Scientific
Research
Publishing

Polyphosphatase PPX1 of *Saccharomyces cerevisiae* as a Tool for Polyphosphate Assay

Lidiya Lichko, Tatiana Kulakovskaya

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Russia
Email: alla@ibpm.pushchino.ru

Abstract

The recombinant exopolyphosphatase PPX1 with a specific activity of ~300 U/mg protein was purified from the strain of *Saccharomyces cerevisiae* with the inactivated *PPN*1 gene transformed by the expression vector carrying the yeast *PPX*1 gene. The recombinant PPX1 was similar to the PPX1 of wild strains in its substrate specificity and requirement for cations. PPX1 had the high substrate specificity to polyphosphates. The preparation was suitable for polyphosphate assay in the presence of orthophosphate and nucleoside phosphates not hydrolyzed by PPX1. The yield of the enzyme preparation was 250 assays per 1 g of the biomass. The recombinant PPX1 was successfully used in polyphosphate assay in different yeast species and some foodstuffs.

Keywords

Saccharomyces cerevisiae, PPX1, Polyphosphate, Polyphosphatase, Assay, Food

1. Introduction

Inorganic polyphosphates (polyP) are the linear polymers containing a few to several hundred orthophosphate residues linked by energy-rich phosphoanhydride bonds. PolyP performs numerous functions in microorganisms: phosphorus and energy storage, cation sequestration, and stress adaptation [1]-[3]. In humans, polyP participates in bone growth and regeneration [4]-[7], in hemostasis, thrombosis and inflammation [8] [9], and in the regulation of calcium level in mitochondria [10]. The multiple effects of polyP on bone growth and regeneration have been demonstrated. PolyP accumulation in osteoblasts is a source of phosphorus for bone apatite formation [6] [7]. The exposure of SaOS-2 osteoblast-like cells to polyP results in their accumulation in mitochondria and simultaneous translocation of the polyP-degrading enzyme alkaline phosphatase to the cell surface [11]. In addition, the treatment of SaOS-2 cells with β-glycerophosphate, ascorbic acid, dexamethasone and polyP leads to a

tenfold intracellular increase in the ATP level [11]. The signaling cascade including polyP, matrix metalloproteinase (MMP)-3, dentin sialophosphoprotein (DSPP), and dentin matrix protein-1 (DMP-1) is involved in the proliferation of odontoblast-like cells [12]. PolyP is believed to be an active scaffold promising for the treatment of bone diseases, 3D cell printing [13], and the development of bioactive hydrogels capable of enhancing bone tissue growth [14]. Platelet polyP plays an essential role in hemostasis, thrombosis and inflammation [15] [16] and is believed to be a therapeutic target during human blood clotting [17]. Polyphosphates are promising candidates in the therapy for bone and blood diseases. They are of interest for the creation of novel bone substitute materials.

PolyP is widely used as a reagent in water treatment, a component of fertilizers, flame retardants and food additives due to its unique properties, inexpensiveness, nontoxicity and biodegradability [18].

Phosphates are normally present in all living cells, being a common component of our diet. The permitted additives (polyphosphates, E452) are widely used to improve the eating quality of many foods [19]. Since polyP is involved in many processes in humans, its consumption has to be controlled. In addition, the survival of some harmful bacteria such as *Campylobacter* in frozen poultry was observed to enhance in the presence of polyP [20]. The methods of polyP detection in foods include chromatography and electrophoresis [21]-[23]. Since foodstuffs (like other biological samples) contain polyP, orthophosphate, pyrophosphate and phosphorous organic compounds, a highly specific and relatively cheap method for polyP analysis in the presence of the above compounds is required.

One of the most specific methods is enzymatic analysis. Specific enzymes hydrolyzing polyP are well known; among them, there is an exopolyphosphatase (polyphosphate phosphohydrolase EC 3.6.1.11) that splits P_i from the end of the polyP chain. The yeast exopolyphosphatase PPX1 has a high specificity to polyP [24]-[26]. Recently, we have obtained a yeast strain with the overproduction of this enzyme [27]. It offers the possibility to develop a specific and cheep assay of polyP in various samples containing pyrophosphate and organic phosphorus compounds.

