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Footnotes:

The abbreviations used are:

P, phosphorous;

P_i, orthophosphate (PO₄³⁻);

polyP_n, inorganic polyphosphate with a chain length of n;

SEM, standard error of the mean.

Abstract

In *Saccharomyces cerevisiae*, inorganic polyphosphate is analyzed by polyphosphate extraction and subsequent quantification. Recently, we developed a method for polyphosphate quantification, and length determination of short chain polyphosphate. However, the lack of a simple, optimized and validated method for analytical polyphosphate extraction has both hindered the advance in this research field, and prevented comparability of results between laboratories. Hence, the goal of this study was to develop an analytical method for polyphosphate extraction from *S. cerevisiae*. Several literature methods were compared with special attention to omission of polyphosphate precipitation steps, because these work neither at low polyphosphate concentrations nor quantitatively. The best literature protocol, which takes 5.5 h and requires five reaction tubes per sample, was optimized here in regards to the amount of extracted polyphosphate and simplification of the work flow. The final protocol extracts 40 % more polyphosphate than the best literature method, takes only 30 min, requires just one reaction tube per sample, and is, therefore, proposed as the new gold standard for analytical polyphosphate extraction from *S. cerevisiae*. In combination with our recently published polyphosphate quantification method, total polyphosphate in *S. cerevisiae* can now be analyzed within 2 h.

Introduction

Inorganic polyphosphate (polyP) is the linear polymer of orthophosphate (P_i) and can be found in all living organisms [1]. In *Saccharomyces cerevisiae*, polyP is analyzed by analytical polyP extraction and subsequent polyP quantification. One of the first analytical polyP extraction methods for *S. cerevisiae* was published in the late 1960s by Langen and Liss [2-7]. The extraction procedure consists of successive treatments of cell mass with different extraction solutions alternated with centrifugation, to separate the extract from the insoluble matter. Cell disruption was achieved using trichloroacetic acid (*i. e.*, a chaotropic agent). The subsequent extractions were done with saturated sodium perchlorate, and NaOH. The different fractions obtained by this protocol allowed some insight in *S. cerevisiae* cell physiology, because with each fraction longer polyP chains were dissolved. A similar protocol, which utilizes perchloric acid instead of trichloroacetic acid for cell disruption, was published by Kulaev *et al.* in 1966 [8]. This protocol is extensively used up to this day [9-13]. In most of the extraction protocols polyP is recovered from the extracts through alcohol precipitation or Ba^{2+} precipitation, which both work neither at low polyP concentrations [14] nor quantitatively [15].

Being an intracellular poly-anion with a phosphate backbone, polyP behaves similarly to DNA and RNA during extraction. For this reason, methods for nucleic acid extraction were adapted to polyP extraction around the turn of the millennium. These include bead beating with acidic phenol followed by chloroform washings, which was originally destined for RNA extraction [16], or cell disruption with chaotropic substances followed by silica spin column purification, which was originally used for total nucleic acid extraction [17, 18]. Both polyP precipitation and silica column purification are used to separate polyP from the extracts and concentrate the polyP. Unfortunately, only polyP with a chain length above 60 to 80 P-subunits binds to the silica columns [17, 19]. PolyP in *S. cerevisiae* occurs also well below this cut off [8, 11-13, 20, 21]. Concluding, due to a lack of a generally accepted gold standard analytical polyP extraction, every laboratory uses its own extraction.

PolyP analysis in *S. cerevisiae* consists of an analytical polyP extraction and polyP quantification. A gold standard for the latter, was recently developed [22]. However, the lack of a simple, optimized and validated method to extract cellular polyP from *S. cerevisiae* has been a drawback, which hindered the advance in this research field. Furthermore, a uniform protocol for polyP extraction, would allow for comparability of results between laboratories. Therefore, the goal of this study was to develop an analytical total polyP extraction method for *S. cerevisiae*.

Materials and methods

Chemicals and enzymes

Phenol saturated in TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.5 – 8.0), and phosphate-free filter paper were acquired from Carl Roth (Germany). PolyP Budit 7 was from Budenheim (Germany). Silicon dioxide nanopowder, and yeast nitrogen base without amino acids were purchased from Sigma-Aldrich (MO, USA). Dow Corning high vacuum grease was from VWR (PA, USA). The Trizol reagent was obtained from Thermo Fisher Scientific (MA, USA). Low-density phase lock gel was prepared by filling high vacuum grease into syringes and autoclaving those [23]. High salt solutions required high-density phase lock gel. High-density phase lock gel was prepared by mixing 1 mass-part silicon dioxide nanopowder with 13 mass-parts high vacuum grease, transferring this mixture into syringes, followed by autoclaving. *S. cerevisiae* exopolyphosphatase 1 and *S. cerevisiae* pyrophosphatase 1 were expressed as double strep tag fusion proteins in *Escherichia coli* and purified through affinity chromatography according to our previous study [22]. All chemicals involved in P_i analysis and fermentations were of analytical grade.

