Title	Quantification of polyphosphate: different sensitivities to short-chain polyphosphate using enzymatic and colorimetric methods as revealed by ion chromatography
Author(s)	Ohtomo, Ryo; Sekiguchi, Yoko; Mimura, Tetsuro; Saito, Masanori; Ezawa, Tatsuhiro
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1 Title: Quantification of polyphosphate: different sensitivity between enzymatic and

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4 Authors: Ryo Ohtomo [1*], Yoko Sekiguchi [2, 3], Tetsuro Mimura [3, 4], Masanori Saito [5] and

5   Tatsuhiro Ezawa [6]

6

7 Address: [1] National Institute of Livestock and Grassland Science, Nishi-nasuno, Tochigi

8   329-2793 Japan, [2] Nippon Dionex K.K., 6-3-14 Nishinakajima, Yokogawa-ku,

9   Osaka 532-0011 Japan, [3] Nara Women's University, Kitauoyanishi-machi,

10   Nara-shi, Nara, 630-8506 Japan, [4] Core Research for Evolutional Science and

11   Technology (CREST), Japan Science and Technology Corporation (JST),

12   Chuou-ku, Tokyo, 113-0027 Japan, [5] National Institute for Agro-Environmental

13   Sciences, 3-1-3 Kannondai, Tsukuba, Ibaraki, 305-8604 Japan, [6] University Farm,

14   Nagoya University, Togo-cho, Aichi 470-0151 Japan

15 *Author for correspondence: Ryo Ohtomo

16   National Institute of Livestock and Grassland Science, Nishi-nasuno,

17   Tochigi 329-2793 Japan, Phone: +81-287-37-7227, FAX: +81-287-36-6629,

18   E-mail: rotm@affrc.go.jp

19

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1 **Title:** Quantification of polyphosphate: different sensitivity between enzymatic and

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5 Tatsuhiro Ezawa [6]

6

7 **Affiliation:** [1] National Institute of Livestock and Grassland Science, Nishi-nasuno, Tochigi

8 329-2793 Japan, [2] Nippon Dionex K.K., 6-3-14 Nishinakajima, Yokogawa-ku, Osaka

9 532-0011 Japan, [3] Nara Women's University, Kitauoyanishi-machi, Nara-shi, Nara, 630-8506

10 Japan, [4] Core Research for Evolutional Science and Technology (CREST), Japan Science and

11 Technology Corporation (JST), Chuou-ku, Tokyo, 113-0027 Japan, [5] National Institute for

12 Agro-Environmental Sciences, 3-1-3 Kannondai, Tsukuba, Ibaraki, 305-8604 Japan, [6]

13 University Farm, Nagoya University, Togo-cho, Aichi 470-0151 Japan

14

Abstract

16 Polyphosphate is ubiquitous and has a variety of biochemical functions. Among

17 polyphosphate quantification methods, an enzymatic assay using a reverse reaction of the

18 *Escherichia coli* polyphosphate kinase (PPK), in which polyphosphate is converted to ATP

19 and quantified by luciferase assay, is the most specific and most sensitive. However,

20 low-sensitivity of PPK to short-chain polyphosphate has been suggested [1]. Ion

21 chromatography equipped with an on-line hydroxide eluent generator enabled us to analyze

22 polyphosphate up to 50 Pi-residues, and we employed this method to investigate the

23 chain-length specificity of PPK in this study. Several fractions of short-chain polyphosphate

24 were prepared by electrophoresis, and the chain-length distribution was analyzed before and

after 1-6 h PPK reaction by ion chromatography. Polyphosphates longer than 23 Pi-residues were processed by PPK completely after 1 h incubation, but complete procession of those between 11-22 Pi-residues required 6 h incubation. Limited procession of polyphosphates of 10 Pi-residues or shorter were observed even after 6 h incubation. Metachromasy of Toluidine blue O, an alternative method for polyphosphate quantification, showed broader chain-length specificity although it was not as sensitive as the enzymatic assay. Combination of these two methods would be practically applicable to analysis of polyphosphate dynamics in living organisms.

Introduction

Inorganic polyphosphate (poly P) is a linear polymer of phosphate (Pi) connected with high-energy bonds. Poly P has been found in all kinds of living cells and is considered to have various biological functions: a Pi reservoir, an alternative source of high-energy bonds, and a buffer against alkali or metals [2]. In addition, it has been known that poly P has some regulatory functions in prokaryotic cells, such as in competence for transformation [3], motility [4, 5], biofilm formation [6], gene expression in stressed conditions [7-9], and protein degradation in amino acid starvation [10, 11]. As more evidence that the simple molecule plays significant roles in cell life accumulates, greater is the demand for a specific and sensitive method to quantify poly P.

Metachromatic reaction of Toluidine blue O (TBO) with poly P [12] is a commonly used method for poly P quantification. Although other polyanions such as nucleic acids interfere with the reaction, this method is simple and practical. Recently, a new method for

poly P quantification using polyphosphate kinase (PPK) has been developed. PPK catalyzes transphosphorylation from poly P to ADP as a reverse reaction of poly P synthesis, then the resultant ATP can be quantified by using the luciferase chemi-luminescence assay [13]. We used both the TBO and PPK methods to investigate the rate of poly P synthesis in arbuscular mycorrhizal fungus [1], which is an obligate plant symbiont that accumulates poly P as a translocation form of Pi [14-16]. The results obtained by these two methods were generally consistent. The PPK method, however, gave a significantly lower value of poly P content in hyphae harvested at an early phase of poly P accumulation in which short-chain poly P would be accumulated. It was suggested that disagreement between the results of the two methods was due to the sensitivity to short-chain poly P being lower in case of PPK than in that of TBO.

