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High-performance liquid chromatographic analysis of low-molecular-mass products synthesized by polynucleotide phosphorylase in polymerization reaction☆

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Abstract

HPLC was used for the simultaneous analysis of low- and high-molecular-mass products of nucleotide polymerization. Oligonucleotides with chain lengths of less than 5 monomer units were found in the reaction mixture at the very beginning of the reaction. These oligomers were not the products of non-specific polymer degradation. An increase in NaCl concentration from 0 to 1 *M* resulted in the appearance of a short lag period and the oligonucleotide accumulation. A simultaneous increase in Mg^{2+} concentration eliminated the lag. It is concluded that high ionic strength affects both the formation of the metal–monomer–polymer complex and the affinity of the polynucleotide phosphorylase molecule for the reaction products.

1. Introduction

Polynucleotide phosphorylase (PNPase) is known to catalyze both the polymerization of ribonucleoside 5'-diphosphates (followed by inorganic phosphate release) and the reverse reaction of phosphorolysis [1]. These reactions proceed in the presence of divalent metal cations, such as Mg^{2+} or Mn^{2+}. PNPase was used for investigations of RNA structure and processing [2,3] and proved to be a suitable tool for the synthesis of a variety of oligo- and polynucleotides that are widely used in many areas of molecular biology [1].

The mechanism of action of the enzyme is "non-synchronous" [1]. In the case of polymerization the polyribonucleotide chain is elongated by successive addition of monomers. If the synthesis is primed, the primer is incorporated into the chain of the product [4]. But data from the literature indicate that the molecular mass distribution (MMD) of the products formed in the absence of primer is a matter of controversy. The conclusion that the MMD is broad was based on the results obtained by electrophoresis in polyacrylamide gel [5]. Although the authors refrained from analyzing products of less than 17 units in length, their data do not allow to exclude the existence of such molecules. On the other hand, it was argued that no oligomers can be detected in the reaction mixture upon de novo

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polymerization; rather, the high-molecular-mass polymer is the only product formed [6]. However, analysis of products formed by oligonucleotide phosphorolysis (the reverse process) indicates that all intermediate oligomer types are present in the reaction mixture [7,8], i.e. the mechanism of oligonucleotide phosphorolysis is rather "synchronous". Besides, the affinity of the enzyme molecule for polynucleotide chains is 10 000-fold higher in the case of a polymer compared to an oligomer [9]. Given that both polymerization and phosphorolysis involve the same steps, the array of short intermediates, whose formation is associated with the phosphorolysis, may be formed during the polymerization as well. Therefore, the presence of intermediates in the reaction mixture cannot be completely excluded.

The methods used until now for the analysis of the whole spectrum of nucleotide polymerization products are time-consuming. Gel permeation [6] and paper chromatography [4] give some idea of the overall distribution of the components of the reaction mixture. Gel electrophoresis [5] demonstrates molecular mass polymorphism of the products of polymerization, but fails to provide accurate data regarding the smallest oligomers. Unfortunately, oligonucleotides (with a chain length of less than 10 monomer units) could not be analyzed by electrophoresis together with polymers, although it is the short oligomer that was of interest to us. In this study we report for the first time on the utilization of an HPLC technique for the analysis of the products of a ribonucleoside 5′-diphosphate polymerization. The method involves simultaneous determination of high- and extremely low-molecular-mass components of the reaction mixture.

2. Experimental

2.1. Polymerization assay and sample preparation

Adenosine 5′-diphosphate (ADP) (87% purity) was purchased from Vector (Berdsk, Russian Federation). The preparation contained adenosine 5′-monophosphate (AMP) and adenosine 5′-triphosphate (ATP) as contaminants. The increase in the content of ATP was determined independently by the bioluminescent method [10].

The preparation of PNPase from the thermophilic microorganism *Thermus thermophilus* ("Biolar", Latvia) had a specific activity of $2.0 \cdot 10^{-2}$ U/ml (0.3 mg protein per ml, 3% of nucleic acids) in the reaction of ADP polymerization (one unit of activity is defined as the amount of the enzyme sufficient to release 1 μmol inorganic phosphate, P_i, per minute at 70°C and pH 8.1).

The polymerization reaction was carried out in 50 m*M* Tris–HCl buffer, pH 8.55 (room temperature), in the presence of 1 m*M* EDTA, 10 m*M* ADP, the enzyme, and the desired concentrations of $MgCl_2 \cdot 6H_2O$ and NaCl (for details, see the captions to relevant figures). The mixture was incubated at 70°C and aliquots were taken to measure the amount of P_i released [11]. In addition to phosphate determination the reaction was monitored by HPLC as well. Samples were diluted 8-fold with the starting buffer (see below) supplemented with 30 m*M* EDTA.

