Liquid Chromatography Purification and Study of Uridilylpolynucleotide-(5′P→O)-Tyrosine Phosphodiesterase

M. B. Viryasov / R. A. Yusupova / G. S. Shatskaya / Yu. F. Drygin*

A. N. Belozersky Institute of Physical and Chemical Biology, Moscow State University, Moscow, 119899, Russia

Key Words

Column liquid chromatography
Size-exclusion chromatography
Anion-exchange chromatography
Hydrophobic interaction HPLC
Phosphodiesterase purification

Summary

LC has been used as a tool for studying uridilylpolynucleotide-(5′P→O)-phosphodiesterase – an enzyme which hydrolyses specifically the phosphodiester bond between picornaviral RNA and viral protein VPg.

According to various chromatographic data, the enzyme forms two types of complex with nucleic acids: weak ones which dissociate in 200 mM KCl, and others which are stable at concentrations up to 900 mM KCl. 2.5-3-fold (preparative) or 6-fold (normal scale) purification of the enzyme was obtained by size-exclusion chromatography (SEC). Cation-exchange separation (4-fold purification) was found to be more suitable as the second enzyme purification step than the earlier anion-exchange method used. Three forms of enzyme activity were discovered by hydrophobic-interaction chromatography on the enzyme preparation obtained by SEC.

Introduction

The eucaryotic enzyme – uridilylpolynucleotide (5′P→O)-tyrosine phosphodiesterase (Y-pUpN PDE) – which specifically hydrolyses the phosphodiester bond between the tyrosine residue of the viral protein VPg and 5′-terminal residue of uridilyc acid of picornaviral RNA, was found in different animal and plant cells [1–5]. The enzyme is active at pH 5.5–8.0, in the presence of 50–200 mM KCl and 0.5–1.5 mM $MgCl_2$ [5].

In previous studies different kinds of liquid chromatography were applied in various combinations for Y-pUpN PDE purification [2, 6, 7]. Conventional size exclusion, anion-exchange and affinity chromatography were tested. However, all procedures used resulted in low yield or inadequate purity of the enzyme for gaining insight into its molecular and kinetic characteristics.

In the present work we examined several modes of HPLC and traditional LC to select those that can be efficient in combination.

Experimental

Isolation of Enzyme

Krebs II ascites carcinoma was maintained in the peritoneal cavity of mice. Ascite cells were collected, twice washed with Earl solution (1:10 v/v, approximately) and stored with 0.25 M sucrose, 10 mM tris-HCl pH 7.5, 1 mM $MgCl_2$ at –75 °C.

Crude enzyme preparation was obtained as described previously [7] by 0.2 M KCl extraction of crude nuclei precipitate by followed sulfate ammonium fractionation (30–60 %) of the nuclear extract. The final precipitate (Sample I) was re-suspended in 65 % ammonium sulfate and stored at –20 °C.

SEC

Low-pressure size exclusion chromatography was performed on ToyoPearl HW55F (Toyo Soda MFG. Co., Ltd., Japan) column (500 × 20 mm, glass) at 4 °C. Elution was carried out with 150 mL of TMM-200 buffer (10 mM Tris-HCl, pH 7.6, 1.5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 200 mM KCl; number after TMM shows KCl concentration in buffer in mM) at the rate of 18 mL h^{-1}. Usually 30–40 mg protein were collected from ammonium sulfate suspension by centrifugation, dissolved in 1–2 mL TMM-200 buffer and applied to the