Optimized analytical polyP extraction for S. cerevisiae

This protocol was developed during this study and is referred to as “optimized protocol”. Solutions were stored in new plastic ware, or in glass ware, which was washed once with 0.1 M HCl and thrice with bidistilled water. All steps were performed at room temperature. *S. cerevisiae* cell mass was washed twice with sterile bidistilled water through repeated centrifugation (5,000 g, 10 min) and resuspension. The cell mass was dried for 5 min at room temperature on phosphate-free filter paper. Content of dry matter was determined through overnight drying at 120°C and subsequent drying / weighing to constant mass at 120°C in a moisture analyzer. For polyP extraction, 24 to 26 mg wet *S. cerevisiae* cell paste, containing ca. 25 % dry matter, was weighed in a 2 ml reaction tube and stored at -20°C until extraction. To the cell mass 400 µl ME-buffer (25 mM MOPS, 2.5 mM Na₂EDTA, pH 7.0 with HCl / NaOH, filtered (0.2 µm pores), storage at 4°C) was added. To fully resuspend the cells, the tube was vortexed 20 sec. Because phenol and chloroform are toxic, the following steps were performed under the fume hood while wearing nitrile gloves and safety glasses. After spinning the tube briefly at 1,000 g, to clear liquid from the tube lid, 300 µl saturated phenol was added. The commercially obtained saturated phenol was already equilibrated with TE-buffer, and stored under a layer of TE-buffer. Saturated phenol was stored at 4°C, and brought to room temperature prior to use. To allow formation of a thorough phenol-water emulsion, the tube was vortexed for 20 sec. Next, the tube was incubated for 10 min at 45°C in an aluminum heating block, and 2 min on ice. The tube was spun briefly at 1,000 g, to clear the lid from liquid. After the addition of 1 ml chloroform, the tube was vortexed for 20 sec, to allow the chloroform to extract any phenol dissolved in the aqueous phase. The tube was spun briefly at 1,000 g, to clear the lid. With a small spatula ca. 200 mg of low-density phase lock gel was applied into the lid of the tube. Between samples, the spatula was wiped with a cloth soaked in 70 % (v / v) ethanol, to avoid inter-sample contamination. The exact amount of phase lock gel was not important. After the tube was centrifuged (12,000 g, 5 min), the top aqueous phase was quantitatively transferred to another reaction tube. The exact volume of the extract was noted (ca. 500 µl). The eluate volume is composed of 400 µl ME-buffer, cell water, and TE-buffer from the phenol. Adjustment of the extract pH was not necessary, because the pH was already neutral through the utilized buffer. Extracts were stored at -20°C until polyP quantification.

PolyP quantification

Extracts were diluted with bidistilled water prior to analysis. PolyP quantification was done according to our previous study [22]. Briefly, nine cavities of a 96-well plate were filled with 100 μ l diluted extract each. Fifty μ l enzyme reaction buffer (15 mM magnesium acetate, 60 mM Tris, 150 mM ammonium acetate, pH 7.5; well called “blank P_i”), 50 μ l enzyme reaction buffer containing 1.1×10^4 U *S. cerevisiae* exopolyphosphatase 1 (called “scPpx1p digestible polyP”), and 50 μ l enzyme reaction buffer containing both 1.1×10^4 U *S. cerevisiae* exopolyphosphatase 1 and 1.3×10^2 U *S. cerevisiae* pyrophosphatase 1 (called “total polyP”) were added to three cavities, respectively. Elsewhere on the 96-well plate, 100 μ l KH₂PO₄ standards (20 to 200 μ M P_i) mixed with 50 μ l enzyme reaction buffer were placed. After 1 h at 37°C, to each cavity 50 μ l P_i detection reagent (2.4 mM ammonium heptamolybdate, 600 mM H₂SO₄, 0.6 mM antimony potassium tartrate, 88 mM ascorbic acid) was added. After 2 min at room temperature, the absorbance, which was indicative of the P_i concentration, was read at 882 nm. The polyP concentration and average chain length were calculated based on the enzymatically released P_i according to Equation 5 and 3 in Ref. [22], respectively. The polyP content in cell dry weight was calculated according to Equation 1. “True polyP” was defined as the measured total polyP minus the blank P_i. PolyP was defined as KPO₃ and, thusly, has a formula weight of 118.07 g * mol⁻¹. If a sample was diluted, for example 100-fold, dilution was given as 100.

$$\text{PolyP (as KPO}_3\text{) content in cell dry weight [\% (w / w)]} = \frac{\text{“true polyP concentration [\mu M P}_i\text{]”} * \text{“formula weight polyP [g * mol}^{-1}\text{]”} * \text{“eluate volume [\mu l]”} * \text{“dilution [times]”}}{\text{“cell wet weight [mg]”} * \text{“content of dry matter [\%]”} * 10^5}$$

