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(54) Title: A THERMOSTABLE HALOARCHAEAL INORGANIC PYROPHOSPHATASE

(57) Abstract: The invention pertains to a PPA from a microorganism belonging to the family *Halobacteriaceae* (HPPA), for example, a PPA from *Haloferax volcanii*. The HPPA provided by the invention is soluble, thermostable and active at high concentrations of salt and/or organic solvent. An embodiment of the invention provides a method of increasing the rate of a reaction by adding an HPPA to the reaction mixture, wherein the reaction produces PPi, for example, an enzymatic reaction, and wherein the reaction is carried out at moderately high temperature and/or low water activity. Further embodiments of the invention provide an assay to detect the PPi released during a reaction which produces PPi by adding an HPPA to convert the PPi in to Pi and measuring the resultant Pi. The invention further pertains to an assay to monitor a reaction which produces PPi in the presence or the absence of an HPPA.



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DESCRIPTION

A THERMOSTABLE HALOARCHAEAL INORGANIC PYROPHOSPHATASE

CROSS-REFERENCE TO A RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application Serial No. 62/243,260, filed October 19, 2015, the disclosure of which is hereby incorporated by reference in its entirety, including all figures, tables and amino acid or nucleic acid sequences.

This invention was made with government support under 2 DE-FG02-05ER15650 awarded by U.S. Department of Energy, Office of Basic Energy Sciences, Division of Chemical Sciences, Geosciences and Biosciences, Physical Biosciences Program and under 2 R01 GM057498-14 awarded by National Institute of General Medical Sciences. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Inorganic pyrophosphatase (PPA) (EC 3.6.1.1) catalyzes the hydrolysis of the phosphoanhydride bond of inorganic pyrophosphate (PPi; $P_2O_7^{4-}$) to form two orthophosphates (Pi; PO_4^{3-}). PPi is a common by-product of biochemical reactions, for example, the biosynthesis of DNA, RNA, protein, peptidoglycan, lipids, cellulose, starch and other biopolymers as well as post-translational modification of proteins, for example, adenylation, uridylation and ubiquitylation of proteins.

The hydrolysis of PPi by PPA releases considerable amount of energy ($\Delta G'^{\circ} = -19.2$ kJ/mol) that can drive unfavorable biochemical transformations to completion. For example, during DNA polymerization, the 3'-hydroxyl group of the nucleotide that resides at the 3' end of the growing DNA strand serves as a nucleophile in the attack of the α phosphorus of the incoming deoxynucleoside 5'-triphosphate (dNTP), thus, releasing PPi. The reaction is endergonic ($\Delta G'^{\circ} = +2.1$ kJ/mol), and, under standard conditions, DNA polymerase alone would drive the conversion of DNA to dNTPs. Thus, the polymerization of DNA is highly dependent on PPA to hydrolyze the energy rich PPi to orthophosphate and drive the polymerization reaction.

Due to their ability to drive such reactions towards the production of PPi, PPAs are used in a wide variety of biotechnological applications. For example, PPAs prevent the

accumulation of PPi during DNA sequencing and PCR reactions. PPAs are also used to remove contaminant PPi prior to single-base-extension to increase the yield of RNA synthesis by *in vitro* transcription and to enable the enzymatic synthesis of guanosine 5'-diphosphate (GDP)-sugars and derivatives. PPAs are also used to measure the kinetics of the reactions that release PPi as a by-product, such as SNP genotyping, RNA synthesis by viral RNA-dependent RNA polymerases and aminoacyl-tRNA synthetase activity. Typically, in these assays, PPA hydrolyzes PPi to 2Pi, which are readily detected by a colorimetric assay.

PPAs of the Class A type (IPR008162 family) are soluble, distributed in diverse organisms and include the thermostable PPAs of Archaea used to enhance the polymerization of DNA (*i.e.*, PPAs of *Thermoplasma acidophilum*, *Pyrococcus horikoshii*, *Sulfolobus* sp., *Methanothermobacter thermautotrophicus* (*Methanobacterium thermoautotrophicum*), and *Thermococcus thioreducens*).