Presented at the 21st ISC held in Stuttgart, Germany, 15th–20th September, 1996

0009-5893/97 133-05 $ 03.00/0

Table I. HPLC conditions

Type of chromatography	Column	Eluent A	Eluent B	Elution conditions	Sample
Size-exclusion	TSK 3000SW, 7.5 × 600 mm	TMM-50, 100, 200 pH 7.6		Isocratic	1–5 mg in 0.1–0.5 mL
Anion-exchange	SynChropack AX 300, 4.6 × 250 mm	TMM-50, pH 7.0	TMM-1000 pH 7.0	3 min eluent A,, 30 min linear gradient to 50 % B, 5 min linear gradient to 100 % B, 5 min isocratic B.	7–9 mg in 0.5 mL
Chromato-focusing	SynChropack AX 300, 4.6 × 250 mm	0.025 M Histidine-HCl, pH 6.2	Polybuffer 74 (1:8) 25 mM KCl	3 min eluent A, 40 min eluent B (pH gradient)	7–9 mg in 0.5 mL
Hydrophobic interaction	SynChropack, HIC-Propyl, 4.6 × 250 mm	TMM-50, pH 7.6, 1 M $(NH_4)_2SO_4$	TMM-50, pH 7.6, 0.05 M $(NH_4)_2SO_4$	3 min eluent A, 30 min linear gradient to 100 % B, 10 min, isocratic B	5–9 mg in 0.5 mL

column. The eluate was collected as 3 mL fractions. The UV absorption of fractions was measured at 235, 260 and 280 nm for calculation of protein content. Active enzyme fractions (Sample II) were pooled. Proteins were precipitated by 65 % ammonium sulfate and stored at –20 °C.

HPLC Equipment and Conditions

PU4100 liquid chromatograph with PU4110 UV-VIS spectrophotometric detector (PyeUnicam, UK) was used in this work. Biocompatible columns with SynChropack adsorbents from Synchrom Inc., USA (Micra Inc. at present time), and exclusion column TSK 3000SW from Beckman, USA were used. Elution conditions are detailed in Table I. All LC separations were performed at room temperature (20 ±° 2 C). In all experiments the sample was applied in starting eluent (A). Chromatography was carried out at 1 mL min^{-1} in each case. UV detection was at 280 or 260 nm.

Cation Exchange Purification

Portions (200 µL) of wet adsorbent SP-Sephadex (Pharmacia Fine Chemicals AB, Sweden) and CM-52 Carboxymethyl Cellulose (Whatman, UK) were equilibrated with TMM buffer (pH 7.0) which contained different concentrations of KCl (50, 100, 200, 400, 600, 800, 1000, 1200 mM). Enzyme samples (200 µL, 0,2 mg protein) in the same solutions were gently mixed with these portions for 15 min, then centrifuged 2 min at 1000 *g*. Supernates were collected for enzymatic activity assay and protein determination.

Determination of Y-pUpN PDE Enzymatic Activity

Aliquots of chromatographic fractions (50 µL) were desalted by micro-spin chromatography on Sephadex G-25, coarse grade (Pharmacia Fine Chemicals AB, Sweden) equilibrated with TMM-200 buffer, pH 7.6 as described in [8].

The assay was performed according to [6] with encephalomyocarditis virus K-peptide-RNA complex, labeled with ^{125}I, as substrate. 4 µL chromatographic fraction and 1 µL ^{125}I-K-peptide-RNA sample (0.02–0.05 µg, 5000 cpm) were used in the reaction. This mixture was incubated for 1 h at 37 °C and the reaction stopped by fast freezing. Then probes were applied to TLC Kieselgel 60 plates (Merck, Germany).TLC was as described [6]. Detection of reaction ^{125}I products was carried out by autoradiography.

In all steps total protein concentration was detected according to Whitaker and Granom [9]. Nucleic acid content was determined by spectrophotometry as described in [10] or by Spirin [11].

Results and Discussion

Despite of almost 20 years history of Y-pUpN PDE since it was discovered [1] (earlier known as an "unlinking" enzyme), little is known about the enzyme itself and the role of this representative of a new class of hydrolases in a living cell.

To date, some enzyme characteristics obtained [1, 2, 6] are still in doubt because traditional methods of liquid chromatography used for isolation resulted in low yield and inadequate purity of the enzyme. In this work we used traditional preparative LC and LC in analytical mode to purify the enzyme under study and to examine its properties.