Accumulation of short-chain poly P in an early phase of poly P synthesis has also been observed in *Saccharomyces cerevisiae* [17]. It has been suggested that short-chain poly P has some important biological functions in cell life based on the observation that endopolyphosphatase activity, which processes long-chain poly P to shorter chain-length poly P, was widely distributed in eucaryotic cells [18]. Indeed, the endopolyphosphatase-defective mutant of *S. cerevisiae*, which could accumulate long-chain poly P but not short-chain poly P, showed slower growth, lower viability in a starved condition [19], and higher sensitivity to calcium ion [20].

Recently, Sekiguchi et al. [21] analyzed short-chain poly P (up to ca. 50 Pi-residues) in food products by using an ion chromatography system connected to an on-line hydroxide eluent generator. Using this system, Pi, pyrophosphate, tripolyphosphate, and tetrapolyphosphate could be quantified in the range of 0.5-500 μM with a correlation factor of more than 0.999. In this study, we applied this chromatographic technique to characterize

PPK reaction with respect to chain-length of poly P; i.e., reactivity of PPK with short-chain poly P (less than 50 Pi-residues) was investigated. We also compared the chain-length specificity of metachromatic reaction between poly P and TBO to establish a systematic method to quantify short- and long- chain poly P.

Materials and Methods

Chemicals and Enzyme

Toluidine blue O (TBO), sodium phosphate (Pi), sodium pyrophosphate (P2) and sodium tripolyphosphate (P3), polyphosphate type 15 and type 75+ were purchased from Sigma Chemicals. The latter two poly P reagents, polyphosphate type 15 and 75+, are the mixtures of a range of poly Ps of which the average chain-lengths are 15 and 79, respectively. ADP (highest purity) was purchased from Roche Diagnostics. *Escherichia coli* polyphosphate kinase (PPK) was purified from PPK overexpressing *E. coli* as previously reported [22]. Specific activity of PPK after purification was 3×10^6 U mg^{-1} protein in which 1 U of activity was designated to phosphorylate 1 pmol ADP min^{-1} at 37˚C using long-chain poly P (ca 750 residues) as a phosphoryl donor. The purified PPK was diluted to 3×10^3 U μL^{-1} in 50% glycerol/ 50 mM Tris-HCl (pH 7.5) and stored at –20˚C.

Fractionation of polyphosphate type 15

To obtain a series of poly P with different chain-lengths, polyphosphate type 15 was fractionated by electrophoresis based on a method as described previously [23]. Eighty μL of 50 mg mL^{-1} of polyphosphate type 15 was applied onto two 15 % polyacrylamide gels (90 x 90 x 1 mm) buffered with 45 mM Tris/ 45 mM boric acid/ 1 mM EDTA (pH 8.3) and run at 10-20 mA constant current until bromophenol blue, a tracking dye, reached 3 cm below the

1 well. The gels were cut into seven serial fractions of 1 cm width from 2 cm below the gel

2 tops, and each gel piece was chopped into 1 mm cubes. Poly P was extracted from the gels by

3 shaking in a 20-fold volume of water (approx. 36 mL) for 2 h at room temperature. The

4 solutions were passed through 0.2 μm membrane filter to remove the gel pieces, freeze-dried

5 and dissolved in one mL distilled water. Five μL of each fraction was electrophoresed, and

6 the gel was stained with 0.05% TBO/ 25% methanol/ 5% glycerol for 30 min and destained

7 in 25% methanol/ 5% glycerol (Fig. 1). Poly P concentration (as Pi) of each fraction was

8 determined by the Mo-blue method (see below).

9

10 *Quantification of poly P*

11   Poly P was quantified by three different methods, including the enzymatic assay by

12 PPK reverse reaction (PPK method), the metachromatic assay with TBO (TBO method), and

13 microphosphate determination by the molybdenum method [24] after acid hydrolysis of poly

14 P (Mo-blue method). In all these methods, the amount of poly P was expressed in terms of Pi.

15   The PPK method was performed as described previously [13]. Two μL of poly P

16 solution was mixed with the final 20 μL of reaction mixture consisting of 50 mM $(NH_4)_2SO_4$,

17 4 mM $MgCl_2$, 40 μM ADP, 40 mM HEPES-KOH pH 7.5, and 3 U μL^{-1} of PPK, and

18 incubated at 37ºC for the prescribed time. Concentration of poly P in the reaction mixture

19 was 5 μM unless otherwise stated. The solution was diluted to 1:100 in the ATP dilution

20 buffer (10mM EDTA and 100 mM Tris-Cl pH 7.5) and 20 μL aliquots were mixed with an

21 equal volume of the CLSII reaction mixture (Roche Diagnostics). Chemi-luminescence was

22 measured by the Luminescencer PSN (ATTO, Japan) as a total luminescent count in 10 sec.