For the raw estimation of the oligomer mass the reaction mixture was desalted by passing it through small Bio-Spin-like columns prepacked with Bio-Gel P-2, P-4, P-6 (Bio-Rad, USA); the supports had exclusion limits of M_r 1800, 4000 and 6000, respectively. High-molecular-mass components, including the enzyme, were eluted without any impediment, while small molecules were retained in the gel pores in accordance with exclusion limits of a particular support. ADP was used as an internal retention standard for low-molecular-mass components.

Phosphorolysis was carried out as follows. The process of polymerization was stopped by cooling the samples on ice. The reaction mixture was desalted as described above. The same volume of the two-fold more concentrated reaction buffer containing P_i instead of ADP was added. The final concentrations of $MgCl_2 \cdot 6H_2O$ and KH_2PO_4 were equal to 25 and 50 m*M*, respectively.

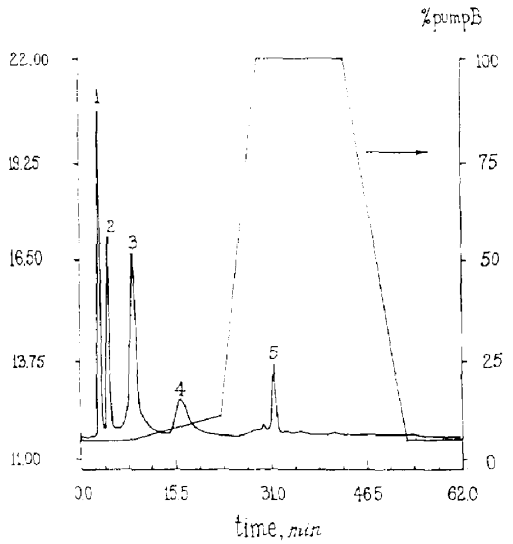

Fig. 1. Ion-exchange HPLC of ADP polymerization mixture and model compounds with our gradient scheme. Values on the left-hand y-axis are percentages of detector 1 V output. Peaks: 1 = adenosine (retention time 3.0 min); 2 = AMP (4.1 min); 3 = ADP (7.2 min); 4 = ATP (16.2 min); 5 = poly(A) (31.0 min).

2.2. High-performance liquid chromatography

The reaction mixture was analyzed at 55°C on a Bio-Rad 800 HRLC gradient system using a Bio-Gel DEAE-5-PW ion-exchange column (75 × 7.5 mm I.D.). The following solutions were used for separating the reaction mixture components: 75 m*M* Tris–HCl (pH 7.6) served as the starting buffer (A) and buffer A containing 1 *M* NH_4Cl, as the eluting buffer (B). The effluent was monitored at 260 nm; the scheme of the gradient is depicted in Fig. 1.

3. Results and discussion

The selected operating conditions made it possible to identify both small molecules (such as adenosine, AMP, ADP, ATP), and derivatives of higher molecular mass (Fig. 1). The distribution of the polynucleotide product with respect to chain lengths was previously shown by gel electrophoresis to range from thousands of monomer units to tens of residues [5]. Nevertheless, the polynucleotide was eluted as a single peak.

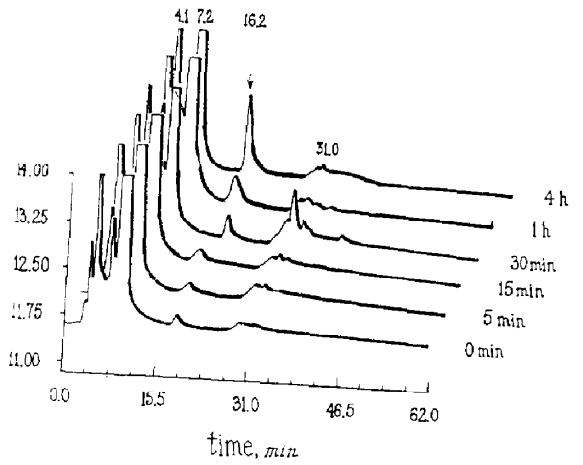

Fig. 2. HPLC separation of the components of the reaction mixture obtained by polymerization of 10 m*M* ADP for varying times. Conditions are specified in the Experimental section; the reaction mixture contained 0.01 U/ml enzyme and 5 m*M* $MgCl_2 \cdot 6H_2O$.

However, a simple modification of the gradient scheme allowed to broaden the poly(A) distribution observed.

