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Endopolyphosphatase activity of RNase A from bovine pancreas

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Endopolyphosphatase activity of RNase A from bovine pancreas

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Abbreviations: polyP – inorganic polyphosphates.
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Inorganic polyphosphate preparations from various biological samples often contain significant RNA contamination, complicating their analysis. The standard method for purifying such preparations from RNA is the treatment with RNases, particularly RNase A. This study demonstrated that commercial preparation of RNAse A, at concentrations used to purify polyphosphate preparations from RNA, exhibits endopolyphosphatase activity against polyphosphates with an average chain length of 65 to 208 phosphate residues, but does not exhibit exopolyphosphatase activity. This activity is inhibited by ribonuclease inhibitor, insensitive to the polyphosphatase inhibitor heparin and does not require divalent metal cations.

Keywords: inorganic polyphosphates, ribonuclease A, endopolyphosphatase

INTRODUCTION

Inorganic polyphosphates (polyP) are linear polymers containing from a few to several hundred orthophosphate residues. They perform numerous functions in the cell, including phosphate and energy storage, cation binding, membrane channel formation, participation in the formation and function of the cell wall, regulation of gene expression and enzyme activity, and adaptation to stress conditions [1, 2, 3, 4, 5]. Due to the increased interest in polyphosphates in mammals, the development of methods for analyzing these compounds is in demand, taking into account the specifics of the object: the relatively low polyP content in mammalian cell preparations and a significant RNA contamination [6]. Therefore, the standard method for purifying such preparations from RNA is treatment with RNases, in particular RNase A [7]. Various enzyme concentrations are used, from 0.05 mg/ml [7] to 0.5 mg/ml [8].

Bovine pancreatic ribonuclease A is one of the most studied [9, 10] and widely used enzymes. It is a small protein of 124 amino acid residues with a molecular weight of 13,686 Da [11] and known for very high stability. RNase A exhibits endoribonuclease activity towards single-stranded RNA, cutting the 3'-end of pyrimidine nucleotides with the formation of 2',3'-cyclic monophosphate [11]. Its optimum pH is 7.6, and its activity is manifested in the pH range of 6-10, the optimal temperature is 60 °C (activity is manifested in the range of 15-70 °C) [9]. Its main physiological function is the destruction of foreign RNA [12]. It has been shown that being lyophilized from an acetic acid solution, RNase A forms dimers and oligomers exhibiting different properties [13].

At the same time, there is information about the possibility of complete hydrolysis of polyP with a chain length of 383 phosphate residues by RNase A at a high concentration of the enzyme [8], which limits the possibility of using this enzyme in the study of polyP.

The question of possible interaction between polyP and RNases has two aspects. On the one hand, it is important to know the conditions under which RNases hydrolyze polyP in order to take this into account when using these enzymes to purify polyP preparations from different biological objects. For example, when polyP from wild-type S. cerevisiae cells were treated with RNase A (0.2 mg/ml), no changes in the content or chain length were recorded, but when polyP from cells of the vtc4 polyphosphate synthetase knockout strain (where the polyP content is very low) were treated, a decrease in the polyP chain length from an average of 75 phosphate residues to 15 phosphate residues was observed [14]. On the other hand, the question of the possible biological significance of polyP-RNA complexes, which are formed during the extraction of RNA from different biological objects, remains unexplored [15]. It should be noted that all data on the possible existence of such complexes in the cells of microorganisms, where polyP content is high, and their possible role date back to the 1970s [16, 17]. Currently, data have been obtained on the important role of polyP in interaction with the RNA-binding protein Hfq and the DNA-binding AlgP [18]. All this allows us to assume that the interaction of polyP, RNA, and RNases may not only be of methodological interest, but also have physiological significance.

The aim of this work was to determine the ability of RNase A to degrade polyphosphates of varying degrees of polymerity and the conditions for the manifestation of such activity.

MATERIALS AND METHODS

Enzyme preparation. The commercial RNase A preparation R5000 (Sigma, USA)

was used in this study. The purity was checked by the company chromatographically and using SDS-PAGE (Ribonuclease A from bovine pancreas Type II-A, Sigma, USA). This RNase A is widely used to remove RNA from various samples, including the cases of analysis of polyP [19].

