on this matrix. Column chromatography on RPC5[13] and BD-cellulose[14] has been successfully used for the purification of specific tRNAs.

It appeared from the experiment of Fig. 1 that the fractionation range of the TSK G 3000 SW column would be optimal for the separation of snRNAs which have chain lengths varying between 100 and 210 nucleotides[1–3,9]. When an extract containing $^3$H-labelled snRNAs was chromatographed on this matrix a quite good separation of the individual snRNAs was obtained. An even better resolution of the snRNA mixture was achieved on a "tandem" system that consisted of TSK G 3000 SW and 2000 SW columns connected in series (Fig. 2A). The residual high-molecular-weight RNA which was not removed during the gradient centrifugation step was eluted in the exclusion volume. Several small RNA peaks appeared in the elution profile besides the prominent 5S and 4S RNA species. The RNA composition of the fractions which were combined as indicated by the bars in Fig. 2A was analysed on a 10% polyacrylamide gel (Fig. 2B). The fluorogram of the gel clearly shows that several low-molecular-weight RNA species, *e.g.*, 7S, U2, 5S and 4S RNA, were highly enriched after a single gel filtration run on this tandem system. In the elution profile a double peak appeared in fraction E which contained two RNA species, 5.8S RNA and U4 snRNA (Fig. 2B) with chain lengths of 165 and 154 nucleotides[9], respectively. These species can be separated by collecting smaller fractions during rechromatography. Approximately 95% of the starting radioactive material was recovered after HPLC gel filtration by precipitation of the eluate in ethanol.

Chromatography of the snRNA mixture under denaturing conditions using buffer C was carried out on a TSK G 4000 SW, a 3000 SW or a "tandem" system consisting of both columns, but the resolution shown in Fig. 2A was not achieved.

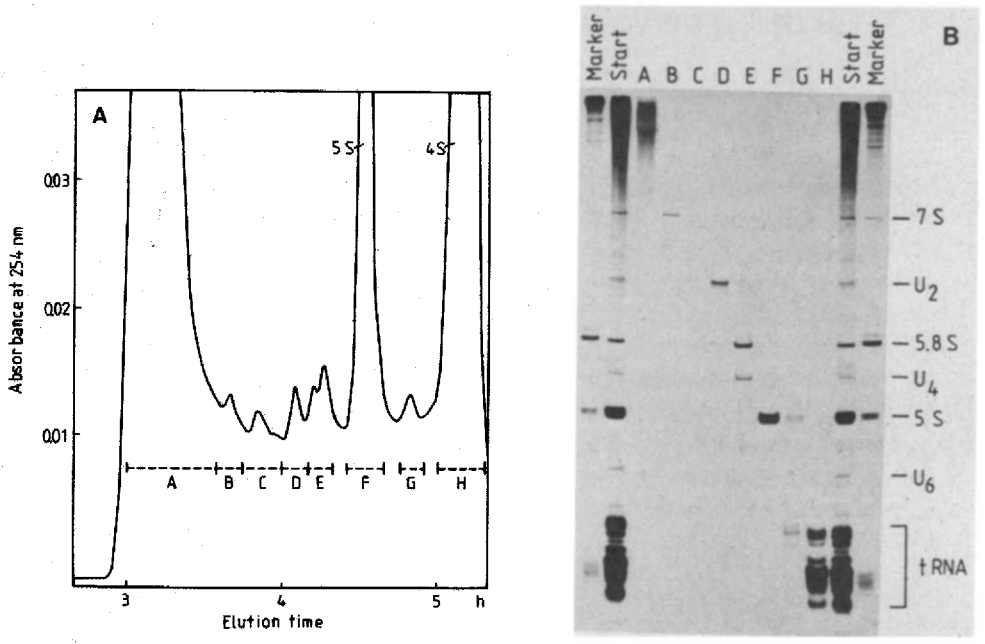


Fig. 2. Purification of small nuclear RNAs. A $^{3}$H-labelled RNA extract ($3 \times 10^{5}$ cpm) from HeLa cell nuclei was prepared as described in Experimental. A RNA fraction containing molecules smaller than 18S (start) was chromatographed on a "tandem" system consisting of one TSK G 3000 SW and one TSK G 2000 SW column (A). Buffer B was used for elution at a flow-rate of 100 $\mu$l/min. The RNA composition of the indicated fractions was analysed on a 10% polyacrylamide gel, which was subsequently fluorographed (B).