Currently used PPAs are inactivated in dose dependent manner by organic solvents, such as ethanol. Solvent tolerant PPAs allow novel synthetic chemistry, alter substrate specificity, ease product recovery, and reduce microbial contamination. Thus, there is a need to identify and produce solvent tolerant PPAs.

BRIEF SUMMARY OF THE INVENTION

The invention provides a PPA from a microorganism belonging to the halophilic archaea (haloarchaea), family *Halobacteriaceae*, hereinafter referred to as an HPPA. An embodiment of the invention provides an HPPA from *Haloferax volcanii*, hereinafter referred to as HvPPA. An embodiment of the invention provides a DNA construct comprising a nucleic acid encoding an HPPA, for example, HvPPA. Another embodiment of the invention provides a recombinant cell containing a nucleic acid encoding an HPPA. A further embodiment of the invention provides a method of expressing and purifying an HPPA.

Since the HPPA provided according to the invention is thermostable and active at moderately high temperature and/or low water activity, an embodiment of the invention provides a method of increasing the rate of a reaction which produces PPi, wherein the reaction is carried out at high temperature and/or low water activity, and wherein the method comprises adding an HPPA to the reaction mixture. Further embodiments of the invention provide an assay to detect PPi released during a reaction which produces PPi by adding an HPPA to the reaction mixture and measuring Pi produced from PPi. Certain embodiments of the invention also provide an assay to monitor the reaction which produces PPi. Even further

embodiment of the invention provides an assay to monitor the reaction which produces PPi in the presence or the absence of an HPPA to determine the effect of HPPA on the reaction.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication, with color drawing(s), will be provided by the Office upon request and payment of the necessary fee.

Figure 1. Evolutionary relationships of archaeal PPA of the IPR008162 family. A phylogenetic tree of amino acid sequences was used to represent the evolutionary relationships of archaeal PPAs. Archaeal PPAs biochemically characterized are highlighted (●) including *Thermoplasma acidophilum* TaPPA (Ta0399), *Pyrococcus horikoshii* PhPPA (PH1907), *Sulfolobus* sp. StPPA (STK_05240) and SaPPA (Saci_0955), *Methanobacterium thermoautotrophicum* MtPPA (MTH_263), and *Thermococcus thio-reducens* TtPPA (H0USY5_9EURY) and *Hfx. volcanii* HvPPA (HVO_0729). The optimal tree with the sum of branch length of 15.65877199 is represented. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. See methods for details.

Figures 2A-2C. Structural comparison of Class A type PPA. A) Multiple amino acid sequence alignment of PPAs. Highlighted are identical (black), functionally similar (grey), Cys-X₆₃-Cys motif (blue) and conserved active site (red) residues. Predicted α -helix and β -sheet structures and Asp residues (*) coordinating Mg²⁺/Mn²⁺ are indicated above the alignment. Abbreviations are as in Fig. 1. B) 3D-structural comparison of PPAs. 3D-structural model of HvPPA (blue ribbon) compared to the X-ray crystal structures of PhPPA (PDB:1UDE) (tan ribbon), SaPPA (PDB:1QEZ), TtPPA (PDB:3R5U) and *Saccharomyces cerevisiae* ScPPA (PDB:1E9G). Mn²⁺ ions (purple ball), phosphate ions (orange and red stick), and water (red ball) ligands are overlaid onto the 3D model. C-terminal (Ct) and N-terminal (Nt) residues are indicated. HvC24 and HvC85 are cysteine residues conserved in all haloarchaeal PPAs. Conserved active site residues analogous to ScPPA include: HvK31 (ScK56), HvE33 (ScE58), HVR45 (ScR78), HvY57 (ScY93), HvD67 (ScD115), HvD69 (ScD117), HvD72 (ScD120), HvD99 (ScD147), HvD104 (ScD152), HvK106 (ScK154), HvY141 (ScY192) and HvK142 (ScK193). HvD67, HvD72 and HvD104 are predicted to coordinate the Mg²⁺ and Mn²⁺ ions. HvPPA 3D-structure was modeled by Phyre2 intensive-mode at a confidence of >90% accuracy for 175 out of 177 residues (99%). C) Comparison

of electrostatic potential of PPAs. Electrostatic potential as represented by Coulombic Surface Coloring with the unit of the potential colored in a range of values -10 (red), 0 (white), and 10 (blue) kcal/mol*e using Chimera v 1.7.