(Eq. 1)

Analytical polyP extraction with the “TEN without precipitation” and TENSX protocol

For the “TEN without precipitation” (inspired by chapter 13.12.2 in Ref. [24]) and TENSX (13.11.1 in Ref. [24]) protocol, to 25 mg *S. cerevisiae* cell wet weight 300 μ l TEN buffer (200 mM Tris, 10 mM EDTA, 0.5 M NaCl, pH 7.5 with HCl / KOH) or 300 μ l TENSX buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, 34.6 mM SDS, 2 % (v / v) Triton X-100, pH 8.0 with HCl / KOH) were added, respectively. After the addition of 300 mg glass beads (500 μ m diameter) and 300 μ l phenol-chloroform-isoamyl alcohol (25:24:1 v:v:v parts), the tube was vortexed for 2 min and centrifuged (12,000 g, 5 min, with phase lock gel). After the supernatant was washed with chloroform, 1/50 volume of phenol red (1.4 mM) was added and the pH titrated to pH 7 to 8 (*i. e.*, red color) with HCl / KOH. Extracts were stored at -20°C.

Analytical polyP extraction with the “Clark without precipitation” protocol inspired by Ref. [14]

Twenty-five mg *S. cerevisiae* wet cell mass was resuspended in 85 μ l trichloroacetic acid (2 % (w / v)), incubated for 5 min, centrifuged (12,000 g, 5 min) and the supernatant transferred to a separate tube. This extraction sequence was repeated once with 85 μ l trichloroacetic acid (2 % (w / v)), and twice with 170 μ l EDTA (2 mM, pH 7.5). Extracts were pooled and washed once with phenol-chloroform-isoamyl alcohol and once with chloroform with the help of phase lock gel. Extracts were titrated to neutral pH before storage at -20°C.

Analytical polyP extraction with the “Langen and Liss without precipitation” protocol inspired by Ref. [2, 6].

All steps were performed on ice. All solutions were ice-cold. Twenty-five mg *S. cerevisiae* cell wet weight was resuspended in 60 μ l trichloroacetic acid (4 % (w / v)), incubated for 15 min, centrifuged (16,900 g, 2 min, 4°C), and the supernatant transferred to a separate tube. This extraction sequence was repeated once with 60 μ l trichloroacetic acid (4 % (w / v)) and no incubation, twice with 60 μ l sodium perchlorate solution (1.7 M NaClO₄, 66 mM trichloroacetic acid) and 5 min incubation,

once with 100 µl ice-cold NaOH (25 mM) and 15 min incubation, once with 50 µl ice-cold bidistilled water and 5 min incubation, and twice with 80 µl NaOH (50 mM) and 15 min incubation. Extracts were pooled and titrated to neutral pH before storage at -20°C.

Analytical polyP extraction “Trizol without precipitation”

Twenty-five mg *S. cerevisiae* wet cell mass was resuspended in 1 ml commercially obtained Trizol reagent and incubated for 5 min. After the addition of 200 µl chloroform and a 3 min incubation period, the aqueous phase was separated from the chloroform-phenol phase through centrifugation (12,000 g, 5 min, with phase lock gel). Extracts were titrated to neutral pH before storage at -20°C.

Analytical polyP extraction according to the Bru et al. protocol and derivatives of the original protocol

The original protocol for analytical polyP extraction from *S. cerevisiae* was taken from Ref. [19]. Briefly, 25 mg cell wet weight was resuspended in 400 µl cold AE-buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.3) and transferred to a screw cap tube containing 300 µl phenol (saturated in TE-buffer) and 40 µl SDS (10 % (w / w)). After inverting the tube four-times and vortexing for 5 sec, the tube was incubated at 5 min for 65°C and 1 min on ice. 300 µl chloroform were added. The tube was inverted four-times, vortexed for 5 sec, and centrifuged (13,000 g, 2 min). The supernatant was transferred to a screw cap tube containing 350 µl chloroform. After inverting the tube four-times and vortexing for 5 sec, the tube was centrifuged (13,000 g, 2 min) and the supernatant transferred to a new tube. Two µl RNase A (50 U * µl⁻¹) and 2 µl DNase (2 U * µl⁻¹) were added, and the tube incubated for 1 h at 37°C. A tube, which contained 1 ml absolute ethanol and 40 µl sodium acetate (3 M, pH 5.3), was prepared and chilled to -20°C. The content was transferred to the prepared tube. After 3 h at -20°C, the tube was centrifuged (13,000 g, 20 min, 4°C). The supernatant was discarded. To the pellet, 500 µl ice-cold ethanol (70 % (v / v)) was added. The tube was centrifuged (13,000 g, 5 min, 4°C) and the supernatant discarded. The pellet was dried, dissolved in 500 µl bidistilled water, and stored at -20°C.