A substance with a retention time close to that of ATP was accumulated (Figs. 2–4, the peak is indicated with an arrow). During the first 15 min its concentration was increased 1.5–2-fold. However, according to the results of a bioluminescence analysis, there was no increase in ATP

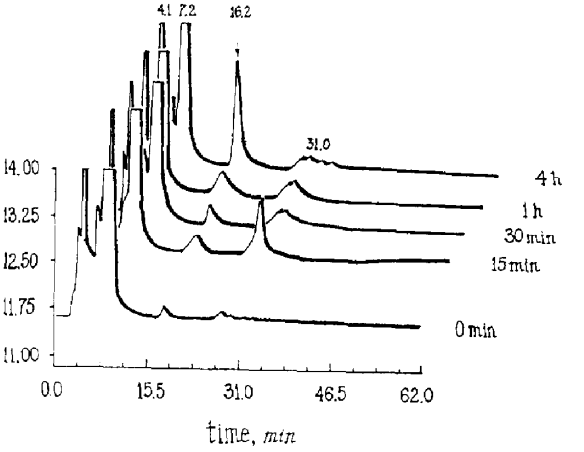

Fig. 3. HPLC separation of the components of the reaction mixture obtained by polymerization of 10 m*M* ADP for varying times. Reaction conditions as in Fig. 2, the medium was supplemented with 1 *M* NaCl. Retention times (min) are presented.

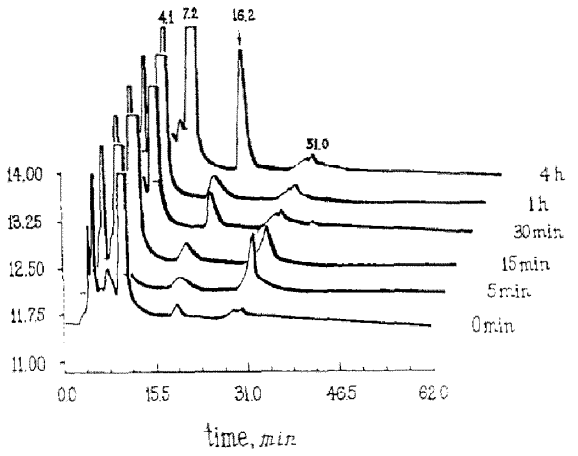

Fig. 4. HPLC separation of the components of the reaction mixture obtained by polymerization of 10 m*M* ADP for varying times. Reaction conditions are specified in the Experimental section, and the reaction mixture contained 0.01 U/ml enzyme, 20 m*M* $MgCl_2 \cdot 6H_2O$ and 1 *M* NaCl.

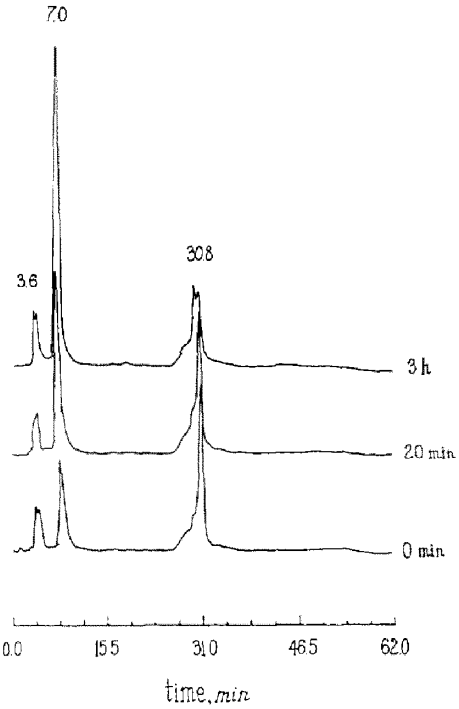

Fig. 5. Reaction of poly(A) phosphorolysis. HPLC separations of the reaction mixture components at indicated times. Conditions as in the Experimental section, except that the mixture contained 0.055 U/ml enzyme.

concentration ($4 \cdot 10^{-4}$ *M*), at least within 1 h of incubation. On the other hand, this substance, as well as ADP, was retained in the gel pores when Bio-Gel P-2 support (exclusion limit of M_r 1800) was used for the separation of the reaction mixture; this was not the case with components of higher molecular mass. Given the correlation between the charge of phosphates and the retention times (Fig. 1), it was reasonable to assume that this substance was an oligonucleotide with a net charge of phosphate groups similar to that of ATP (-4 under these conditions). It should be noted that a 5′-pyrophosphate-terminated product was not detected in the polymerization mixture [5]. Judging from the results of desalting experiments, the molecular mass of this substance did not exceed 1800 and corresponded to a chain of no more than 5 residues in length (provided that the molecular mass of the monomer is equal to that of AMP). Furthermore, the dinucleotide was shown to be resistant to PNPase-catalyzed phosphorolysis [9]. Since it was not the peak under consideration that was finally increased in the course of the reversed phosphorolysis reaction (Fig. 5), the oligonucleotide was not a dimer. Therefore, the component appeared to be a short oligomer (3 to 5 monomer units), most likely, a trimer.