Yeast strain. The cells of Saccharomyces cerevisiae strain BY4741 from the commercial collection of Dharmacon (USA) was maintained on YPD agar medium. For polyP extraction, cells were grown to stationary growth stage in YPD medium (1% yeast extract (Difco, USA), 2% peptone (Dia-M, Russia), 2% glucose) on a shaker (145 rpm) at 29 °C, pelleted by centrifugation at 5000 g for 5 minutes, and washed twice with water.

Extraction of polyP. PolyP was extracted from cells using the Langen and Liss method modified by Kulaev as described previously [19]. The following polyP fractions were obtained: acid-soluble (extracted with 0.5 M HClO4 at 0 °C); salt-soluble (extracted with a saturated NaClO4 solution at 0 °C) and alkali-soluble (extracted with a NaOH solution, pH 9-10, at 0 °C). For further work, we used the alkali-soluble fraction, purified with Norit M activated carbon (Sigma-Aldrich, USA) to remove possible RNA contamination as described previously [14].

Exopolyphosphatase activity was determined by the rate of phosphate (Pi) release. Preparations of polyP with a medium chain length of 208 (polyP208) (Monsanto, USA) were purified from pyrophosphate and orthophosphate impurities by gel filtration as described previously [21]. The reaction was carried out at 30 °C for 120 min in 1 ml of a reaction mixture containing 1.0 μM polyP208, 90 μg/ml RNase A in 50 mM Tris-HCl with pH 7.6. The released phosphate was measured colorimetrically with ammonium molybdate and ascorbic acid [22]**.**

Endopolyphosphatase activity was determined by the decrease in the chain length of polyphosphates. The reaction mixture contained 50 mM Tris-HCl pH 7.6, 1.0 μM polyP (concentration of polymer molecules) with an average chain length of: 15 (polyP15) (Sigma, USA), 65 (polyP65) (Reakhim, Russia), 208 (Monsanto, USA) or polyP of the alkali-soluble fraction extracted from yeast cells, RNase A at a concentration of 90 μg/ml. Incubated for 30, 60 or 120 minutes at 30°C. Then cooled, mixed with glycerol and subjected to electrophoresis according to the method described previously [22] with the following modification: 15% polyacrylamide gel without the presence of urea was used. Gels were stained with toluidine blue [22] and staining intensity was analyzed using ImageJ software.

RESULTS AND DISCUSSION

The presence of endopolyphosphatase activity is most often determined by a decrease in the polyP chain length, detected using PAGE [24,25] due to the fact that methods for quantitative analysis of this activity require labor-intensive synthesis of fluorescent polyP derivatives [26]. To detect endopolyphosphatase activity, a commercial polyP208 preparation was incubated with RNase A for varying periods of time and subjected to electrophoresis. The electropherogram is shown in Fig. 1. As can be seen from the figure, a distinct decrease in the average chain length is observed proportionally to the incubation time. As in the experiments of Labberton et al [8], a diffuse band with a lower mobility than the control appears on the electropherogram. A sample containing the enzyme but without the substrate does not stain (not shown). If, after incubation, perchloric acid is added to the reaction mixture to coagulate the protein, the mixture is centrifuged, and the pH of the supernatant is returned to 7.6 by adding NaOH, this band does not appear on the electropherogram. It can be assumed then the aggregates of the protein and the substrate are formed, which stain like the substrate but have a lower mobility.

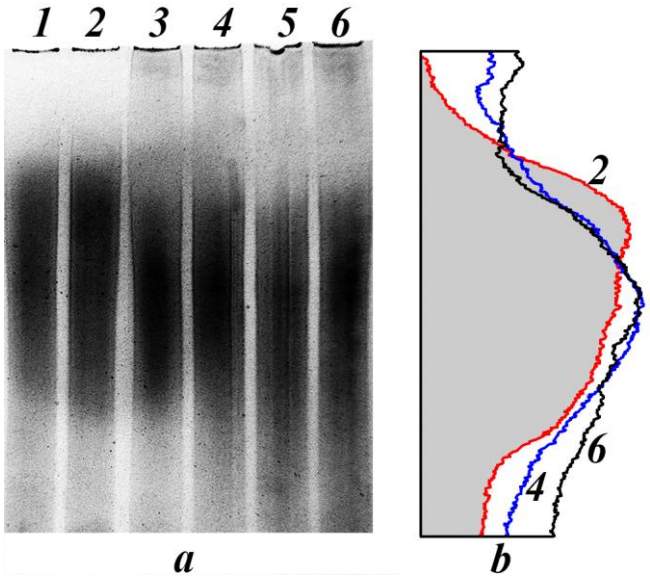


Fig.1. Reduction of the chain length of a polyphosphate preparation with an average chain length of 208 phosphate residues depending on the incubation time with RNase.
a – electropherograms, b - staining intensity graphs, 1 – polyP without the addition of RNase (control) 0 min, 2 – polyP without the addition of RNase (control) 120 min, 3 – polyP with the addition of RNase 0 min, 4 – polyP with the addition of RNase 30 min, 5 – polyP with the addition of RNase 60 min, 6 – polyP with the addition of RNase 120 min