The protocol “Bru without ethanol precipitation” was performed as follows. To 25 mg wet *S. cerevisiae* cell mass, 400 µl AE-buffer and 40 µl SDS (10 % (w / w)) were added. The tube was vortexed for 20 sec, incubated for 5 min at 65°C and 1 min on ice, and briefly spun down. After the addition of 300 µl chloroform, the tube was vortexed for 20 sec and spun down briefly. Two-hundred mg of low-density phase lock gel were added, and the tube centrifuged (12,000 g, 5 min). The supernatant was transferred to a new tube and 500 µl chloroform was added. After 20 sec of vortexing and a brief spin, 200 mg low-density phase lock gel was added, and the tube centrifuged (12,000 g, 1 min). The aqueous phase was transferred to a new tube, whereas the volume was noted. Two µl RNase A (50 U * µl⁻¹) and 2 µl DNase (2 U * µl⁻¹) were added, and the tube incubated for 1 h at 37°C. Extracts were titrated to neutral pH before storage at -20°C.

The protocol “Bru without chloroform washing, nuclease treatment and ethanol precipitation” was the same as “Bru without ethanol precipitation”, but with the chloroform washing and the nuclease treatment omitted. The optimized protocol can be found above.

Comparison of different protocols for analytical polyP extraction from S. cerevisiae

S. cerevisiae VH2.200 (an industrial yeast strain used for baker’s yeast and yeast extract production) cell mass was produced by batch cultivation in synthetic defined medium (SD-medium, 0.68 % (w / w) yeast nitrogen base without amino acids, 2 % (w / w) glucose, pH 5.4, filtered (0.2 µm pores)). Thereof, polyP-rich cells were prepared according to a protocol from Liss and Langen [7]. Briefly, cells were starved at 2.5 g cell wet weight * L⁻¹ and 200 RPM for 8 h at 30°C in a filtered (0.2 µm pores) medium containing 40 mM (NH₄)₂SO₄, 13.3 mM KCl, 8 mM MgSO₄, 4 mM CaCl₂, 12.5 mM sodium succinate, 66.6 mM glucose, pH 5.0. Afterwards cells were incubated at 30 g cell wet weight * L⁻¹ and 250 RPM in a filtered (0.2 µm pores) medium containing 28.6 mM KH₂PO₄, 20 mM MgCl₂, 0.25 M glucose, pH 6.0 for 2 h at 30°C. Cells were washed twice with bidistilled water and 25 mg aliquots were stored in 2 ml tubes at -20°C. **Sample storage at -20°C did not suffice to prevent over time polyP degradation. Hence, minimization of sample storage before polyP extraction is**

243 **recommended.** Cells were extracted with the “TEN without precipitation”, TENSX, “Langen and Liss
244 without precipitation”, “Clark without precipitation”, “Trizol without precipitation”, and “Bru without
245 ethanol precipitation” extraction protocols. Afterwards polyP was quantified, as mentioned above.

Results

An analytical polyP extraction is here defined as a laboratory, wet-chemical method to extract total polyP from *S. cerevisiae*. In comparison to a *preparative* extraction (*i. e.*, industrial-scale substance extraction), number, amount and cost of required extractants, extractant toxicity, and required time are of subordinate importance for an analytical extraction. The most important performance parameter for an analytical extraction was the amount of extracted polyP. Because there is no reference method for absolute determination of polyP in living cells, different extraction procedures from literature were compared and the one, which extracted the most polyP from *S. cerevisiae* cells, was chosen for further experiments. Afterwards, this method was optimized in terms of the amount of extracted polyP and simplification of the work flow. Emphasis was put on finding an extraction method, which does not require ethanol or barium salt precipitation of the polyP. Because potassium is by far the most abundant cation in *S. cerevisiae* vacuoles, where most of the polyP is stored, and PO_3^- is the chemical formula of longer chain polyP, polyP is reported as KPO_3 in this study [25, 26].

To identify an analytical polyP extraction, which extracts the most polyP from yeast cells, literature was searched for suitable methods. Extractions with yeast cell digesting enzymes (*e. g.*, zymolyase) were not considered due to the requirement for long incubation times, during which cellular polyP content would potentially decrease. The frequently used polyP extraction method utilizing silica spin columns was also not included, because polyP with a chain length shorter than 60 to 80 does not bind quantitatively to silica. Finally, six extraction methods from literature were chosen. The “Clark without precipitation” and “Langen and Liss without precipitation” protocol yield several polyP fractions. Because only total cellular polyP content was of interest, polyP fractions from these analytical polyP extractions were pooled. *S. cerevisiae* VH2.200 was chosen as the model organism. Thereof, polyP-rich cells were prepared according to Liss and Langen [7].