This study was aimed at developing a polyphosphate assay with the recombinant polyphosphatase PPX1 of *S. cerevisiae*.

2. Materials and Methods

2.1. Strains and Culture Conditions

The strain of *S. cerevisiae* CRN/pMB1_PPX1 Sc with the overexpression of PPX1 was obtained and cultivated as described in detail in our previous publications [27] [28]. The culture was grown to the stationary growth phase for 24 h and used to obtain the preparation of polyphosphatase PPX1.

The yeast strains used for polyphosphate analysis were as follows: *Saccharomyces cerevisiae* strain CRY [25], *Cryptococcus curvatus* VKM Y-2236, *Lindnera fabianii* VKM Y-1450, and *Kuraishia capsulata* VKM Y-2514 (All-Russian Collection of Microorganisms). The CRY strain was maintained on agarized YPD medium; other yeast strains were maintained on malt agar slants at 29°C. The yeasts *S. cerevisiae*, *L. fabianii* and *K. capsulata* were grown in the liquid YPD medium (2% glucose, 1% yeast extract, 2% peptone); *Cr. curvatus* was grown in the medium with 1% glucose, 0.4% yeast extract, and 0.5% peptone. The cultures were grown at 29°C in flasks with 250 ml of the medium on a shaker at 145 rpm to the stationary growth phase for 24 h.

2.2. Purification of Recombinant Polyphosphatase

The enzyme preparation was obtained from ~4 g of wet biomass of CRN/pMB1_PPX1 Sc. The spheroplasts were obtained as described earlier [27]. All further steps of enzyme purification were carried out at 4°C. The spheroplasts were lysed in a glass homogenizer in 30 ml of 25 mM Tris-HCl, pH 7.2, with 0.1 M sorbitol and 0.5 mM phenylmethylsulfonyl fluoride (Sigma, USA) followed by centrifugation at 15,000 g for 60 min. The stages of enzyme purification are shown in **Table 1**. The supernatant (cell-free extract) was supplemented with ammonium sulfate to 50% saturation. After 30-min stirring, the precipitate was removed by centrifugation (9000×g for 30 min). The supernatant (30 ml) was loaded into a column (1.6 × 7 cm) with Butyl-Toyopearl (Toson, Japan) equilibrated with 25 mM Tris-HCl, pH 7.2, containing 50% ammonium sulfate. The column was washed with 20 ml of 25%, 10%, and 0% ammonium sulfate in 25 mM Tris-HCl, pH 7.2. The fractions with polyphosphatase activity (10% and 0% ammonium sulfate) were pooled and subjected to ultrafiltration using the

Table 1. Purification of recombinant polyphosphatase PPX1 from *S. cerevisiae* CRN/pMB1_PPX1 Sc.

Purification stage	Activity	
	Total activity, U	Specific activity, U/mg protein
Cytosol	344	5.0
Ammonium sulfate, 50% saturation	268	8.4
Butyl-Toyopearl	261	10.4
DEAE-Toyopearl 650 M	177	63.0
Heparin-agarose	150	294.0

The enzyme activity was assayed in 50 mM Tris-HCl, pH 7.2, supplemented with 2.5 mM Mg^{2+}, 200 mM NH_4Cl, and 2 mM $polyP_{15}$.

Amicon cell (YM-10 membrane). The preparation (~ 8 ml) was washed with buffer A (25 mM Tris-HCl, pH 7.2, with 5 mM $MgSO_4$, 0.5 mM EDTA, and 0.1% Triton x-100) and loaded into a column (1.6 × 7) with DEAE-Toyopearl 650 M (Toson, Japan) equilibrated with buffer A. The column was washed with 100 ml of buffer A, and polyphosphatase was eluted at a flow rate of 30 ml/h with increasing KCl concentrations (0 - 0.4 M) in buffer A. The gradient volume was 200 ml.

The fractions with polyphosphatase activity eluted at 0.25 M KCl were pooled and loaded into a column (1.6 × 7 cm) with heparin-agarose (Sigma). After washing the column with 50 ml of buffer A, the enzyme was eluted at a flow rate of 15 ml/h with a step gradient, using 0.4, 0.5, 0.6, and 0.7 M KCl solutions in buffer A (10 ml of each). The fractions with polyphosphatase activity eluted at 0.5 and 0.6 M KCl were pooled and subjected to ultrafiltration using the Amicon cell (YM-10 membrane) and buffer A. As a result, we obtained 3 ml of the partially purified polyphosphatase PPX1. The preparation was divided into 100 μl portions and stored at −70˚C.