When polyP208 was incubated with RNase under these conditions, no increase in orthophosphate was observed, indicating the absence of exopolyphosphatase activity.

Consequently, RNase A preparation exhibits only endopolyphosphatase activity, and the enzyme preparation does not contain contaminants of enzymes with exopolyphosphatase activity (exopolyphosphatases, pyrophosphatases, and nonspecific phosphatases).

It cannot be ruled out that this activity could be exhibited by some minor impurity protein that was not detected by the purity analysis methods used by the supplier. To ensure that the observed activity was attributed specifically to RNase and not to possible contaminants of other enzymes, an experiment was conducted using the commercial ribonuclease inhibitor RiboCare (Evrogen)(https://evrogen.ru/products/isolation/ribocare-rnase-inhibitor). The reaction was carried out in 10 µl reaction mixture containing 50 mM Tris-HCl pH 7.6, 1.0 µM polyP208, 0.45 µg RNase and 120 units of ribonuclease inhibitor for 180 minutes.

The results are presented in Fig. 2. As can be seen from the figure, the ribonuclease inhibitor suppressed endopolyphosphatase activity.

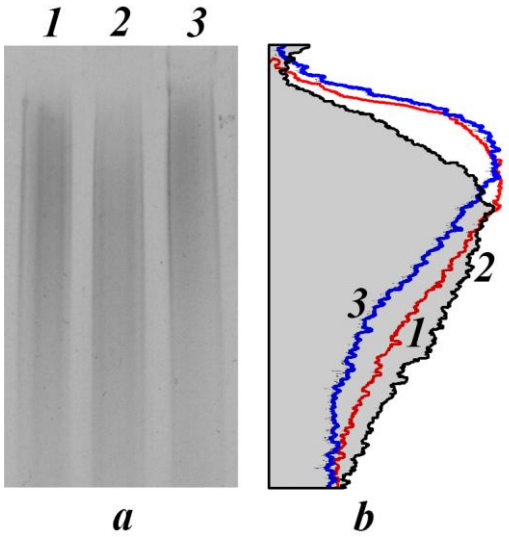


Fig. 2. Reduction of the chain length of polyphosphate preparation with an average chain length of 208 phosphate residues during incubation with RNase A in the absence and presence of ribonuclease inhibitor RiboCare. a – electropherograms, b - staining intensity graphs, 1 – polyP without the addition of RNase (control), 2 – polyP with the addition of RNase, 3– polyP with the addition of RNase and inhibitor

Known endopolyphosphatases are inhibited by heparin [28]. In this regard, we checked the effect of heparin on the observed activity. For this we incubated polyP208 with the enzyme in the absence and presence of heparin at a concentration of 0.25 mg/ml (Fig. 3). As can be seen from the figure, heparin did not inhibit the activity.