In our previous work SEC of the crude enzyme preparation was performed on Sephadex G-100 [7]. When elution was carried out with TMM-50 buffer the active enzyme was eluted along with high molecular mass components (nucleic acids, presumably). We proposed that Y-pUpN PDE in crude cell extracts forms non-specific complex with nucleic acids.

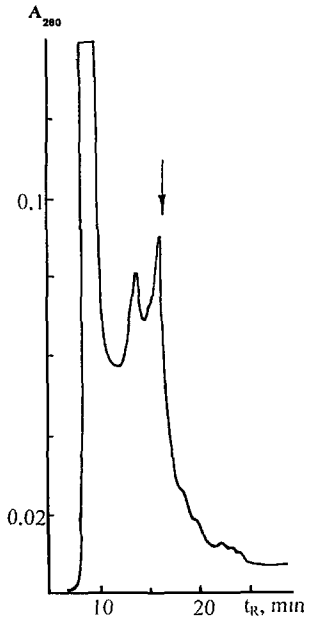

Figure 1
SEC of enzyme Sample I on TSK 3000SW column, TMM-200 eluent. Arrow indicates enzymatic activity maximum.

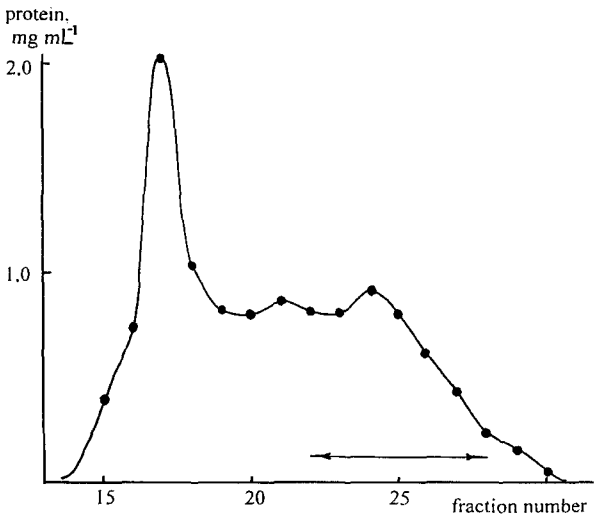

Figure 3
SEC of Sample I on Toyopearl HW55F column; TMM-200 buffer. Active fractions indicated by arrows.

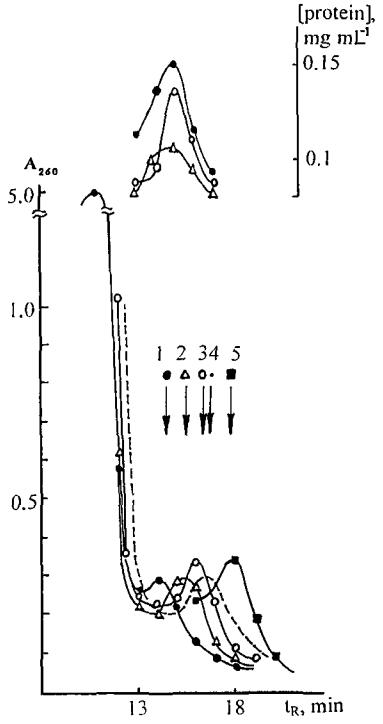

Figure 2
SEC of Sample I on TSK 3000SW column; TMM-200 eluent containing 50 (**1**), 100 (**2**), 150 (**3**), 200 (**4**) and 900 (**5**) mM KCl. Arrows indicate positions of maximum enzymatic activity in each case. Curves of protein content in chromatographic fractions with different ionic strength eluents depicted above.