2.3. Polyphosphatase Activity Assay

Polyphosphatase activity was assayed by Pi release as described previously [27]. The incubation medium (1 ml) contained 50 mM Tris-HCL (pH 7.2), 2.5 mM $MgSO_4$, 200 mM NH_4Cl, and 2 mM polyP (as P_i) with an average chain length of 15 phosphate residues ($polyP_{15}$). The cases when other polyP and cations were used are indicated specially. PolyP (Monsanto, USA) was purified from pyrophosphate and orthophosphate as described previously [24]. The amount of the enzyme forming 1 μmol P_i per minute was taken as a unit of enzyme activity (U). P_i was determined by the method described previously [28].

Endopolyphosphatase activity was estimated by fragmentation of $polyP_{208}$. The medium (1 ml) containing 50 mM Tris-HCl, pH 7.2, 5 mM $polyP_{208}$, 2.5 mM $MgSO_4$, and the enzyme preparation (~30 mU of polyphosphatase activity) was incubated for 30 - 120 min at 30˚C. The reaction was stopped by adding 60% $HClO_4$ up to 0.5 N followed by centrifugation at 14000 ×g for 2 min, and then 6 N NaOH was added to the supernatant up to the neutral pH. The samples (20 μl) were subjected to 20% PAGE in the presence of 7 M urea; the gels were stained with toluidine blue [29].

2.4. Preparation of the Samples for polyP Analysis

The biomass was separated by centrifugation at 5000 g for 30 min, washed twice with distilled water, and ~1 g of wet biomass of each culture was used for extraction. The acid-soluble fraction of phosphorus compounds was obtained by the twofold treatment of biomass samples with 10 ml 0.5 N $HClO_4$ at 0˚C for 15 min under stirring with subsequent centrifugation. The supernatant pH was adjusted to 7.5 with KOH. The samples were treated with Norit A charcoal to remove phosphorus-containing organic compounds [30].

For obtaining the acid-insoluble fraction of phosphorus compounds, the remaining precipitate of biomass after the extraction with 0.5 N $HClO_4$ was suspended in H_2O (~10 ml) and its pH was adjusted to 7.5.

Two samples of frozen fish fillet (toothfish and cod) and one sample of cheese ("Seven towns", Russia) were extracted with cold distilled water (4 ml per 1 g of samples) at 0˚C for 70 min and 20 h, respectively. Then the samples were filtered and the filtrates were used for the assay.

2.5. P_i and Labile Phosphorus Assay

P_i was determined in all extracts by the method described previously [28]. For labile phosphorus assay, the equal volumes of 2 N HCl were added to the samples, and the latter were treated at 100°C for 10 min. Then the acid-insoluble fractions were centrifuged at 5000 g for 10 min, and the supernatants were analyzed. The labile phosphorus content was defined as a difference in the P_i content before and after the hydrolysis [30].

2.6. Enzymatic Assay of Polyphosphates

The content of P_i was measured before and after the incubation of the samples with PPX1. The assay buffer for the measurement of polyP with PPX1 contained 50 mM Tris-HCl (pH 7.2), 2.5 mM $MgSO_4$, 200 mM NH_4Cl, 100 - 300 μl of the analyzed sample, and 20 mU of the purified PPX1 at a final volume of 200 - 400 μl. The incubations were carried out for 30 min at 30°C and stopped by adding P_i-assay reagent as in 2.3.

3. Results and Discussion

3.1. The Yield, Specific Activity, and Preparation Stability of PPX1

The employment of the PPX1 overproducer resulted in the enhanced yield of PPX1 compared to the wild strain. We obtained 38 U per 1 g wet biomass through four stages of purification (Table 1). The similar specific activity (283 U/mg protein) of the wild strain could be obtained after 5 stages of purification, with the yield of 0.1 U per 1 g wet biomass [31]. The SDS-PAGE of the PPX1 preparation (not shown) demonstrated one major protein band of 45 kD corresponding to the molecular mass of wild type enzyme [25] [26].