23   The TBO method was based on decrease in absorbance at 620 nm by metachromatic

24 reaction of TBO with poly P [12]. Ten μL poly P solution was mixed with 500-750 μL TBO

1　assay solution (15 mg mL^{-1} TBO and 0.1 N acetic acid), left for 15min at room temperature

2　and absorbance at 620 nm was measured within 30 min.

3　　　　For the Mo-Blue method, 5 µL poly P solution was mixed with an equal volume of 2

4　N HCl and heated at 95ºC for 30 min. Then the solution was diluted to 300 µL with distilled

5　water, mixed with 700 µL of the Mo-blue assay solution (6 parts of 0.42% $(NH_4)_6Mo_7O_{24} \cdot$

6　$4H_2O$ in 1 N H_2SO_4 and 1 part of 10% ascorbate), and incubated at 45ºC for 20 min. Pi

7　concentration was determined based on absorbance at 820 nm with reference to the standard

8　curve of 0-10 mM phosphate.

9

10　***Characterization of PPK reaction by ion chromatography***

11　　　　To characterize the reactivity of PPK to short-chain poly P, the PPK reaction

12　mixtures before and after incubation were analyzed by ion chromatography (IC). The

13　fractions 1 and 7 from polyphosphate type 15 were used as substrates, and a higher

14　concentration of poly P (5 mM as Pi) and ADP (20 mM) in a final volume of 50 µL was

15　necessary for this purpose. For the control sample (0 h incubation), heat-inactivated (95˚C

16　for 5 min) PPK was added to the reaction mixture. After incubation for 1-6 h, the reaction

17　was terminated by heating at 95˚C for 5 min, then the mixtures were stored at −20˚C.

18　Before IC analysis, the samples were thawed and diluted to 1:20 with distilled water, and

19　25 µL of the diluted sample was introduced to the anion exchange column as described

20　previously [21]. Dionex IonPac AS11 (2 x 250 mm) and AG11 (2 x 50 mm) were used as

21　the analytical columns with a potassium hydroxide gradient of 30-200 mM over 30 min.

22　Poly P was detected by a conductivity detector with the Dionex ASRS-ULTRA suppressor.

23　　　　Concentration of each poly P species was quantified based on the peak area. The

24　conversion factor from the area to concentration was 0.351 µS · min nmol^{-1} Pi-residues

1   which was obtained from IC analysis of the standard Pi, P2 and P3. In the case of IC

2   analyses of the PPK reaction mixtures, attenuation of signal intensity of poly P was

3   observed. Therefore, net concentration of poly P in the solution was determined by dividing

4   the peak areas by the "attenuation coefficients" which were calculated for individual poly P

5   species. Concentrations of ADP and ATP were determined using the conversion factors of

6   0.601 and 1.07 $\mu S \cdot min\ nmol^{-1}$, respectively. ATP concentration in the reaction mixture

7   was also determined using the CLSII kit as described above after 1:40,000 dilution with the

8   ATP dilution buffer.

9

10

11   **Results**

12   ***Lower reactivity of PPK to short-chain poly P***

13   Reactivity of PPK with short-chain poly P was investigated using the fractions 1 to 7

14   as substrates. The reactivity was expressed as the percentage of reacted poly P compared with

15   the absolute concentration as determined by the Mo-blue method. After 40 min incubation,

16   the reactivity of PPK decreased as the chain-length became shorter, that is, the reactivity was

17   highest (60%) with the fraction 1, the longest poly P fraction, and lowest (20%) with the

18   fraction 7, the shortest poly P fraction (Fig. 2).

19   Figure 3 indicates the time course analysis of PPK reaction using the fractions 1 and 7

20   as substrates. Percentage of reacted poly P after 1 h incubation was 70% and 25% in the

21   fractions 1 and 7, respectively. About 85% and 45% of poly P was reacted after 6 h

22   incubation in the fractions 1 and 7, respectively. Further incubation up to 24 h did not

23   improve the reactivity (data not shown).

24   Precise chain-length recognized by PPK was determined by IC using the fractions 1

1 and 7. Although some unidentified peaks were observed, it was possible to identify most of

2 the poly P species by fitting a logarithmic curve based on the retention time of Pi, P2, and P3

3 standards (data not shown). In the case of identification of poly P species in the PPK reaction

4 mixture, the peaks of poly P shorter than P8 were hardly identified because of interference by

5 other anionic compounds in the reaction mixture (Fig. 4B-D and 5B-D). Distribution of poly

6 P chain-lengths in the fraction 1 was from P10 to P40 (or longer) (Fig. 4A). Using this

7 fraction as substrate, it was revealed that poly Ps longer than P23 were diminished after 1 h

8 incubation (Fig. 4C), and that poly Ps longer than P10 were diminished after 6 h incubation

9 (Fig. 4D). P2 and P10 appeared to increase as the PPK reaction proceeded. Chain-length

10 distribution in the fraction 7 was from Pi to P32 (Fig. 5A and Table 2). After 1 h incubation

11 with the fraction 7, poly Ps longer than P10 decreased and those longer than P23 disappeared

12 (Fig. 5C and Table 2). It took 6 h to process most of the poly Ps longer than P10 (Fig. 5D and

13 Table 2). At this time, however, about half of the P8-P10 species remained unprocessed. An

14 increase in the P2 signal was observed as the PPK reaction proceeded (Fig. 5B-D). The total

15 decrease in poly P was in good agreement with the decrease of ADP and increase of ATP

16 (Table 2). These results were consistent with those of ATP determination by the

17 luciferine-luciferase system (data not shown).