Figures 3A-3B. Class A type inorganic pyrophosphatase purified from *Haloferax volcanii* by tandem affinity and size exclusion chromatography. A) HvPPA fractions analyzed by SDS-PAGE. *Hfx. volcanii* H26 (lane 1) and H26-pJAM2920 expressing His₆-HvPPA (lane 2) applied at OD₆₀₀ of 0.065 cells per lane. Ni²⁺-Sepharose (lane 3) and Superdex 200 GL10/300 (lane 4) chromatography fractions of HvPPA applied at 1 µg protein per lane. Protein was separated by reducing 10% SDS-PAGE and analyzed by Coomassie Blue R-250 staining (upper) and anti-His6 immunoblotting (lower). B) HvPPA analyzed by Superdex 200 30/100 GL size exclusion chromatography. Column fractions are represented by a semi-log plot of molecular weight (Mr in kDa) verses Kav with molecular mass standards (●) and HvPPA hexamer (□) and trimer (○) indicated.

Figures 4A-4D. Effect of pH, temperature, salt and divalent cations on *Haloferax volcanii* inorganic pyrophosphatase (HvPPA) activity. A-C) HvPPA was equilibrated for 10 min at the pH, temperature, and NaCl concentrations indicated prior to the addition of PPI substrate (100 µM). MgCl₂ (10 mM) was included in the activity assays. For pH optimum, assays were supplemented with 3 M NaCl and included 20 mM buffers of sodium acetate at pH 4-5, MES at pH 6-6.5, Tris-Cl at pH 7-9 and CAPS at pH 10. For temperature optimum assays, reactions were in 20 mM Tris-Cl pH 8 supplemented with 3 M NaCl. For salt optimum, HvPPA was diluted from 20 mM Tris-Cl pH 8 containing 3 M NaCl into the same buffer with NaCl concentrations as indicated. D) To test the influence of cations, HvPPA was dialyzed sequentially against 500 ml of buffer (20 mM Tris-Cl pH 8, 2 M NaCl and 1 µM EDTA) (4 h at 4°C) and the same buffer with EDTA omitted (4 h at 4°C). Reactions for panel D contained HvPPA (0.93 µg), 20 mM Tris-Cl pH 8, 2 M NaCl and divalent metal at the concentration indicated. Metals used were: CaCl₂·2H₂O, ZnCl₂, CoCl₂·6H₂O, MnCl₂·4H₂O, NiCl₂·6H₂O and MgCl₂·6H₂O. Reactions were monitored for 10 min at RT unless otherwise indicated.

Figures 5A-5B. Sodium fluoride-based inhibition of *Haloferax volcanii* inorganic pyrophosphatase (HvPPA). A) HvPPA (1.3 µg) was assayed in 1 ml reaction volume containing 1 mM PPI, 10 mM MgCl₂, 3 M NaCl and 20 mM Tris-Cl buffer pH 7.5 supplemented with NaF as indicated. Reactions were monitored for 15 min at RT. B) Amino acid residues of HvPPA predicted to interact with F⁻, PPI, H₂O and Mg²⁺ bound molecules as

determined by modeling compared to the x-ray crystal structure of *E. coli* PPA (PDB: 2AUU).