The PPX1 enzyme had a specific exopolyphosphatase activity of 294 U/mg protein with $polyP_{15}$ (Table 1). The specific activities of the known preparations of purified PPX1 determined under the same conditions were 204 [24], 283 [31], and 202 U/mg protein [32]. Therefore, the preparation that we have obtained is one of the most active preparations described earlier.

The amount of PPX1 preparation was sufficient to analyze ~1000 samples by the method of enzymatic polyP detection. The purified preparation retained about 100 % of its initial activity after 5-month storage in 25 mM Tris-HCl, pH 7.2, with 5 mM $MgSO_4$, 0.5 mM EDTA, and 0.1% Triton x-100 at −70°C (not shown).

3.2. Properties of Recombinant PPX1

Some properties of the recombinant PPX1 have been studied to be compared with those of the known preparations from wild yeast strains. The notable property of the enzyme preparation intended for specific polyP assay is its substrate specificity. The preparation had a high specificity to polyP and was almost inactive with AMP, ADP, ATP, PCR-mixture dNTP at 1 mM concentration, as well as 2 mM pyrophosphate. The enzyme was more active with tripolyphosphate and short-chain polyP than with long-chain polyP (Table 2).

The substrate specificity of the recombinant polyphosphatase PPX1 was very similar to that of the purified cytosol polyphosphatase obtained from the wild yeast strain [31].

The yeast polyphosphatase PPN1 is inhibited by ATP and stimulated by ADP [33]. The effects of AMP, ADP and ATP on the activity of the recombinant PPX1 with $polyP_{15}$ as a substrate were insignificant (Table 3).

Table 2. Substrate specificity of recombinant PPX1.

Substrate	Activity	
	Specific activity, U/mg protein (with 2 mM of substrate, (for 15 min)	Amount of substrate hydrolyzed, % (with 1 mM of substrate for 120 min
PP_i	0	0
$PolyP_3$	278 ± 3	100%
$PolyP_{15}$	241 ± 2	87%
$PolyP_{208}$	207 ± 4	57%

The enzyme activity was assayed in 50 mM Tris-HCl, pH 7.2, supplemented with 2.5 mM Mg^{2+} and 200 mM NH_4Cl.

Table 3. Effects of nucleoside phosphates on the exopolyphosphatase activity of purified PPX1.

Effectors concentration, mM	Activity, U/mg protein
Control, without additions	240 ± 1
AMP, 1.0	233 ± 2
AMP, 0.5	216 ± 1
AMP, 0.1	230 ± 4
ADP, 1.0	206 ± 4
ADP, 0.5	280 ± 5
ADP, 0.1	240 ± 1
ATP, 1.0	233 ± 2
ATP, 0.5	254 ± 5
ATP, 0.1	240 ± 1

The enzyme activity was assayed in 50 mM Tris-HCl, pH 7.2, supplemented with 2.5 mM Mg^{2+}, 200 mM NH_4Cl, and 2 mM $polyP_{15}$.

We have used PAGE to assess the changes in the chain length of $polyP_{208}$ under the treatment with the PPX1 preparation (**Figure 1**). As one can easily see, the shorter-chain polyP disappeared first, and there was no indication of substrate depolymerization. The most high-molecular weight polyP was not hydrolyzed even after 120-min incubation. Thus, the recombinant PPX1 had no endopolyphosphatase activity, in contrast to PPN1 [33].

We have examined the effects of magnesium, cobalt and ammonium ions in the concentrations used earlier for the PPX1 enzyme obtained from the wild strain [31] on the activity of recombinant PPX1 (**Table 4**). The PPX1 enzyme was inactive in the absence of divalent cations. Magnesium or cobalt ions were necessary for the enzyme activity, and ammonium ions had an additional stimulating effect (**Table 4**). The stimulating effects of Mg^{2+}, Co^{2+} and NH_4^+ were observed for the PPX1 from the wild yeast strain [31].

The addition of 1 mM EDTA to the incubation medium stimulated the enzyme activity in the presence of 2.5 mM Mg^{2+} similar to the wild type of enzyme [31]. This effect of EDTA is explained by blocking of inhibitory influence of impurities of heavy metal cations [31].