This oligomer was unlikely to arise from either phosphorolysis or non-specific degradation [12] of the produced polynucleotide and the endogenous primer. First, the primer content of the enzyme preparation was low (3%, or 5 μg/ml). Judging from the chromatographic data, the yield was at least 10 times higher than it would have been observed, had the product been formed by the primer decomposition in 5 min. Secondly, the amount of inorganic phosphate accumulated 5 min after the onset of the reaction would be insufficient to start the reverse process of phosphorolysis resulting in the formation of a detectable amount of the product (Figs. 2–4). Since endogenous primers were reported [13] to exceed in length the studied oligonucleotide, it can be concluded from this work that an extremely short oligonucleotide synthesized de

novo was found in the reaction mixture soon after the start of the polymerization.

At high NaCl concentration the appearance of detectable phosphate in the medium was preceded by a short lag period (Fig. 6, curve 2), as reported previously [1,14]. The same effect was observed in the presence of low Mg^{2+} concentrations [15]. The simultaneous accumulation of the oligomer was more pronounced at 1 *M* NaCl (Fig. 3) than in media with low ionic strength (Fig. 2), in spite of the decrease in the overall rate of the reaction (Fig. 6, curves 2 and 1, respectively). This could be expected, given the electrostatic nature of enzyme–nucleic acid interactions. In other words, an increase in NaCl concentration resulted in the dissociation of the complex formed by the enzyme molecule and short oligonucleotide chains. The accumulation of longer oligomers and short polymers takes place at low ionic strengths as well [5]. This was demonstrated by gel electrophoresis with other preparations of the enzyme [5], suggesting that high salt concentrations only promoted the process.

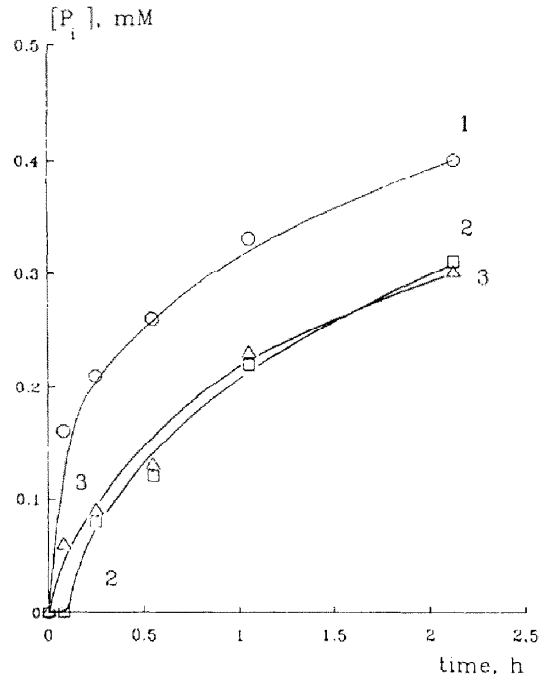

Fig. 6. Release of inorganic phosphate in the course of ADP polymerization. Reaction conditions are specified in the Experimental section. The reaction mixture contained 0.01 U/ml enzyme and 10 m*M* ADP. Additions: (1) 5 m*M* $MgCl_2 \cdot 6H_2O$; (2) 5 m*M* $MgCl_2 \cdot 6H_2O$ and 1 *M* NaCl; (3) 20 m*M* $MgCl_2 \cdot 6H_2O$ and 1 *M* NaCl.

If the level of Mg^{2+} in the medium containing 1 *M* NaCl was increased, the lag was eliminated (Fig. 6, curve 3) and the elongation of the polynucleotide chain became more efficient (Fig. 4, the peak with a retention time of 31.0 min). Given the importance of magnesium ions for the initial step of the reaction [15,16], it can be suggested that the divalent cation takes part in the formation of the active complexes at either the initiation step (monomer–monomer interactions) or during the process of elongation (the interaction of the monomer and the growing chain), as is the case of other polymerases [17]. The appearance of the lag is likely due to the need for the accumulation of a certain amount of oligomers, which would be sufficient for further elongation of the chain [1]. Of course, the phosphorolysis also requires Mg^{2+} ions, so the latter may facilitate the adjustment of the long charged chain on the enzyme molecule by neutralizing the charge. Thus, high salt concentration affects the metal–monomer–chain complex and the affinity of the enzyme molecule for the reaction products.

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