18

19 *Broad chain-length specificity of TBO metachromasy*

20 Influence of chain-length on metachromatic reaction of TBO was investigated using

21 various substrates. First the reactivity was examined using the fractions 1 to 7 (Fig. 6A).

22 Although fraction 7 showed the lowest reactivity, the value was more than 85% of that of

23 fraction 1. No apparent decrease in reactivity against decrease in chain-length was observed.

24 Although TBO was absolutely insensitive to P3, it was found that polyphosphate types 15

1   and 75+ (heterogeneous poly P) showed similar reactivity to TBO (Fig. 6A). It was also

2   shown that the metachromatic reaction of TBO was not affected by a high concentration of

3   urea (Fig. 6C).

4

5   **Discussion**

6   The PPK assay is undoubtedly the most sensitive and specific method of those

7   investigated. For example, the detection limit of the PPK method is around 0.5 pmol poly P

8   (in terms of Pi-residues) μL^{-1} or even lower, whereas that of the TBO method is more than

9   100 pmol poly P μL^{-1}. Furthermore, the PPK method is not affected by polyanionic

10   macromolecules such as nucleic acids, which are rich in cell extracts and interfere with the

11   metachromatic reaction of TBO. It was unclear, however, whether short-chain poly P could

12   be quantified by PPK. In the present study, it was demonstrated that poly Ps longer than 22

13   residues could be converted to ATP completely within 1 h under the specified conditions, but

14   the conversion of those of 22 residues and the shorter ones was circumstantial. Poly Ps

15   shorter than 10 residues appeared almost insensitive to PPK reaction. Quantitative correlation

16   between the increase in ATP and the decrease in total poly Ps longer than P7 implies that

17   poly Ps shorter than P8 might remain unprocessed, although the corresponding peaks of these

18   poly Ps were hardly defined on the IC charts.

19   It has been shown that the PPK reverse reaction was highly processive as it is in the

20   case of forward (poly P-generating) reaction [25]. In this system, the procession of the

21   substrate proceeds very rapidly without dissociation of substrate from the catalytic site of the

22   enzyme. Therefore, the late limiting step of the reaction is formation of an enzyme-substrate

23   complex. One possible mechanism of low-reactivity of PPK to short-chain poly P is due to

24   low-affinity of the substrate-binding site of PPK to the substrate. This idea is supported by

1   the fact that no ATP-generating reaction occurred with the shortest poly P, tripolyphosphate

2   (P3) (unpublished result). Another possibility is that low PPK reactivity is due to incomplete

3   conversion of poly P to ATP in the reverse reaction. Interestingly, accumulation of P2 during

4   the reaction was observed in the present study. It was supposed that P2 would be a final

5   product of the PPK reverse reaction, in which case a significant amount of Pi-residues would

6   escape from the PPK procession when short-chain poly P is to be quantified. For example,

7   10% underestimation will occur with P20 substrate. But this "un-accessible ratio" will

8   become negligible when much longer poly P is to be quantified.

9   High-resolution ion chromatography enables us to quantify individual poly P species

10   shorter than P50. It would be difficult, however, to apply this technique to direct

11   measurement of poly P in cell extract because of the high background level of other anions.

12   The titanium column, a specialized component for chromatographic analysis of phospho-ester

13   compound, appears promising, and optimization of analytical conditions is under way.

14   The TBO method showed chain-length specificity broader than that of the PPK

15   method. In the case of the PPK method, poly P type 75+ showed higher reactivity than type

16   15, although they showed similar reactivity by the TBO method. That chain-length specificity

17   of TBO was broader than that of PPK was confirmed by the observation that fractions 1 and 7

18   after PPK reaction still contained short-chain poly Ps that were detectable by the TBO

19   method (unpublished results). Another advantage of the TBO method is that the

20   metachromatic reaction was not affected by urea. This means that the purification step

21   (exclusion of the protein denaturing reagent from cell extract) in which loss of short-chain

22   poly P occurs is not required. Although there are some disadvantages of the TBO method,

23   such as lower specificity and higher detection limit than that of the PPK method, a

24   combination of the PPK and TBO methods would be very practical for comprehensive

quantification of short- and long-chain poly P.

References

[1] T. Ezawa, T.R. Cavagnaro, S.E. Smith, F.A. Smith, R. Ohtomo, Rapid accumulation of polyphosphate in extraradical hyphae of arbuscular mycorrhizal fungus as revealed by histochemistry and polyphosphate kinase/ luciferase system, New Phytol. 161 (2003) 387-392.

[2] A. Kornberg, Inorganic polyphosphate: a molecule of many functions, Prog. Mol. Subcell. Biol. 23 (1999) 1-18.

[3] C.E. Castuma, R. Huang, A. Kornberg, R.N. Reusch, Inorganic polyphosphates in the acquisition of competence in *Escherichia coli*, J. Biol. Chem. 270 (1995) 12980-12983.

[4] M.H. Rashid, A. Kornberg, Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 4885-4890.