To allow better separation of the phenol-chloroform and aqueous phase during the extractions, a phase lock gel was added before centrifugation [23]. With phase lock gel, it was possible to transfer the entire aqueous phase after centrifugation. Contamination of the aqueous phase with cell debris and the interphase was avoided, because both were located below the phase lock gel. High vacuum grease could be used as low-density phase lock gel. The high-density version for high salt solutions was prepared by mixing the grease with silicon dioxide. This self-made phase lock gel offered a cheap alternative to commercial phase lock gel. The manufacturer of the high vacuum grease did not matter, because both Dow Corning and Beckman high vacuum grease performed well. Commercially available phase lock gel is oftentimes already filled into reaction tubes. In this study phase lock gel was added directly into the lid of a reaction tube prior to centrifugation, to avoid liquid loss through transfer to a tube already containing phase lock gel. During assay development, to avoid hydrolysis of polyP during storage, polyP extracts were mixed with phenol red solution as pH indicator and titrated to neutral pH (*i. e.*, red color) with HCl or KOH.

Two protocols involving mechanical cell disruption were investigated (*i. e.*, “TEN without precipitation” and TENSX protocol) [24]. Originally, the protocols were destined for RNA and plasmid DNA preparation, respectively. Two protocols involving chemical extraction with several aqueous extractants applied one after another to a cell pellet were utilized: the “Langen and Liss without precipitation” (inspired by Ref. [2, 6]) and the “Clark without precipitation” (inspired by Ref. [14]) protocol. The last tested extraction (*i. e.*, Bru without ethanol precipitation) was adapted from Bru *et al.* [19]. All six extraction methods were applied to aliquots of one batch of polyP-rich *S. cerevisiae* cells (Fig. 1). The results must be interpreted as both amount of total extracted polyP and inhibition of the polyP quantification method. Both effects could not be separated in the here presented experiments. Extracts were diluted 20 to 200-fold for polyP quantification depending on the polyP concentration in the extract. This dilution reduced the impact of inhibitory substances in the extracts on polyP quantification. As mentioned above, an extraction method was sought, which works without precipitation of the polyP. Therefore, precipitation steps in the TEN, Trizol, Clark, Langen and Liss, and Bru protocol were omitted. When interpreting the results of Fig. 1, it should be noted that precipitations probably would have removed substances, which inhibited polyP quantification. The best example is the “Trizol without precipitation” method, which performed unsatisfactory (first bar in Fig. 1). The “Trizol without precipitation” extraction solution contained around 4 M chaotropic substances,

which completely inhibited polyP quantification and would have been removed through precipitation of polyP. Chemical cell disruption with trichloroacetic acid in the “Clark without precipitation” and “Langen and Liss without precipitation” protocol was probably insufficient, resulting in low amounts of extracted polyP (second and forth bar in Fig. 1). After the trichloroacetic acid cell disruption step, “Clark without precipitation” follows up with an EDTA extraction, whereas “Langen and Liss without precipitation” extract with sodium perchlorate and NaOH. In comparison, the latter protocol was seven-times more efficient. With cell disruption through bead beating at high salinity (*i. e.*, “TEN without precipitation”, third bar Fig. 1) even less polyP than with the “Langen and Liss without precipitation” protocol was extracted, probably due to inhibition of polyP quantification through high salinity in the extracts. Bead beating at low salinity (*i. e.*, TENSX, fifth bar in Fig. 1) took second place. In the end, the “Bru without ethanol precipitation” was chosen for further optimization, because it extracted, by far, the most polyP (sixth bar in Fig. 1). This success can be attributed to phenol as the cell disrupting agent in combination with EDTA. EDTA helped to mask divalent cations in the extract, which could precipitate polyP.

Fig. 1. Amount of extracted polyP with six extraction methods. After extraction of polyP with the methods indicated on the x-axis (without precipitation) from polyP-rich *S. cerevisiae* cells, polyP content (as KPO₃) in cell dry weight was determined. All extractions were done from one batch of cells. Mean values from two extractions done on separate days with one replicate each are shown. Error bars indicate the SEM between the two extractions done on separate days. Abbreviation: Bru w/o ppt, Bru protocol without ethanol precipitation.

The protocol “Bru without ethanol precipitation” was already a modified version of the original Bru *et al.* protocol, which can be seen in Fig. 2A. The modifications were as follows. Instead of $1-2 \times 10^7$ cells, 24 to 26 mg cell wet weight (ca. 5 - 7 mg cell dry weight) were used per extraction, to allow deduction of the biotechnologically valuable parameter “polyP mass in cell dry weight” and remove time consuming microscopic cell counting. Reagents listed for tube two were directly added to tube one, to avoid loss of cells through transferring of the cell suspension. Normal reaction tubes performed well and were, thusly, used instead of screw cap tubes. Inversion of reaction tubes for mixing was deemed redundant and, therefore, omitted. Vortex times were increased from 5 to 20 sec to allow proper formation of phenol-water and phenol-chloroform-water emulsions. The phase lock gel was introduced, to allow better separation of the chloroform-phenol and aqueous phase. At last, the ethanol precipitation step was omitted. Basically, in “Bru without ethanol precipitation”, in comparison to the original Bru *et al.* protocol, tubes 1 and 2 in Fig. 2A were merged and tube 5 omitted.