Size Exclusion Chromatography

Crude enzyme preparation (Sample I) was used for SEC. A typical chromatogram of this separation is shown in Figure 1. In buffer containing 200 mM KCl most of the active enzyme was eluted along with 30 kDa apparent mass proteins. The yield of the active enzyme was 80 % with 5.7-fold decrease in protein and 50-fold decrease in nucleic acid content in the preparation. The enzyme recovery time was found to depend on KCl molarity in the eluent (Figure 2). Raising KCl concentration to 900 mM caused an increase in enzyme retention time. In all experiments the enzyme activity maximum coincided with a small absorption peak at 260 nm, which corresponded to a nucleic acid (determined according to [11]). The position of the protein content was independent of change in KCl concentration. Notice that in the presence of 50 mM KCl active enzyme was eluted along with high molecular mass components [7].

Preparative SEC was performed on ToyoPearl HW-55F column with TMM-200 eluent (Figure 3). The enzyme yield was about 80 % with 2.5-fold purification of protein content and 11-fold purification of nucleic acids. This preparation was precipitated with 65 % ammonium sulfate and then used in following experiments (Sample II).

In this work using SEC we found that increase in KCl concentration in the eluent to 200 mM resulted in

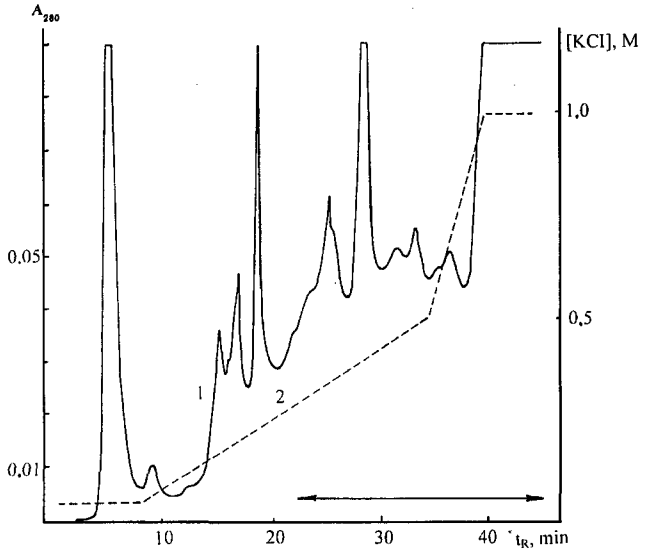

Figure 4
Anion-exchange separation of Sample II on SynChropack AX 300 column. **1** = UV detection 280 nm; **2** = KCl concentration in eluent. Arrows indicates fractions with enzymatic activity.

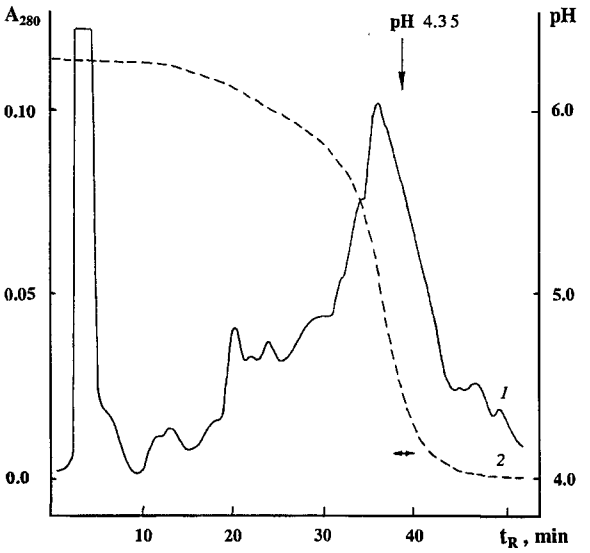

Figure 5
Chromatofocusing separation of Sample II on SynChropack AX 300 column. **1** = UV detection 260 nm; **2** = pH gradient profile. Arrows at bottom indicate active enzyme fractions.

migration of the enzyme towards the zone of free cell protein retention, between BSA and myoglobin. Thus we found conditions for the isolation of the unbound form of the enzyme. Nevertheless, some material of nucleic origin was discovered in the active enzyme fractions even in the presence of 900 mM KCl, so we propose that in a cell extract Y-pUpN PDE forms a stable (specific?) complex with a low molecular mass nucleic acid. It looks realistic because after SEC on Toypearl HW55F at low pressure with TMM-200 most of the enzyme is also separated from high molecular mass nucleic acids.