[5] M.H. Rashid, N.N. Rao, A. Kornberg, Inorganic polyphosphate is required for motility of bacterial pathogens, J. Bacteriol. 182 (2000) 225-227.

1   [6] M.H. Rashid, K. Rumbaugh, L. Passador, D.G. Davies, A.N. Hamood, B.H. Iglewski, A.

2   Kornberg, Polyphosphate kinase is essential for biofilm development, quorum sensing,

3   and virulence of *Pseudomonas aeruginosa*, Proc. Natl. Acad. Sci. U.S.A. 97 (2000)

4   9636-9641.

5   [7] T. Shiba, K. Tsutsumi, H. Yano, Y. Ihara, A. Kameda, K. Tanaka, H. Takahashi, M.

6   Munekata, N.N. Rao, A. Kornberg, Inorganic polyphosphate and the induction of *rpoS*

7   expression, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 11210-11215.

8   [8] N.N. Rao, S. Liu, A. Kornberg, Inorganic polyphosphate in *Escherichia coli*: the

9   phosphate regulon and the stringent response, J. Bacteriol. 180 (1998) 2186-2193.

10   [9] K.S. Kim, N.N. Rao, C.D. Fraley, A. Kornberg, Inorganic polyphosphate is essential for

11   long-term survival and virulence factors in *Shigella* and *Salmonella* spp., Proc. Natl.

12   Acad. Sci. U.S.A. 99 (2002) 7675-7680.

13   [10] A. Kuroda, S. Tanaka, T. Ikeda, J. Kato, N. Takiguchi, H. Ohtake, Inorganic

14   polyphosphate kinase is required to stimulate protein degradation and adaptation to

15   amino acid starvation in *Escherichia coli*, Proc. Natl. Acad. Sci. U.S.A. 96 (1999)

16   14264-14269.

17   [11] A. Kuroda, K. Nomura, R. Ohtomo, J. Kato, T. Ikeda, N. Takiguchi, H. Ohtake, A.

18   Kornberg, Role of inorganic polyphosphate in promoting ribosomal protein

19   degradation by the Lon protease in *E. coli*, Science 293 (2001) 705-708.

20   [12] J. Griffin, N. Davidian, R. Penniall, Studies of phosphorus metabolism by isolated

21   nuclei. VII. Identification of polyphosphate as a product, J. Biol. Chem. 240 (1965)

22   4427-4434.

23   [13] D. Ault-Riche, C.D. Fraley, C.M. Tzeng, A. Kornberg, Novel assay reveals multiple

24   pathways regulating stress-induced accumulations of inorganic polyphosphate in

Escherichia coli, J. Bacteriol. 180 (1998) 1841-1847.

[14] J.A. Callow, L.C.M. Capaccio, G. Parish, P.B. Tinker, Detection and estimation of polyphosphate in vesicular-arbuscular mycorrhizas, New Phytol. 80 (1978) 125-134.

[15] G. Cox, K.J. Moran, F. Sanders, C. Nockolds, P.B. Tinker, Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. III. Polyphosphate granules and phosphorus translocation, New Phytol. 84 (1980) 649-659.

[16] M.Z. Solaiman, T. Ezawa, T. Kojima, M. Saito, Polyphosphate in intraradical and extraradical hyphae of an arbuscular mycorrhizal fungus, *Gigaspora margarita*, Appl. Environ. Microbiol. 65 (1999) 5604-5606.

[17] J. Schuddemat, R. De-Boo, C. Van-Leeuwen, P. Van-Den-Broek, J. Van-Steveninck, Polyphosphate synthesis in yeast, Biochim. Biophys. Acta 1010 (1989) 191-198.

[18] K.D. Kumble, A. Kornberg, Endopolyphosphatases for long chain inorganic polyphosphate in yeast and mammals, J. Biol. Chem. 271 (1996) 27146-27151.

[19] A. Sethuraman, N.N. Rao, A. Kornberg, The endopolyphosphatase gene: essential in *Saccharomyces cerevisiae*, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 8542-8547.

[20] N. Ogawa, J. DeRisi, P.O. Brown, New components of a system for phosphate accumulation and polyphosphate metabolism in *Saccharomyces cerevisiae* revealed by genomic expression analysis, Mol. Biol. Cell 11 (2000) 4309-4321.

[21] Y. Sekiguchi, A. Matsunaga, A. Yamamoto, Y. Inoue, Analysis of condensed phosphates in food products by ion chromatography with an on-line hydroxide eluent generator, J. Chromatogr. A 881 (2000) 639-644.

[22] K. Ahn, A. Kornberg, Polyphosphate kinase from *Escherichia coli*. Purification and demonstration of a phosphoenzyme intermediate, J. Biol. Chem. 265 (1990) 11734-11739.

1 [23] T. Ezawa, S. Kuwahara, K. Sakamoto, T. Yoshida, M. Saito, Specific inhibitor and

2   substrate specificity of alkaline phosphatase expressed in the symbiotic phase of the

3   arbuscular mycorrhizal fungus, *Glomus etunicatum*, Mycologia 91 (1999) 636-641.

4 [24] P.S. Chen, T.Y. Toribara, H. Warner, Microdetermination of phosphorus, Anal. Chem.

5   28 (1956) 1756-1758.