A) Analytical polyP extraction from Bru *et al.* (2016)

B) Analytical polyP extraction from this study

Fig. 2. Workflow of the original protocol for *S. cerevisiae* analytical polyP extraction from Bru *et al.* [19] (A) in comparison to the optimized *S. cerevisiae* analytical polyP extraction from this study (B). Each frame indicates the steps performed in one of the successively employed reaction tubes.

To quantify the effects of the changes applied to the original Bru *et al.* protocol, the original Bru *et al.* protocol was compared to “Bru without precipitation” (first and second bar in Fig. 3, respectively). For the original protocol, only the used cell mass was changed to 25 mg cell wet weight, instead of the 1-2*10⁷ cells. “Bru without precipitation” showed a 30 % increase in extracted polyP in comparison to the original Bru *et al.* protocol. This increase can be ascribed to the omission of the precipitation and the use of phase lock gel. Notably, only very little blank P_i could be detected in the original Bru *et al.* extracts in comparison to the “Bru without precipitation” extracts. It can be concluded that P_i and probably short chain polyP were removed by ethanol precipitation, which confirmed our initial hypothesis that precipitation of polyP is not quantitative. The use of phase lock gel allowed the quantitative transfer of the aqueous phase after chloroform washing. As the next step in optimization, the chloroform washing and nuclease treatment (tube three and four in Fig. 2A) were omitted from the “Bru without precipitation” protocol (termed “Bru without chloroform washing, nuclease treatment, and ethanol precipitation”; third bar in Fig. 3). This led to an increase of 2.3 % extracted polyP. Bru *et al.* included the nuclease treatment, because they found inhibitory effects of DNA and RNA on polyP quantification. Here and in our recent study on polyP quantification [22], no such effect was observed. The increase in extracted polyP was probably due to volume loss during the chloroform washing step.

Fig. 3. Comparison of different analytical polyP extractions derived from the original Bru *et al.* protocol [19]. The workflow of the original Bru *et al.* protocol (first bar) and the optimized protocol (fourth bar) can be seen in Fig. 2A and Fig. 2B, respectively. The most important changes from the original protocol (first bar) to “Bru without ethanol precipitation” (second bar, abbreviated as “Bru w/o ppt”) were as follows. Phase lock gel was introduced, to separate the phenol-chloroform and aqueous phase during centrifugation. The ethanol precipitation step of the polyP was omitted (*i. e.*, tube five in Fig. 2A). For “Bru without chloroform washing, nuclease treatment, and ethanol precipitation” (third bar, abbreviated as “Bru w/o CHCl₃ washing, nuclease, ppt”) the second chloroform washing step (*i. e.*, tube three in Fig. 1A) and the nuclease treatment (*i. e.*, tube four in Fig. 1A) were omitted. At last, reaction conditions were optimized according to Fig. 4 (termed “optimized protocol”; fourth bar). All extractions displayed were done from one batch of cells. Mean values from two experiments with three replicates each are shown. Error bars indicate the SEM between both experiments.