Figures 6A-6D. Effect of salt, solvent and temperature on *Haloferax volcanii* inorganic pyrophosphatase (HvPPA). A) HvPPA thermostability. HvPPA was incubated at 42°C and 65°C as indicated with enzyme at 0.093 mg/ml of buffer (20 mM Tris-Cl pH 8, 2.5 mM MgCl₂ and 3 M NaCl). HvPPA was diluted to 0.28 µg per 100 µl buffer (with MgCl₂ increased to 10 mM) and assayed by addition of 1 mM PPi substrate (10 min, RT). Activity is % relative to samples incubated on ice. B) HvPPA stability in 50% (v/v) solvent. HvPPA was incubated for 2 h (on ice) at 0.47 mg/ml buffer (20 mM Tris-Cl pH 8 and 2 M NaCl) supplemented with solvent as indicated. HvPPA was diluted to 2.4 µg per 100 µl buffer (20 mM Tris-Cl pH 8, 2.5 mM MgCl₂ and 2 M NaCl) and assayed by addition of 50 µM PPi substrate (10 min, RT). Activity is % relative to samples incubated with no solvent. C) HvPPA activity in 25% (v/v) solvent. Reactions were 500 µl with 2.8 µg HvPPA, 1.5 M NaCl, 1.5 mM PPi, 10 mM MgCl₂. Reaction occurred at 10 minutes at RT. D) HvPPA stability in salt. HvPPA was incubated for 2 h (on ice) at 0.3 mg per ml of buffer (20 mM Tris-Cl pH 8) supplemented with NaCl at the concentrations indicated. HvPPA was diluted to 0.87 µg per 100 µl reaction buffer as in Figure 6B. Activity is % relative to samples incubated in buffer supplemented with 3M NaCl.

Figures 7A-7B. on *Haloferax volcanii* inorganic pyrophosphatase (HvPPA) activity solvent and coupled adenylation assay at high temperature and low water activity. A) Schematic of the coupled assay. Adenylation of the ubiquitin-like SAMP by the E1-like enzyme (UbaA) was monitored by HvPPA mediated hydrolysis of the PPi (P₂O₇⁴⁻) by-product to 2 Pi (2 moles HPO₄²⁻) at 50°C. B) Generation of Pi correlated with the addition of ATP, UbaA, HvPPA and SAMP1 to the assay buffer. ΔGG, C-terminal diglycine residue deletion.

BRIEF DESCRIPTION OF SEQUENCES

SEQ ID NO: 1: Nucleotide sequence encoding HvPPA.

SEQ ID NO: 2: Amino acid sequence of HvPPA.

SEQ ID NOs: 3 to 108: Amino acid sequences of various HPAA.

SEQ ID NO: 109: Amino acid sequence of FLAG-tag.

SEQ ID NO: 110: PPA NdeI forward.

SEQ ID NO: 111: PPA BlpI reverse.

DETAILED DISCLOSURE OF THE INVENTION

As used herein, the singular forms “a”, “an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms “including”, “includes”, “having”, “has”, “with”, or variants thereof are used in either the detailed description and/or the claims, such terms are intended to be inclusive in a manner similar to the term “comprising”. The transitional terms/phrases (and any grammatical variations thereof) “comprising”, “comprises”, “comprise”, “consisting essentially of”, “consists essentially of”, “consisting” and “consists” can be used interchangeably.

The term “about” or “approximately” means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system. For example, “about” can mean within 1 or more than 1 standard deviation, per the practice in the art. Alternatively, “about” can mean a range of up to 0-20%, 0 to 10%, 0 to 5%, or up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated the term “about” meaning within an acceptable error range for the particular value should be assumed. In the context of compositions containing amounts of ingredients where the terms “about” or “approximately” are used, these compositions contain the stated amount of the ingredient with a variation (error range) of 0-10% around the value ($X \pm 10\%$).

In the present disclosure, ranges are stated in shorthand, so as to avoid having to set out at length and describe each and every value within the range. Any appropriate value within the range can be selected, where appropriate, as the upper value, lower value, or the terminus of the range. For example, a range of 0.1-1.0 represents the terminal values of 0.1 and 1.0, as well as the intermediate values of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and all intermediate ranges encompassed within 0.1-1.0, such as 0.2-0.5, 0.2-0.8, 0.7-1.0, etc.