Cation-Exchange Purification

Recently in our laboratory the isoelectric point of the enzyme (pI 4.6) was determined. According to this data we propose that the enzyme should breakthrough the cation-exchange resin at pH > 6 along with acidic proteins, while most neutral and basic proteins will be adsorbed. Thus, the cation-exchangers, SP-Sephadex and CM-cellulose were used for these experiments. The active enzyme was recovered almost totally at all KCl concentrations used, even in 50 mM KCl. The most pure enzyme preparation was obtained in TMM-200 buffer. We obtained about 50–60 % loss in total protein content when enzyme preparations were incubated with gels (SP-Sephadex or CM-cellulose) in TMM-200 buffer. The successive usage of CM-cellulose and SP-Sephadex resulted in 4-fold purification with minimum lost of enzyme. In spite of only moderate purification this method was approved as the second step in the enzyme isolation because of good yield and simplicity.

Anion-Exchange and Chromatofocusing

Previously anion-exchange chromatography on DEAE-Sephadex A-25 was used as the first step in enzyme purification [5] or as the second step after SEC on Sephadex G-100 [7]. In both cases the active enzyme was eluted in stepwise fashion over a wide range of KCl concentrations. Thus, it results in a poor yield of the active enzyme. We suppose it can be explained by interaction of the Y-pUpN PDE with a nucleic acid(s) which, in turn, has adsorbed firmly on the anion-exchanger.

Two different modes of ion-exchange chromatography (IEC) were probed at the next step of enzyme purification in this work. The chromatogram of Sample II obtained on a SynChropack AX-300 column is shown in Figure 4. The enzyme activity was recovered over a wide range of KCl concentrations (0.3–1 M), despite the sample containing a minute amount of nucleic acids according to UV absorption at 260 nm. Part of the active enzyme was strongly bound to the adsorbent and only eluted using a large amount of TMM-1000. This fact can be explained by the assumption that even a low nucleic acid content of the enzyme preparation is critical for anion-exchange chromatography. Thus, we considered that standard anion-exchange chromatography is not appropriate for the third step of enzyme purification.

Another mode of anion-exchange separation is chromatofocusing which is superior to the first above. Chromatofocusing was performed at room temperature as described in [12]. 25 mM KCl was added to the eluent to increase the gradient . The active enzyme was eluted at pH 4.3 (Figure 5). The yield of active enzyme was rather low, not more than 10 %. The addition of 2-mercaptoethanol to the eluent did not improve results. We suggest that most of the enzyme during separation at room tem-

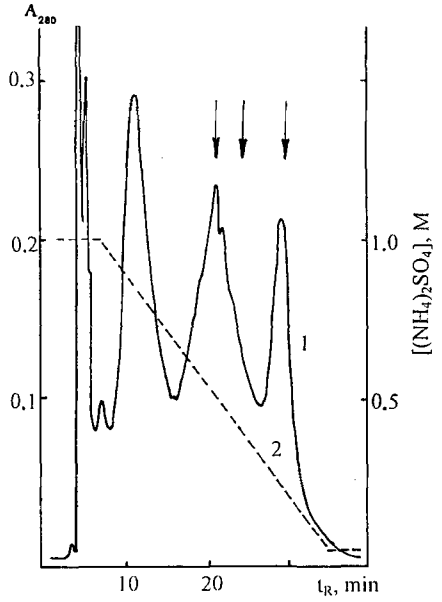

Figure 6
Separation of Sample II by hydrophobic interaction chromatography on SynChropack HIC-Propyl column. **1** = UV detection 280 nm; **2** = ammonium sulfate concentration. Arrows indicate positions of enzymatic activity maxima.

perature and low pH (in combination) is inactivated irreversibly. We hope to obtain a significant increase in the active enzyme yield performing this procedure at 4 °C.