The next step in optimization involved the single variation of each process parameter during extraction (Fig. 4). The goal was, on the one hand, to increase extraction efficiency, and, on the other hand, get a better understanding of all process parameters and their degree of impact on polyP extraction. The arrows in Fig. 4 indicate the chosen process parameter. The SDS-volume was looked at first, because preliminary experiments showed an inhibitory effect of SDS on polyP quantification (data not shown). The addition of any amount of SDS had no positive effect on polyP extraction (Fig. 4A). Also, SDS destabilized the phase lock gel. Notably, at 160 μ l SDS polyP quantification was fully inhibited. Consequently, SDS was omitted from the protocol. Phenol was varied next, because it seemed to have a major impact as the cell disrupting agent (Fig. 4B). At volumes higher than 300 μ l phenol, the chloroform volume was increased proportionally, to allow proper removal of the phenol from the aqueous phase. Between 0 and 150 μ l phenol a dose-dependent response was measured. At increased volumes, no effect on polyP extraction was measured. The fact that only the heat treatment without phenol and chloroform extracted 25 % of the polyP is of interest for *preparative* polyP extractions in a biotechnological setting, where the use of toxic chemicals must be avoided (compare bar 1 and 5 in Fig. 4B). The use of only chloroform and no phenol extracted 42 % of the polyP, probably due to the denaturation of polyP degrading enzymes. Although 150 μ l phenol and 300 μ l chloroform did suffice for this cell batch, 300 μ l phenol and 300 μ l chloroform were chosen, to accommodate also slightly higher biomass amounts. The incubation time at 65°C had little impact on polyP extraction (Fig. 4C). Again, to accommodate more difficult to open cells including higher cell amounts, 10 min incubation time were chosen over the previously employed 5 min. Heating of the sample after addition of phenol was unnecessary (Fig. 4D). Even without heating (*i. e.*, at room temperature, 23°C) all polyP was extracted (bar 1 in Fig. 4D). Furthermore, a loss of 0.14 % polyP in cell dry weight per 20°C was calculated. To set a definite temperature, rather than room temperature, 45°C was chosen. The pH of the extraction buffer had no impact on polyP extraction, as long as the pH was between 5.3 and 7.5 (Fig. 4E). To attain the different pH values, different buffering agents with different pK_a values were tested (*i. e.*, acetate, 2-(N-morpholino)ethanesulfonic acid (MES), Tris). The buffer MES and a pH of 6.3 were chosen for further studies. PolyP can be considered as a soluble cation exchanger. Should polyP be bound as insoluble magnesium or calcium polyP, sodium ions should competitively replace the divalent cations and allow polyP to dissolve. To test this hypothesis, the addition of NaCl to the extraction buffer was tested (Fig. 4F). However, even at the lowest tested concentration of 5 mM NaCl

an inhibitory effect was observed. Therefore, NaCl was not included in the protocol. This fact should be considered for other studies, where extracts rich in NaCl are analyzed. The EDTA concentration was varied between 1 and 80 mM (Fig. 4G). EDTA binds divalent cations and should help polyP to dissolve. However, magnesium is required for polyP quantification. Indeed, too much EDTA had a negative effect on polyP extraction, probably due to the complexation of magnesium during polyP quantification. However, lower concentrations of EDTA had a positive effect on polyP extraction. Although 1 mM EDTA would have sufficed for this batch of cells, 2.5 mM EDTA was chosen for the protocol, to accommodate for higher divalent cation concentrations. It should also be considered that the phenol is saturated in TE-buffer. With 300 μ l phenol and a water content of about 23 % (v / v) in the phenol, approximately 69 μ l of TE-buffer is supplied additionally per sample. During assay development, to attain storage stability, extracts were titrated to pH 7 to 8 with the help of phenol red. This step could be omitted through exchange of pH 6.3 MES to pH 7.0 MOPS (3-(N-morpholino)propanesulfonic acid) as the buffering agent in the extraction buffer. MOPS was chosen over MES, because of its more neutral pK_a value. Together with this buffering agent exchange, the buffer concentration was studied (Fig. 4H). The buffer concentration had no impact on polyP extraction. Twenty-five mM MOPS was chosen for further experiments. The analytical polyP extraction should be capable of extracting polyP even at cellular concentrations of 20, 30, and 40 % polyP (as KPO_3) in cell dry weight. However, only cell mass with ca. 10 % polyP was available. Therefore, extraction solution volumes (*i. e.*, extraction buffer, phenol and chloroform) were scaled down by factor 0.5, 0.33, and 0.25, to simulate higher cellular polyP concentrations of 10, 20, and 40 % polyP in cell dry weight, respectively (Fig. 4I). The extraction dealt well with this challenge. An increase in extracted polyP was even observed up to a scaling of volumes to the factor 0.33. This effect could probably be explained by the higher dilution of the extracts and, thusly, lower concentration of inhibitory substances during polyP quantification. Even at these unrealistically high cell debris to polyP ratios, the assay performed well and should, therefore, be capable to detect at least 40 % polyP in cell dry weight. At last, the chloroform volume was varied (Fig. 4J), to allow a maximum removal of phenol from the aqueous phase for analytical and work safety purposes. The chloroform volume had no impact on extraction efficiency and was set at 1,000 μ l for further studies.

The workflow of this optimized protocol can be seen in Fig. 2B. Considering only incubation and centrifugation steps, the original Bru *et al.* protocol took 4:36 h (ca. 5.5 h including pipetting), whereas the optimized protocol took 17 min (ca. 30 min including pipetting). Furthermore, the optimized protocol required only one instead of five reaction tubes per sample in comparison to the original Bru *et al.* protocol. In the end, this optimized protocol for analytical polyP extraction was compared to the other versions of the Bru *et al.* extraction (fourth bar in Fig. 3). The optimization process, seen in Fig. 4, resulted in a 4.7 % increase of extracted polyP in comparison with the “Bru without chloroform washing, nuclease treatment, and ethanol precipitation” protocol. Overall, with the optimized protocol, the extracted mass of polyP from the same cells was increased by 40 % in comparison to the original Bru *et al.* protocol (bar one and four in Fig. 3, respectively).