The invention demonstrates that a Class A type HPPA, for example, HvPPA, is thermostable and catalytically active in extreme conditions, for example, in the presence of 2-3 M salt (NaCl) and/or 25% (v/v) organic solvent (dimethylsulfoxide, N,N-

dimethylformamide, ethanol or methanol). Accordingly, the invention provides an HPPA which is active in the conditions unfavorable to the activity of PPAs from other organisms. The invention also provides a method of facilitating a reaction which produces PPi, the method comprising adding an HPPA for example, HvPPA, in to the reaction mixture.

5 Further, an embodiment of the invention provides an assay based on the HPPA mediated production of Pi from PPi to monitor and/or measure the kinetics of a reaction which produces PPi.

An embodiment of the invention provides HvPPA which is encoded by a nucleotide having the sequence of SEQ ID NO: 1 and has the amino acid sequence of SEQ ID NO: 2.

10 “Nucleotide sequence”, “polynucleotide” or “nucleic acid” can be used interchangeably and are understood to mean, according to the present invention, either a double-stranded DNA, a single-stranded DNA or products of transcription of the said DNAs (e.g., RNA molecules). The present invention does not relate to genomic polynucleotide sequences in their natural environment or natural state. The nucleic acid, polynucleotide, or

15 nucleotide sequences of the invention can be isolated, purified (or partially purified), by separation methods including, but not limited to, ion-exchange chromatography, molecular size exclusion chromatography, or by genetic engineering methods such as amplification, subtractive hybridization, cloning, subcloning or chemical synthesis, or combinations of these genetic engineering methods.

20 Nucleotide and proteins having the sequences that are at least 40%, 50%, 60%, 65%, 70%, generally at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, to 95%, 96%, 97%, 98%, 99% or more sequence identity to the sequences described in the invention are also provided. The sequence identity can be determined by the sequence alignment programs that are well known in the art. Non-limiting examples of such sequence alignment program

25 include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson and Lipman, 1988, *Proc. Natl. Acad. Sci. USA* 85(8):2444-2448; Altschul *et al.*, 1990, *J. Mol. Biol.* 215(3):403-410; Thompson *et al.*, 1994, *Nucleic Acids Res.* 22(2):4673-4680; Higgins *et al.*, 1996, *Methods Enzymol.* 266:383-402; Altschul *et al.*, 1990, *J. Mol. Biol.* 215(3):403-410; Altschul *et al.*, 1993, *Nature Genetics* 3:266-272).

30 Sequence comparisons are, typically, conducted using default parameters provided by the vendor or using those parameters set forth in the above-identified references, which are hereby incorporated by reference in their entireties.

In one embodiment, a nucleotide having the sequence which is at least 40%, 50%, 60%, 65%, 70%, generally at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, to 95%, 96%, 97%, 98%, 99% or more sequence identity to the sequence of SEQ ID NO: 1 is provided.

5 Similarly, a protein having the sequence which is at least 40%, 50%, 60%, 65%, 70%, generally at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, to 95%, 96%, 97%, 98%, 99% or more sequence identity to the sequence of SEQ ID NO: 2 is also provided.