Thus, the recombinant PPX1 preparation was similar in its physicochemical properties to the purified PPX1 preparations from the wild yeast strains [24] [31].

3.3. Polyphosphate Assay with PPX1

One of the simplest and cheapest ways of assessing polyP content in microbial cells is the determination of labile phosphorus [30]. However, the data of **Table 5** demonstrate that not all labile phosphorus is represented by enzymatic determined polyP. The relationship between the content of labile phosphorus and polyP depends both on the yeast species and on the analyzed fraction. In *S. cerevisiae*, ~50% and 100% of labile phosphorus of acid-soluble and acid-insoluble fraction, respectively, was identified as polyP by the enzymatic method (**Table 5**). It is known that the acid-insoluble fraction of *S. cerevisiae* has polyP with the longest chains [30]. Nevertheless, they were completely hydrolyzed by PPX1.

Nucleoside phosphates were no more than 10% of labile phosphorus of the acid-soluble fraction, and the amount of pyrophosphate in *S. cerevisiae* was insignificant compared to polyP [34]. Therefore, the nature of labile phosphorus, which was not hydrolyzed by PPX1 in the acid-soluble fraction, is still an open question. It is not improbable that *S. cerevisiae* contains polyP not hydrolyzed by PPX1 due to some unknown structural peculiarities. There are no data on the content of nucleoside phosphates, pyrophosphate and organic phosphate compounds in other yeast species under study. The enzymatic method makes it possible to determine the polyP content in these species.

The polyP/pyrophosphate mixture has been used commercially in fish fillet [35] and cheese [36] production. The enzymatic analysis shows that polyP makes no less than 10% of the phosphorus content in food samples (**Table 6**). The remainder of the labile phosphorus most likely represents the pyrophosphate.
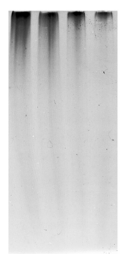
0 60 90 120

Figure 1. The chain length of polyP208 under treatment with the PPX1 preparation, 20% PAGE in the presence of 0.7 M urea. The numbers indicate the time of incubation of $polyP_{208}$ with PPX1.

Table 4. Effects of cations on the exopolyphosphatase activity of purified PPX1.

Additions	Specific activity, U/ mg protein
No additions	0
2.5 mM Mg^{2+}	200 ± 5
2.5 mM Mg^{2+}, 200 mM NH_4^+	250 ± 10
2.5 mM Mg^{2+}, 200 mM NH_4^+, 1 mM EDTA	398 ± 2
0.1 mM Co^{2+}	346 ± 3
0.1 mM Co^{2+}, 200 mM NH_4^+	448 ± 5

The enzyme activity was assayed in 50 mM Tris-HCl, pH 7.2, supplemented with 2 mM $polyP_{15}$.

Table 5. P_i, labile phosphorus and enzymatic detected polyP in yeast species.

Yeast species	Phosphorus compounds, μmol/g wet biomass				
	P_i	Acid-soluble fraction, labile phosphorus	Acid-soluble fraction, enzymatic detected polyP	Acid-insoluble fraction, labile phosphorus	Acid-insoluble fraction, enzymatic detected polyP
S. cerevisiae	12.6 ± 0.7	15.2 ± 0.3	7.3 ± 0.5	36.0 ± 0.7	36.0 ± 0.5
L. fabianii	6.0 ± 1.0	4.3 ± 0.2	2.5 ± 0.5	16.0 ± 2.6	15.1 ± 3.0
Cr. curvatus	12.0 ± 0.5	3.3 ± 0.1	2.4 ± 0.2	11.0 ± 2.0	4.6 ± 0.6
K. capsulata	8.0 ± 1.7	6.8 ± 0.3	6.0 ± 0.6	11.8 ± 3.9	3.5 ± 0.2

Table 6. P_i, labile phosphorus and enzymatic detected polyP in food samples.