Hydrophobic Interaction Chromatography (HIC)

Sample II was applied in TMM-50 buffer with 15 % ammonium sulfate, despite the upper gradient limit being 1 M (25 %) ammonium sulfate. While the sample was injected in TMM-50 without ammonium sulfate the results were similar. The active enzyme was eluted in three peaks (Figure 6) over a gradient range 0.65–0.2 M (16–5 %) ammonium sulfate. The yield of total enzyme activity was not less 50 %, purification was about 4-fold.

The peculiarity of the enzyme under study is that its precipitation proceeds over a wide range of ammonium sulfate concentrations and depends on protein concentration. We have obtained three maxima in enzyme activity by hydrophobic interaction chromatography. We attribute this result to the dissociation of some enzyme complexes during chromatographic separation. As more than 50 % of the active enzyme was recovered, this method is suitable for enzyme isolation. HIC analysis of the enzyme preparation obtained by chromatofocusing is in progress.

Conclusions

Summing up results of this study we conclude the following:

According to traditional and SEC and normal LC or anion-exchange data, Y-pUpN PDE forms two types of complex with nucleic acids: weak ones which dissociate in 200 mM KCl, and others which are stable up to 900 mM KCl. 2.5–3-fold (preparative LC) or 6-fold (normal LC) purification of the enzyme was obtained by size-exclusion chromatography.

Cation-exchange purification was found to be more suitable at the second enzyme purification step. It results in 4-fold purification and in a minor loss of the enzyme.

Complex formation of the enzyme with cell nucleic acids results in poor separation and low yield after anion-exchange chromatography of the enzyme preparation obtained by SEC.

Three maxima in the active enzyme content were discovered by HIC in the enzyme preparation obtained by SEC. This necessitates additional purification of the enzyme preparation for HIC. We propose to use HIC for enzyme isolation as the last step of purification procedure.

Acknowledgements

This work was supported in parts by the Russian Foundation for Fundamental Research (Grant 94-04-12938) and International Scientific Foundation of G. Soros (Grant M5Z000).

References

[1] *V. Ambros, R. F. Petterson, D. Baltimore*, Cell **15**, 1439 (1978).
[2] *V. Ambros, D. Baltimore*, J. Biol. Chem. **255**, 6739 (1980).
[3] *D. V. Sangar, J. Bryant, T. J. R. Harris, F. Brown, D. J. Rowlands*, J. Virol. **39**, 67 (1981).
[4] *A. J. Dorner, P. G. Rothberg, E. Wimmer*, FEBS Letters **132**, 219 (1981).
[5] *Yu. F. Drygin, E. Y. Siyanova*, Biokhimia **51**, 249 (1986).
[6] *Y. Drygin, A. Shabanov, A. Bogdanov*, FEBS Letters **239**, 343 (1988).
[7] *Yu. F. Drygin, A. A. Shabanov, A. A. Bogdanov*, Biokhimia **53**, 1382 (1988).
[8] *E. Helmerhorst, G. B. Stokes*, Anal. Biochem. **104**, 130 (1980).
[9] *J. R. Whitaker, P. E. Granom*, Anal. Biochem. **109**, 156 (1980).
[10] *R. M. C. Dawson, D. C. Elliott, W. H. Elliott, K. M. Jones*, Data for biochemical research, 3rd ed., Clareton Press, Oxford, 1986.
[11] *A. S. Spirin*, Biokhimia **23**, 656 (1958).
[12] In: "Short protocols in molecular biology", F. M. Ausubel, ed. 2nd ed., John Wiley & Sons, New York, 1992, p.10–52.

Received: Sep 16, 1996
Revised manuscript received: Nov 26, 1996
Accepted: Dec 11, 1996