Further non-limiting examples an HPPA include proteins identified by UniProt entry numbers L5NDU6 (SEQ ID NO: 3), M0GG38 (SEQ ID NO: 4), M0I975 (SEQ ID NO: 5),
10 M0FUM4 (SEQ ID NO: 6), M0FFB2 (SEQ ID NO: 7), M0FTS8 (SEQ ID NO: 8), M0GLF4 (SEQ ID NO: 9), M0HK17 (SEQ ID NO: 10), M0GRW8 (SEQ ID NO: 11), A0A0D6JT27 (SEQ ID NO: 12), M0HPW6 (SEQ ID NO: 13), M0IJ12 (SEQ ID NO: 14), M0J1D0 (SEQ ID NO: 15), M0I755 (SEQ ID NO: 16), M0FHY5 (SEQ ID NO: 17), M0DSG1 (SEQ ID NO: 18), I3R2F0 (SEQ ID NO: 19), A0A0F8AXZ4 (SEQ ID NO: 20), A0A063ZSK5 (SEQ ID NO: 21), V6DYW8 (SEQ ID NO: 22), M0AI50 (SEQ ID NO: 23), M0ACD9 (SEQ ID NO: 24), W0JSC4 (SEQ ID NO: 25), M0L713 (SEQ ID NO: 26), L9ZZ93 (SEQ ID NO: 27), M0B0N2 (SEQ ID NO: 28), L0JJS8 (SEQ ID NO: 29), M0CDJ7 (SEQ ID NO: 30), M0BJC3 (SEQ ID NO: 31), B9LS63 (SEQ ID NO: 32), M0E9P1 (SEQ ID NO: 33), D3SX83 (SEQ ID NO: 34), D2RW38 (SEQ ID NO: 35), M0C3G1 (SEQ ID NO: 36), M0JY31 (SEQ ID NO: 37), L9WPM3 (SEQ ID NO: 38), M0PRE6 (SEQ ID NO: 39), L9XRM4 (SEQ ID NO: 40), F8D7Q3 (SEQ ID NO: 41), M0NY18 (SEQ ID NO: 42), M0KHY3 (SEQ ID NO: 43), A0A0B5H0E2 (SEQ ID NO: 44), M0L599 (SEQ ID NO: 45), M0KLP2 (SEQ ID NO: 46), M0F4A3 (SEQ ID NO: 47), M0NU13 (SEQ ID NO: 48), M0ENH7 (SEQ ID NO: 49), M0IVY2 (SEQ ID NO: 50), M0DG34 (SEQ ID NO: 51), C7P1S8 (SEQ ID NO: 52),
25 V5TIQ9 (SEQ ID NO: 53), G0HUD5 (SEQ ID NO: 54), M0EUQ2 (SEQ ID NO: 55), M0NWL5 (SEQ ID NO: 56), I7BTX4 (SEQ ID NO: 57), L9Z9R6 (SEQ ID NO: 58), L9Z019 (SEQ ID NO: 59), L9ZID4 (SEQ ID NO: 60), M0K9U1 (SEQ ID NO: 61), M0DQG4 (SEQ ID NO: 62), Q5UXJ0 (SEQ ID NO: 63), L0IDF8 (SEQ ID NO: 64), L9X8I7 (SEQ ID NO: 65), L0AKK7 (SEQ ID NO: 66), M0BBH8 (SEQ ID NO: 67), Q3IMH1 (SEQ ID NO: 68),
30 L0K0M8 (SEQ ID NO: 69), M1XNP8 (SEQ ID NO: 70), E7QS08 (SEQ ID NO: 71), J3EYU6 (SEQ ID NO: 72), L9X0H4 (SEQ ID NO: 73), M0CLI0 (SEQ ID NO: 74), L9W889 (SEQ ID NO: 75), D8J8G3 (SEQ ID NO: 76), M0NAM6 (SEQ ID NO: 77), M0N5K4 (SEQ ID NO: 78), M0P5M8 (SEQ ID NO: 79), M0MFR0 (SEQ ID NO: 80), M0MGM0 (SEQ ID

NO: 81), W0K0R9 (SEQ ID NO: 82), E4NRZ6 (SEQ ID NO: 83), V4ZPA9 (SEQ ID NO: 84), U1QCV5 (SEQ ID NO: 85), V4Y944 (SEQ ID NO: 86), G2MJ96 (SEQ ID NO: 87), M0CVD7 (SEQ ID NO: 88), C7NMR7 (SEQ ID NO: 89), M0M2R7 (SEQ ID NO: 90), F7PQH1 (SEQ ID NO: 91), R4W5L5 (SEQ ID NO: 92), V4HJG7 (SEQ ID NO: 93),
5 U1PEB6 (SEQ ID NO: 94), U1QT16 (SEQ ID NO: 95), A0A0F7PBR3 (SEQ ID NO: 96), Q18KT4 (SEQ ID NO: 97), G0LHI3 (SEQ ID NO: 98), B0R2Z8 (SEQ ID NO: 99), Q9HSF3 (SEQ ID NO: 100), U1PVH2 (SEQ ID NO: 101), U1NPX9 (SEQ ID NO: 102), U1NUP6 (SEQ ID NO: 103), V4YFB8 (SEQ ID NO: 104), U2YXF9 (SEQ ID NO: 105), U1PS97 (SEQ ID NO: 106), V4XHA0 (SEQ ID NO: 107) or U1P365 (SEQ ID NO: 108). Additional
10 examples of an HPPA are well known to a person of ordinary skill in the art and such embodiments are within the scope of the invention.