Samples	Phosphorus compounds, μmol/g wet mass		
	P_i	P labile	PolyP, enzymatic assay
Frozen fish fillet, toothfish	13.0 ± 0.6	2.5 ± 0.6	1.8 ± 0.3
Frozen fish fillet, cod	9.4 ± 0.9	2.0 ± 0.1	0.97 ± 0.03
Cheese	12.9 ± 2.9	6.5 ± 0.6	1.58 ± 0.02

4. Conclusion

The yeast strain overproducing PPX1 allowed us to develop a simple method for preparing this enzyme. The yield of the enzyme preparation was 250 assays per 1 g of the biomass. The PPX1 preparation has high substrate specificity and is suitable for polyP assay in the presence of orthophosphate, pyrophosphate and nucleoside phosphates. The method of enzymatic polyP detection is applied to polyP measurement in biomass extracts of some yeast species and foodstuffs. The data on polyP content in different yeast species have been revised.

Acknowledgements

References

[1] Kornberg, A., Rao, N.N. and Ault-Riché, D. (1999) Inorganic Polyphosphate: A Molecule with Many Functions. *Annual Review of Biochemistry*, **68**, 89-125. http://dx.doi.org/10.1146/annurev.biochem.68.1.89

[2] Kulaev, I.S., Vagabov, V.M. and Kulakovskaya, T.V. (2004) The Biochemistry of Inorganic Polyphosphates. John Wiley and Sons Ltd., Chichester. http://dx.doi.org/10.1002/0470858192

[3] Rao, N.N., Gómez-García, M.R. and Kornberg, A. (2009) Inorganic Polyphosphate: Essential for Growth and Survival. *Annual Review of Biochemistry*, **78**, 605-647. http://dx.doi.org/10.1146/annurev.biochem.77.083007.093039

[4] Leyhausen, G., Lorenz, B., Zhu, H., Geurtsen, W., Bohnensack, R., Müller, W.E.G. and Schröder, H.C. (1998) Inorganic Poly-Phosphate in Human Osteoblast-Like Cells. *Journal of Bone and Mineral Metabolism*, **13**, 803-812. http://dx.doi.org/10.1359/jbmr.1998.13.5.803

[5] Morimoto, D., Tomita, T., Kuroda, S., Higuchi, C., Rato, S., Shiba, T., Nakagami, H., Morishita, R. and Yoshikawa, H. (2010) In-Organic Polyphosphate Differentiates Human Mesenchymal Stem Cells into Osteoblastic Cells. *Journal of Bone and Mineral Metabolism*, **28**, 418-423. http://dx.doi.org/10.1007/s00774-010-0157-4

[6] Müller, W.E., Wang, X., Diehl-Seifert, B., Kropf, K., Schloßmacher, U., Lieberwirth, I., Glasser, G., Wiens, M. and Schröder, H.C. (2011) Inorganic Polymeric Phosphate/Polyphosphate as an Inducer of Alkaline Phosphatase and a Modulator of Intracellular Ca(2+) Level in Osteoblasts (SaOS-2 Cells) *in Vitro*. *Acta Biomaterialia*, **7**, 2661-2671. http://dx.doi.org/10.1016/j.actbio.2011.03.007

[7] Omelon, S., Georgiou, J., Henneman, Z.J., Wise, L.M., Sukhu, B., Hant, T., Wynnyckyj, S., Holmyard, D., Bielecki, R. and Grynpas, M.D. (2009) Control of Vertebrate Skeletal Mineralization by Polyphosphates. *PLoS ONE*, **4**, e5634 http://dx.doi.org/10.1371/journal.pone.0005634

[8] Smith, S.A. and Morrissey, J.H. (2008) Polyphosphate as a General Procoagulant Agent. *Journal of Thrombosis and Haemostasis*, **6**, 1750-1756. http://dx.doi.org/10.1111/j.1538-7836.2008.03104.x

[9] Travers, R.J., Smith, S.A. and Morrissey, J.H. (2015) Polyphosphate, Platelets, and Coagulation. *International Journal of Laboratory Hematology*, **37**, 31-35. http://dx.doi.org/10.1111/ijlh.12349

[10] Abramov, A.Y., Fraley, C., Diao, C.T., Winkfein, R., Colicos, M.A., Duchen, M.R., French, R.J. and Pavlov, E. (2007) Targeted Polyphosphatase Expression Alters Mitochondrial Metabolism and Inhibits Calcium-Dependent Cell Death. *Proceedings of the National Academy of Sciences of the United States of America*, **13**, 18091-18096. http://dx.doi.org/10.1073/pnas.0708959104