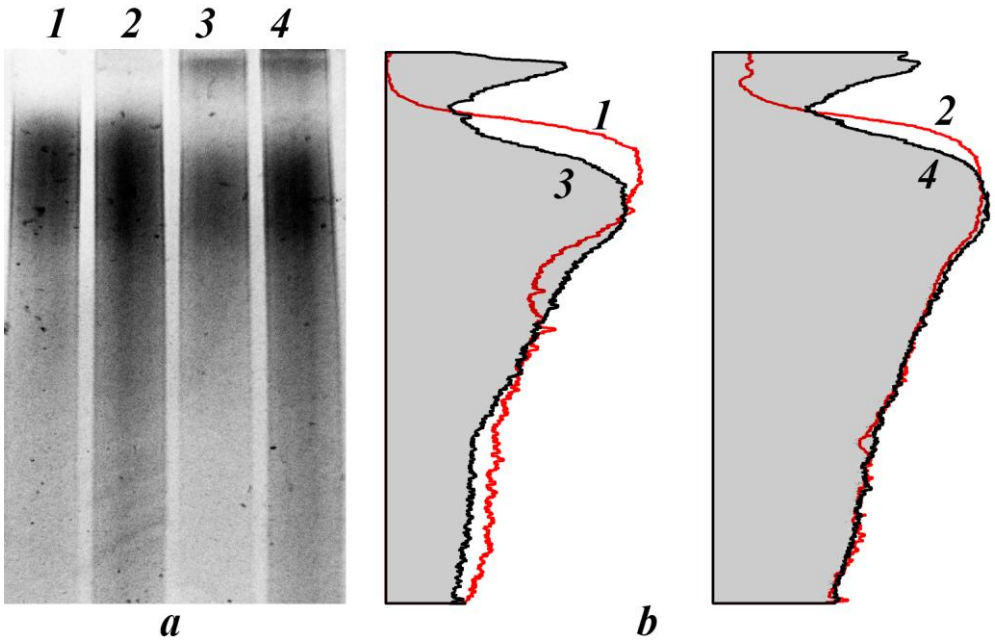


Fig. 3. The effect of heparin at a concentration of 0.25 mg/ml on the activity toward polyphosphates with an average chain length of 208 phosphate. a – electropherograms, b – staining intensity graphs, 1 – polyP208 without the addition of RNase, 2 – polyP208 without the addition of RNase, but with haperin, 3 – polyP208 with the addition of RNase, 4 – polyP208 with the addition of RNase and heparin

Experiments were also conducted with polyP of other chain lengths as substrates. No activity was detected with polyP15 (not shown), while in case of polyP65 a decrease in chain length was observed, similar to polyP208 (Fig. 4).

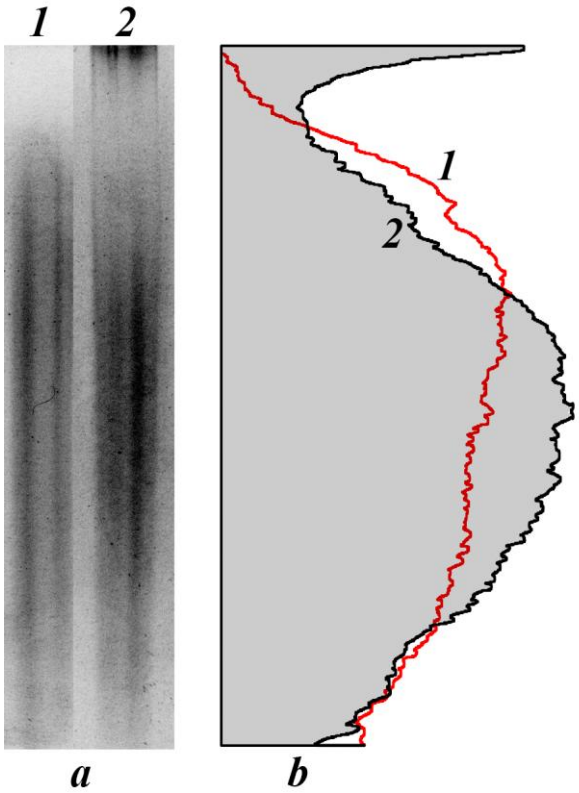


Fig 4. Reduction of the chain length of a polyphosphate preparation with an average chain length of 65 phosphate residues during incubation with RNase A.
a – electropherograms, b - staining intensity graphs, 1 – polyP without the addition of RNase (control), 2 –polyP with the addition of RNase

No effect of cations such as Mg^{2+} and Zn^{2+} at concentrations of 2 mM on the endopolyphosphatase activity was found (not shown). These cations, especially Zn, are necessary for the activity of currently known endopolyphosphatases [27].

RNase A is used, among other things, to purify polyP preparations obtained from biomass. To test its activity against such polyPs, we extracted polyphosphates from yeast cells. Since activity with short-chain polyP15 was not observed, for this experiment we used an alkali-soluble polyP fraction with an average chain length of ~100 phosphate residues. which is relatively abundant within biomass [1] The extract was adjusted to neutral pH and purified from possible RNA contamination with Norit M activated carbon. The electropherogram and staining intensity graph are shown in Fig. 5. A result similar to that observed with polyP65 was observed but with less pronounced reduction of the chain length.