6 [25] C.-M. Tzeng, A. Kornberg, The multiple activities of polyphosphate kinase of

7   *Escherichia coli* and their subunit structure determined by radiation target analysis, J.

8   Biol. Chem. 275 (2000) 3977-3983.

9

1

Figure 1

2
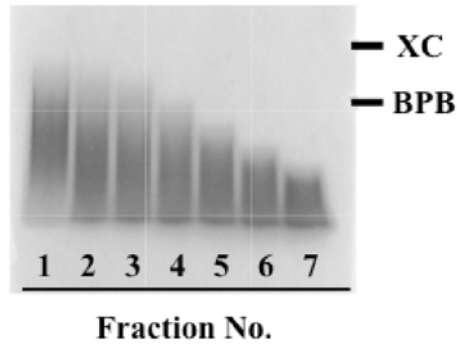
Fig. 1. Electrophoretic analysis of fractionated polyphosphate type 15

Four mg polyphosphate type 15 was fractionated by polyacrylamide gel electrophoresis, and 5 µL of the each fraction was loaded onto 15% polyacrylamide gel and stained with Toluidine blue O (see Materials and methods). Positions of the tracking dyes, xylen cyanol (XC) and bromophenol blue (BPB), are indicated.

Figure 2
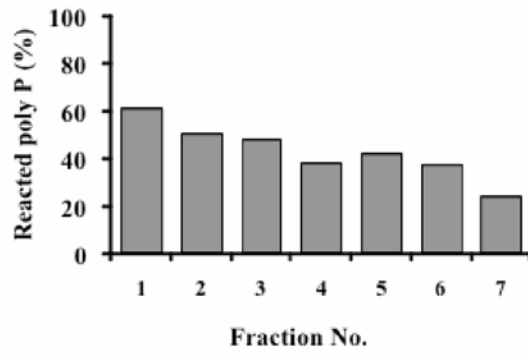
1

2   **Fig. 2. Reactivity of polyphosphate kinase (PPK) to short-chain poly P (fractions 1 to 7)**

3   Concentration of substrate in the reaction mixtures was 5 µM (as Pi). The mixtures

4   were incubated for 40 min at 37˚C, and ATP concentration was determined by the

5   luciferin-luciferase system. Reactivity of PPK is expressed as the percentage of poly P

6   converted to ATP by PPK reverse reaction.
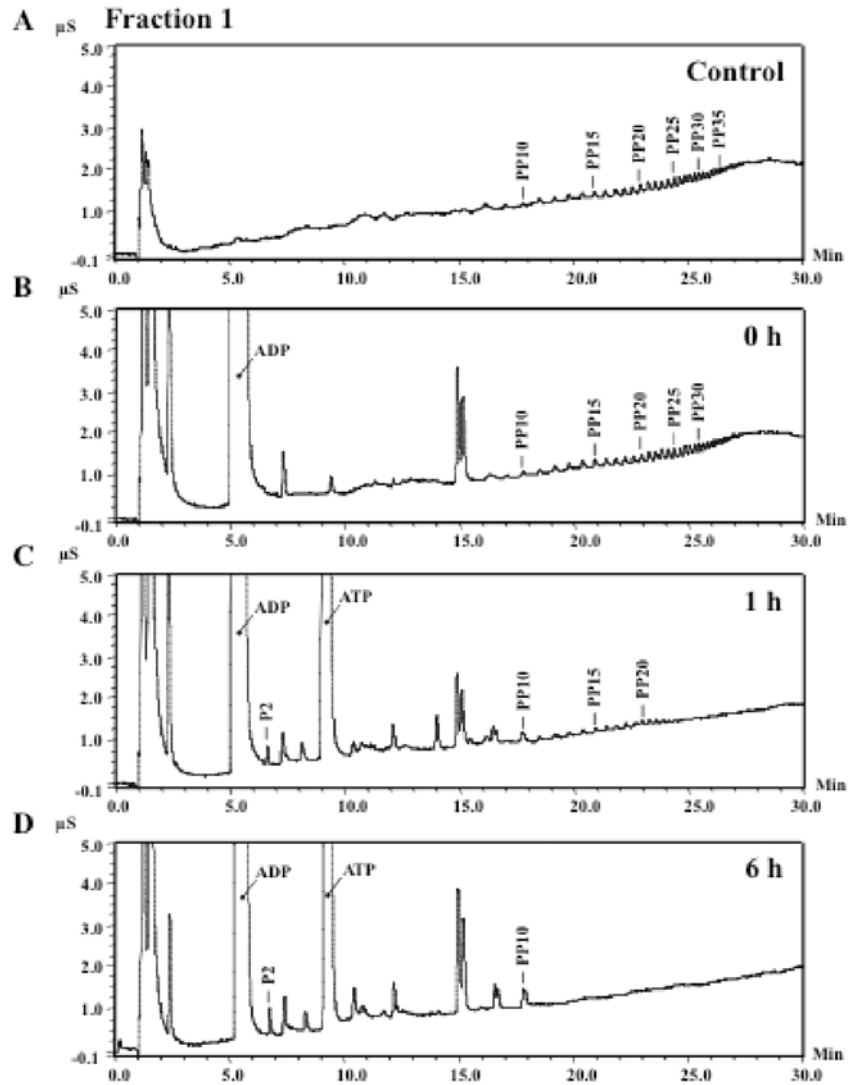
Figure 3
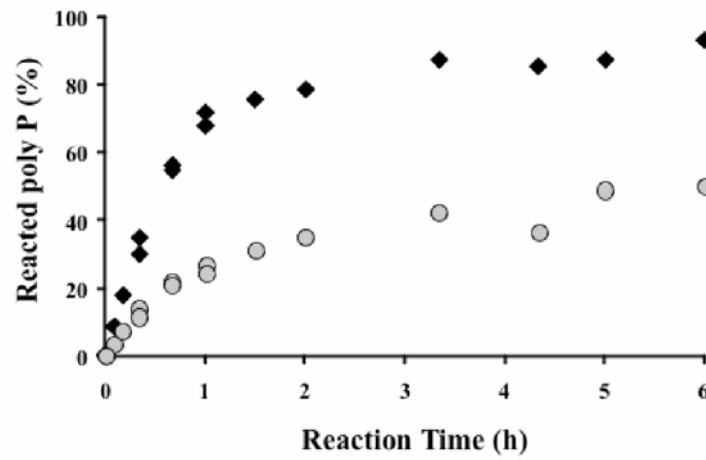
1