Bar four in Fig. 3 shows the results from the extraction of polyP-rich *S. cerevisiae* cells with the optimized extraction protocol. Mean values from two experiments done on separate days are shown. In each experiment three extractions from the same batch of cells were performed. Concluding, six extractions were compared for this bar ($n = 6$). Results obtained were: 10.6, 11.1, 11.3, 10.7, 10.5, and 11.2 % polyP (m / m) in cell dry weight. Over the six measurements, an average of 10.9 ± 0.1 % polyP (mean $\pm$ SEM) was obtained. Between the two experiments, an average of 10.9 ± 0.0 % (mean $\pm$ SEM) was obtained. This precision should suffice for biological experiments.

Fig. 4. Optimization of analytical polyP extraction. Measured polyP (as KPO_3) in cell dry weight [% (w / w)] is reported on the y-axis. The extraction protocol “Bru without chloroform washing, nuclease treatment, and ethanol precipitation” was the

starting point for this optimization. Starting from this protocol the SDS volume (A), the phenol-chloroform volume (B), the incubation time at 65°C (C), the incubation temperature (D), the pH (E), the addition of NaCl (F), the EDTA concentration (G), the buffer concentration (H), and the chloroform volume (J) were varied one after another. Arrows indicate which parameter was chosen for the next step. In (H) 50 mM MES pH 6.3 is included as the reference. To test if the method could potentially extract polyP at higher cellular concentrations (*i. e.*, 20, 30 and 40 % polyP (as KPO₃) in cell dry weight), extraction volumes were reduced by the factors 0.5, 0.33 and 0.25, respectively (I). Every bar graph represents one experiment with one replicate per bar. Abbreviations: Ac, acetate; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

Discussion

Our goal was to develop a simple, optimized, and validated method for analytical polyP extraction from *S. cerevisiae*. There is no method available to determine the absolute amount of polyP in living cells. Therefore, the analytical polyP extraction, which extracts the most polyP from a given cell mass, must be considered the gold standard. Bru *et al.* compared their analytical polyP extraction with acid-phenol-bead beating cell lysis in LETS buffer (0.1 M LiCl, 10 mM EDTA, 10 mM Tris, 0.5 SDS, pH 8.0) followed by chloroform washing and ethanol precipitation [16], perchloric acid-bead beating extraction [27], and sulfuric acid extraction followed by silica spin column purification [18, 28]. Bru *et al.* reported that their extraction scored, by far, highest in terms of extracted polyP, followed by the other extractions in the mentioned order (see Fig. 5A in Ref. [19]). In comparison to this study, Bru *et al.* strictly adhered to the literature protocols and included precipitations and silica column purification, when prescribed. Bru *et al.* covered all relevant analytical polyP extraction methods from literature. Thusly, the extraction procedure from Bru *et al.* was considered – up to this study – as the gold standard for analytical polyP extraction from *S. cerevisiae*. We can confirm the outstanding performance of the Bru *et al.* protocol. In our study, bead beating protocols, and protocols, which rely on chaotropic agents (*i. e.*, trichloroacetic acid or guanidinium thiocyanate) for cell lysis, also performed worse than the protocol from Bru *et al.* We optimized the original Bru *et al.* protocol and present a protocol, which extracts 40 % more polyP than the original Bru *et al.* protocol. Therefore, we propose our method as the new gold standard for analytical polyP extraction from *S. cerevisiae*. Our method takes about 30 minutes in comparison to the 5.5 h required for the original Bru *et al.* protocol. A few further advantages over the original Bru *et al.* protocol include extracts, which are already at neutral pH and stabilized with EDTA for storage, and work safety, because phenol handling time is reduced and phase lock gel locks away chloroform and phenol at the bottom of the reaction tube. Recently, we developed a method for comprehensive polyP quantification, and short chain polyP length determination [22]. The combination of our analytical polyP extraction from this study and the polyP quantification represents an almost complete workflow for polyP analysis in *S. cerevisiae*. Only the length determination of long chain polyP (above 60 P-subunits) is missing. The new workflow allows comprehensive polyP quantification in *S. cerevisiae* within 2 h. It must be noted that although our polyP quantification method is species-independent, our analytical polyP extraction is tested with and optimized for *S. cerevisiae*. Furthermore, our analytical polyP extraction is specifically designed for use in combination with our polyP quantification. Other down-stream applications, which rely on higher extract purity, might require different extraction protocols. The envisaged applications of the assay presented here lie within the entire field of *S. cerevisiae* polyP research.

Conflicts of interest

The authors declare that they have no conflicts of interest with the content of this article.

Author contribution

JJC conducted the experiments. Data interpretation and writing of the paper was done by LMB and JJC. LMB initiated and coordinated the study. All authors read and approved the final manuscript.

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