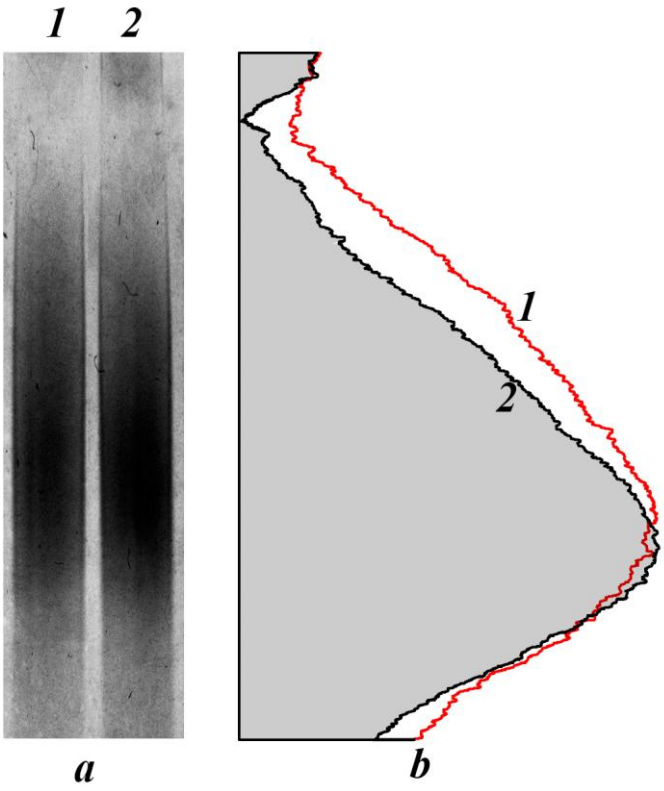


Fig. 5. Reduction of the chain length of polyphosphates of the alkali-soluble fraction obtained from yeast cells (average chain length of about 100 phosphate residues) upon incubation with RNase A. a – electropherograms, b – staining intensity graphs. 1 – polyP without the addition of RNase, 2 – polyP with the addition of RNase

Thus, commercial RNase A preparation, at the concentrations used to purify polyP preparations from RNA, is capable of cleaving long-chain polyP into shorter ones without releasing orthophosphate. The work of Labberton et al. [8], which served as a prerequisite for our interest in the endopolyphosphatase activity of this RNase, provides an electropherogram of polyP with a chain length of 383 phosphate residues after treatment with this RNase. The authors used the enzyme in significantly larger quantities than used in our work, and all polyphosphates in their experiment degraded to sizes that were not stained with DAPI [8]. It should be noted that DAPI stains polyP only when the chain length exceeds 15 phosphate residues [29]. No other information about the detected activity is provided. In our work, we used RNase in the amount used to purify polyP from RNA contaminants [7], carried out an inhibitory analysis and assessed the conditions for the manifestation of endopolyphosphatase activity using commercial polyP preparations with different chain lengths as substrates. The possibility that the observed activity may belong to this RNase is supported by its inhibition by a specific inhibitor (Fig.2). This endopolyphosphatase activity was not inhibited by heparin and was not influenced by

divalent cations. In mammals, the known enzymes with endopolyphosphatase activity belong to the family of diphosphoinositol polyphosphate phosphohydrolases (DIPPs)[5]; they require divalent cations [27] and are inhibited by heparin[28]. It can be assumed that DIPPs cannot be responsible for endopolyphosphatase activity in this case. It cannot be ruled out that mammals may have other enzymes with endopolyphosphatase activity that differ in their properties from DIPPs, and it is the admixture of such an unknown enzyme that determines the endopolyphosphatase activity of the RNase preparation. Additional studies, for example, studying the effect of site-directed mutagenesis of key residues of RNase on RNase and endopolyphosphatase activities, analysis of the three-dimensional structure of RNase using polyP docking and molecular dynamics are necessary for a more rigorous statement about the presence of endopolyphosphatase activity in RNase A. However, the presence of endopolyphosphatase activity in a commercial RNase preparation with a standard level of purity in the concentrations used in the purification of polyP preparations from RNA is of interest both for research practice and for the further search for new mammalian enzymes involved in polyP metabolism.

Contributions. T.V. Kulakovskaya – conceived and supervised the study, edited the manuscript; A.A. Tomashevsky – carried out experiments, wrote the manuscript

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Conflict of Interest. The authors declare that they have no conflicts of interest.

Compliance with ethical standards. This article does not contain any studies involving human participants or animals performed by any of the authors.

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