2 **Fig. 3. Time course analysis of PPK reaction using the fractions 1 and 7 as substrate**

3   Conditions of the experiment were the same as those described in Fig. 2 except for the

4 incubation times (0-6 h). The fraction 1, diamonds; the fraction 7, circles.
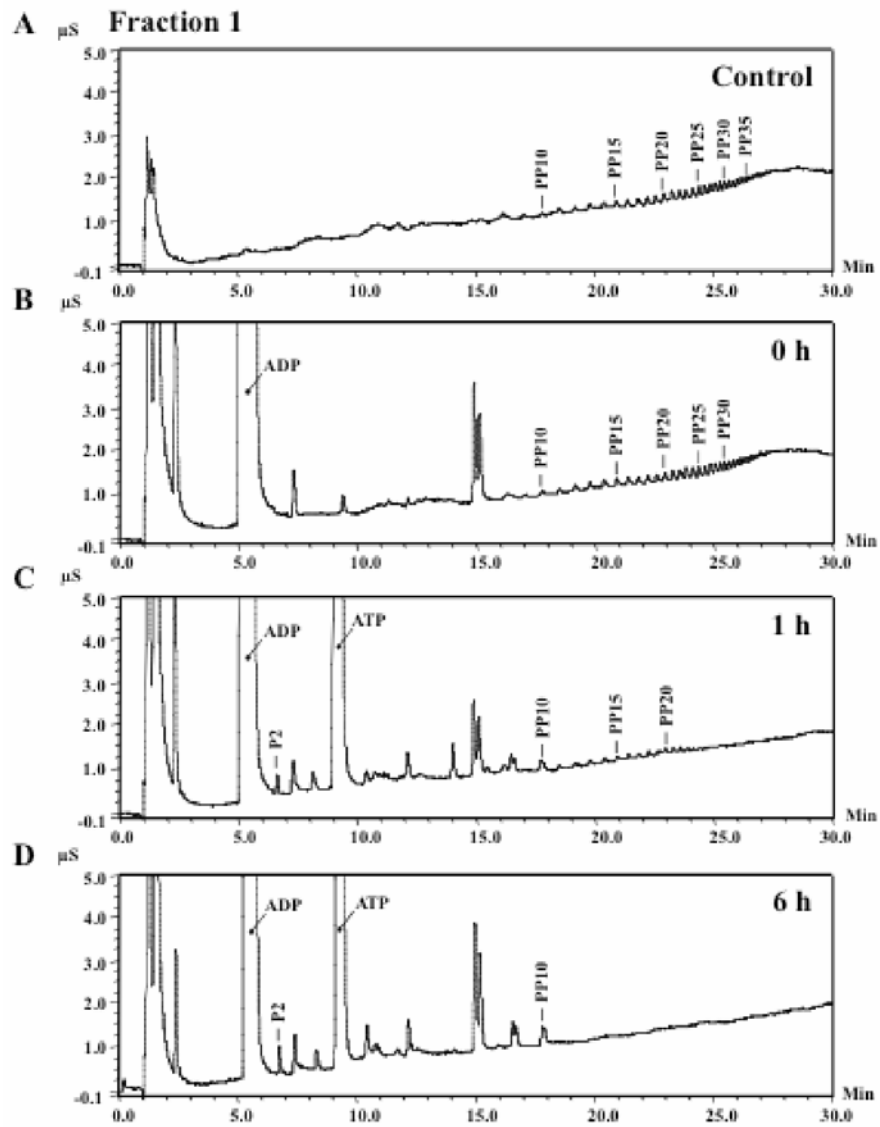
Fig. 4. Ion chromatographic analysis of PPK reaction with the fraction 1

A (Control): 25 μL of 5 mM poly P (fraction 1) was analyzed without PPK reaction.