[11] Müller, W.E., Tolba, E., Feng, Q., Schröder, H.C., Markl, J.S., Kokkinopoulou, M. and Wang, X. (2015) Amorphous Ca^{2+} Poly-Phosphate Nanoparticles Regulate the ATP Level in Bone-Like SaOS-2 Cells. *Journal of Cell Science*, **128**, 2202-2207. http://dx.doi.org/10.1242/jcs.170605

[12] Ozeki, N., Hase, N., Yamaguchi, H., Hiyama, T., Kawai, R., Kondo, A., Nakata, K. and Mogi, M. (2015) Polyphosphate Induces Matrix Metalloproteinase-3-Mediated Proliferation of Odontoblast-Like Cells Derived from Induced Pluripotent Stem Cells. *Experimental Cell Research*, **333**, 303-315. http://dx.doi.org/10.1016/j.yexcr.2015.01.007

[13] Müller, W.E., Tolba, E., Schröder, H.C. and Wang, X. (2015) Polyphosphate: A Morphogenetically Active Implant Material Serving as Metabolic Fuel for Bone Regeneration. *Macromolecular Bioscience*, **15**, 1182-1197. http://dx.doi.org/10.1002/mabi.201500100

[14] Wu, A.T.H., Aoki, T., Sakoda, M., Ohta, S., Ichimura, S., Ito, T., Ushida, T. and Furukawa, K.S. (2015) Enhancing Osteogenic Differentiation of MC3T3-E1 Cells by Immobilizing Inorganic Polyphosphate onto Hyaluronic Acid Hydrogel. *Biomacromolecules*, **16**, 166-173. http://dx.doi.org/10.1021/bm501356c

[15] Müller, F. and Renné, T. (2011) Platelet Polyphosphates: The Nexus of Primary and Secondary Hemostasis. *Scandinavian Journal of Clinical and Laboratory Investigation*, **71**, 82-86.

[16] Travers, R.J., Smith, S.A. and Morrissey, J.H. (2015) Polyphosphate, Platelets, and Coagulation. *International Journal of Laboratory Hematology*, **37**, 31-35. http://dx.doi.org/10.1111/ijlh.12349

[17] Zhu, S., Travers, R.J., Morrissey, J.H. and Diamond, S.L. (2015) FXIa and Platelet Polyphosphate as Therapeutic Targets during Human Blood Clotting on Collagen/Tissue Factor Surfaces under Flow. *Blood*, **126**, 1494-1502. http://dx.doi.org/10.1182/blood-2015-04-641472

[18] Kulakovskaya, T.V., Vagabov, V.M. and Kulaev, I.S. (2012) Inorganic Polyphosphate in Industry, Agriculture and Medicine: Modern State and Outlook. *Process Biochemistry*, **47**, 1-10. http://dx.doi.org/10.1016/j.procbio.2011.10.028

[19] Smith, J. and Hong-Shum, L., Eds. (2003) Food Additives Data Book. Wiley-Blackwell, Oxford. http://dx.doi.org/10.1002/9780470995327

[20] Gunther IV, N.W., Rajkowski, K.T. and Sommers, C. (2015) Survival after Cryogenic Freezing of *Campylobacter* Species in Ground Turkey Patties Treated with Polyphosphates. *Journal of Food Protection*, **78**, 419-423. http://dx.doi.org/10.4315/0362-028X.JFP-14-301

[21] Sekiguchi, Y., Matsunaga, A., Yamamoto, A. and Inoue, Y. (2000) Analysis of Condensed Phosphates in Food Products by Ion Chromatography with an Online Hydroxide Eluent Generator. *Journal of Chromatography*, **881**, 639-644. http://dx.doi.org/10.1016/S0021-9673(99)01278-9

[22] Kaufmann, M., Maden, K., Leisser, W., Matera, M. and Gude, T. (2005) Analysis of Polyphosphates in Fish and Shrimps Tissues by Two Different Chromatography Methods: Implication of False-Negative and -Positive Findings. *Food Additives & Contaminants*, **22**, 1073-1082. http://dx.doi.org/10.1080/02652030500239565