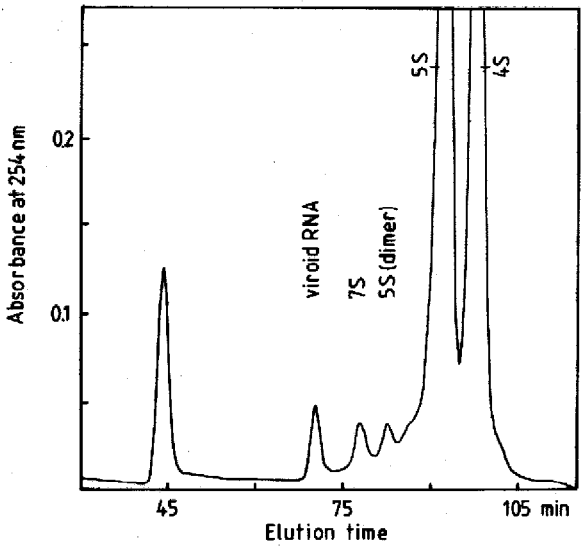


Fig. 3. Analytical separation of viroid RNA-containing plant extract. A RNA extract from potato spindle tuber viroid-infected leaves (70 $\mu$g) was applied onto a TSK G 4000 SW column ($60 \times 0.75$ cm) and eluted at a flow-rate of 200 $\mu$l/min with buffer B.

A biologically interesting group of RNAs are plant viroids. They consist of single-stranded, covalently closed, circular RNA molecules having a chain length of approximately 360 nucleotides[4]. These molecules exist in their native state as rod-like structures. We succeeded in separating the viroid RNA from other cellular contaminants (Fig. 3). The viroid RNA eluted from a TSK G 4000 SW column as a well defined peak which was immediately followed by the 7S ribosomal RNA species. Large quantities, approximately 20 mg of RNA extract from viroid-infected tomato leaves, were successfully fractionated on a preparative TSK G 4000 SW column (60 × 2.15 cm) (not illustrated). The elution profile of the preparative column was similar to that in Fig. 3. The viroid RNA fraction appeared already highly enriched after a single chromatographic run. Small quantities of contaminating RNAs which were only detectable in polyacrylamide gels after silver staining could easily be removed by rechromatography (data not shown).

## CONCLUSION

Gel permeation chromatography on TSK columns is an excellent preparative method for the purification of specific RNAs of low molecular weight. The isolation of specific small nuclear RNAs as well as the purification of plant viroid RNA can be achieved in high yield.

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## REFERENCES

1 G. Zieve and S. Penman, *Cell*, 8 (1976) 19.
2 M. R. Lerner and J. A. Steitz, *Proc. Nat. Acad. Sci. U.S.*, 76 (1979) 5495.
3 M. R. Lerner and J. A. Steitz, *Cell*, 25 (1981) 298.
4 H. L. Sänger, G. Klotz, D. Riesner, H. J. Gross and A. K. Kleinschmidt, *Proc. Nat. Acad. Sci. U.S.*, 73 (1976) 3852.
5 D. Grierson, in D. Rickwood and B. D. Hames (Editors), *Gel Electrophoresis of Nucleic Acids*, IRL Press, Oxford, 1982, p. 1.
6 T. Ikemura and J. E. Dahlberg, *J. Biol. Chem.*, 248 (1973) 5024.
7 F. Varricchio and H. J. Ernst, *Anal. Biochem.*, 68 (1975) 485.
8 L. Graeve, W. Goemann, P. Földi and J. Kruppa, *Biochem. Biophys. Res. Commun.*, 107 (1982) 1559.
9 L. Ireland, J. Szyszko and M. Krause, *Mol. Biol. Rep.*, 8 (1982) 97.
10 A. C. Peacock and C. W. Dingman, *Biochemistry*, 8 (1969) 608.
11 R. A. Laskey, *Methods Enzymol.*, 65 (1980) 363.
12 Y. Kato, K. Komiya, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 193 (1980) 29.
13 A. D. Kelmers, G. D. Novelli and M. P. Stuhlberg, *J. Biol. Chem.*, 240 (1965) 3979.
14 F. Fittler, J. Kruppa and H. G. Zachau, *Biochim. Biophys. Acta*, 277 (1972) 513.