B-D: Concentration of poly P (fraction 1) and ADP in the reaction mixture was 5 mM and 20

mM, respectively, and the mixtures were incubated for 0 h (B), 1 h (C), and 6 h (D) before

the analyses. Note that PPK in the mixture analyzed at 0 h was heat-inactivated.
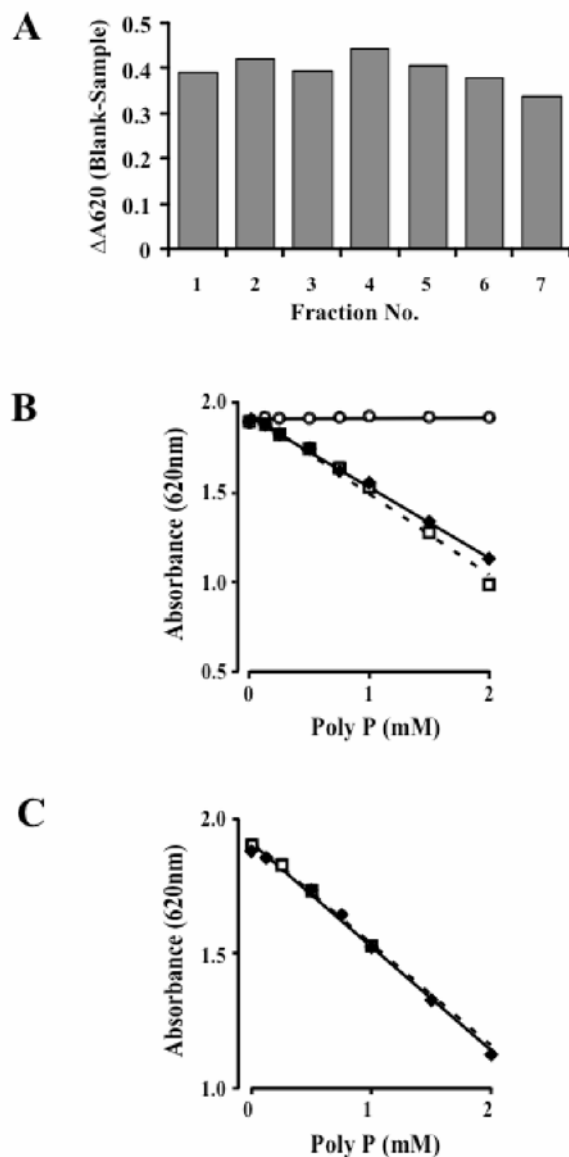
Fig. 5. Ion chromatographic analysis of PPK reaction with the fraction 7

A-D: Conditions of the analyses were the same as those described in Fig. 4 except for substrate (fraction 7) of the PPK reaction.

1

2   **Fig. 6. Characterization of Toluidine blue O (TBO) metachromatic reaction**

3   A: Reactivity of TBO with the fractions 1 to 7 obtained from polyphosphate type 15

4   (Sigma Chemicals). Concentration of poly P in the reaction mixtures was 1 mM (as Pi).

5   Reactivity is expressed as the decrease in absorbance at 620 nm (ΔA620: A620$_{blank}$ −

6   A620$_{sample}$). B: Reactivity of TBO with tripolyphosphate (circles, solid line), polyphosphate

7   type 15 (diamonds, dotted line), and type 75+ (squares, solid line). C: Reactivity of TBO

8   with polyphosphate type 15 in the presence (squares, solid line) and absence (diamonds,

9   dotted line) of 4 M urea.

21

**Table 1. Ion chromatographic analysis of poly P in fraction 7
before and after the PPK reaction.**

Peak	Concentration (mM)				% Reacted with PPK [1]	
	Control	0 h	1 h	6 h	1 h	6 h
Pi	0.07	1.67	1.25	0.67		
P2	0.12	0.12	0.25	0.46		
P3	0.08					
P4	0.18					
P5	0.17					
P6	0.23					
P7	0.26					
P8	0.27	0.27	0.24	0.12	13	56
P9	0.26	0.26	0.23	0.11	10	57
P10	0.27	0.27	0.21	0.16	23	39
P11	0.27	0.27	0.19	0.05	32	83
P12	0.26	0.26	0.16	0.02	39	93
P13	0.30	0.30	0.18	0.02	39	92
P14	0.24	0.24	0.14		40	100
P15	0.25	0.25	0.12		51	100
P16	0.24	0.24	0.12		50	100
P17	0.22	0.22	0.09		61	100
P18	0.21	0.21	0.07		66	100
P19	0.19	0.19	0.06		69	100
P20	0.18	0.18	0.05		73	100
P21	0.16	0.16	0.04		78	100
P22	0.14	0.14	0.02		87	100
P23	0.12	0.12			100	100
P24	0.10	0.10			100	100
P25	0.08	0.08			100	100
P26	0.07	0.07			100	100
P27	0.06	0.06			100	100
P28	0.05	0.05			100	100
P29	0.03	0.03			100	100
P30	0.02	0.02			100	100
P31	0.01					
P32	0.01					
P33	0.01					
Total Poly P (>P7) [2]	3.99	3.97	1.90	0.48		
ΔPoly P after Incubation [3]			**2.07**	**3.49**		
ADP	0	25.6	23.1	21.7	10	15
ATP	0	0.0	**2.1**	**3.2**		
ADP + ATP	0	25.6	25.1	24.9		

[1] Percentage of poly P reacted with PPK after 1 or 6 h incubation.
[2] Concentration or percentage of total poly P longer than P7.
[3] Decrease in total poly P (>P7) after 1 or 6 h incubation.