[23] Wang, L., Li, J. and Zhang, L. (2015) Determination of Polyphosphates in Fish and Shrimp Muscles by Capillary Electrophoresis with Indirect UV Detection after Phosphatase Inhibition Using High Pressure Pretreatment. *Food Chemistry*, **185**, 349-354. http://dx.doi.org/10.1016/j.foodchem.2015.04.008

[24] Andreeva, N.A. and Okorokov, L.A. (1993) Purification and Characterization of Highly Active and Stable Polyphosphatase from *Saccharomyces cerevisiae* Cell Envelope. *Yeast*, **9**, 127-139. http://dx.doi.org/10.1002/yea.320090204

[25] Wurst, H., Shiba, T. and Kornberg, A. (1995) The Gene for a Major Exopolyphosphatase of *Saccharomyces cerevisiae*. *Journal of Bacteriology*, **177**, 898-906.

[26] Andreeva, N.A., Kulakovskaya, T.V., Karpov, A.V., Sidorov, I.A. and Kulaev, I.S. (1998) Purification and Properties of Polyphosphatase from *Saccharomyces cerevisiae* Cytosol. *Yeast*, **14**, 383-390. http://dx.doi.org/10.1002/(SICI)1097-0061(19980315)14:4<383::AID-YEA232>3.3.CO;2-J

[27] Lichko, L.P., Eldarov, M.A., Dumina, M.V. and Kulakovskaya, T.V. (2014) PPX1 Gene Overexpression Has No Influence on Polyphosphates in *Saccharomyces cerevisiae*. *Biochemistry*, **79**, 1211-1215. http://dx.doi.org/10.1134/S000629791411008X

[28] Kulakovskaya, T.V., Andreeva, N.A., Karpov, A.V., Sidorov, I.A. and Kulaev, I.S. (1999) Hydrolysis of Tripolyphosphate by Purified Exopolyphosphatase of *Saccharomyces cerevisiae* Cytosol: Kinetic Model. *Biochemistry* (*Moscow*), **64**, 990-993.

[29] Kumble, K.D. and Kornberg, A. (1995) Inorganic Polyphosphate in Mammalian Cells and Tissues. *Journal of Biological Chemistry*, **270**, 5818-5822. http://dx.doi.org/10.1074/jbc.270.11.5818

[30] Vagabov, V.M., Trilisenko, L.V. and Kulaev, I.S. (2000) Dependence of Inorganic Polyphosphate Chain Length on the Orthophosphate Content in the Culture Medium of the Yeast *Saccharomyces cerevisiae*. *Biochemistry* (*Moscow*), **65**, 349-355.

[31] Andreeva, N.A., Kulakovskaya, T.V. and Kulaev, I.S. (1996) Purification and Characterization of Polyphosphatase from *Saccharomyces cerevisiae* Cytosol. *Biochemistry* (*Moscow*), **61**, 1213-1220.

[32] Lorenz, B., Müller, W.G.E., Kulaev, I.S. and Schröder, H.C.J. (1994) Purification and Characterization of an Exopolyphosphatase from *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, **269**, 22198-22204.

[33] Andreeva, N., Trilisenko, L., Eldarov, M. and Kulakovskaya, T. (2015) Polyphosphatase PPN1 of *Saccharomyces cerevisiae*: Switching of Exopolyphosphatase and Endopolyphosphatase Activities. *PLoS ONE*, **10**, e0119594. http://dx.doi.org/10.1371/journal.pone.0119594

[34] Kulaev, I.S. (1979) The Biochemistry of Inorganic Polyphosphates. Wiley, New York.

[35] Wang, L., Li, J. and Zhang, L. (2015) Determination of Polyphosphates in Fish and Shrimp Muscles by Capillary Electro-Phoresis with Indirect UV Detection after Phosphatase Inhibition Using High Pressure Pretreatment. *Food Chemistry*, **185**, 349-354. http://dx.doi.org/10.1016/j.foodchem.2015.04.008

[36] Nagyová, G., Buňka, F., Salek, R.N., Černíková, M., Mančík, P., Grůber, T. and Kuchař, D. (2014) Use of Sodium Polyphosphates with Different Linear Lengths in the Production of Spreadable Processed Cheese. *Journal of Dairy Science*, **97**, 111-122. http://dx.doi.org/10.3168/jds.2013-7210