Certain embodiment of the invention provide a DNA construct (sometimes referred to as nucleotide constructs or vectors) comprising a nucleotide encoding an HPPA. A typical vector contains an origin of replication, a promoter which drives the expression of an
15 operably connected nucleotide and one or more selection markers. Vectors appropriate for use according to the instant invention are well known to a person of ordinary skill in the art and such embodiments are within the purview of the invention.

One embodiment of the invention provides a cell, for example, bacterial cell, archaeal cell, fungal cell, plant cell or animal cell, containing a nucleotide encoding an HPPA. The
20 nucleotide encoding an HPPA can be in a vector or incorporated in to the genome of the cell. In one embodiment, the invention provides a cell, for example, bacterial cell, archaeal cell, fungal cell, plant cell or animal cell, containing a nucleotide encoding HvPPA, either in a plasmid or incorporated in to the genome of the cell.

A further embodiment of the invention provides a method of expressing an HPPA in a
25 cell, for example, bacterial cell, archaeal cell, fungal cell, plant cell or animal cell, the method comprising transforming the cell with a nucleotide encoding an HPPA, culturing the cell under conditions which allows the expression of the HPPA, and optionally, purifying the HPPA from the culture. In one embodiment, the cell expressing an HPAA is *Escherichia coli*.

30 In one embodiment, the purification of an HPPA is facilitated by conjugating the HPPA with a protein or peptide tag. Non-limiting examples of a protein or peptide tag which facilitates the purification of the HPPA protein include 6x histidine (His₆), maltose binding protein, calmodulin binding peptide, covalent yet dissociable NorpD peptide, Strep-TagTM II,

FLAG-Tag (amino acid sequence: Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (SEQ ID NO: 109), heavy chain of protein C or Glutathione S-transferase. Additional examples of protein or peptide tags useful for purification of HPPA are well known to the person of ordinary skill in the art and such embodiments are within the purview of the invention.

5 Various methods of expressing and purifying a protein in a bacterial cell, archaeal cell, fungal cell, plant cell or animal cell are well known to a person of ordinary skill in the art and such embodiments are within the purview of the invention.

10 An embodiment of the invention further provides a method of driving a reaction which produces PPi towards the production of PPi. For the purpose of this invention, the phrase “driving a reaction towards the production of PPi” indicates increasing the rate of the reaction in the direction of the production of PPi. For example, if a reaction involving the production of PPi is reversible, the reaction can lead to the production of PPi or to the utilization of PPi. In such condition, “driving a reaction towards the production of PPi” indicates increasing the rate of the reaction in the direction which leads to the production of PPi.

15 The method of driving a reaction which produces PPi towards the production of PPi comprises adding an HPPA to the reaction mixture of the reaction which produces PPi. In an embodiment, the reaction which produces PPi is an enzymatic reaction. In a further embodiment, the reaction which produces PPi takes place under the conditions of high temperature and/or low water activity.

20 For the purpose of this invention, the term “moderately high temperature” indicates that the temperature is typically favorable for thermophilic enzymes. Temperatures favorable for a thermophilic enzyme is about 40°C to about 100°C, about 40°C to about 80°C, about 40°C to about 60°C, about 40°C to about 50°C or about 42°C.

25 For the purpose of the invention, the term “water activity” refers to the amount of water associated with an enzyme in a reaction mixture rather than the total amount of water in the reaction mixture. Therefore, the term “low water activity” refers to the conditions where the amount of water associated with an enzyme in a reaction mixture is low compared to the total amount of water in the reaction mixture. The conditions which affect water activity include, but are not limited to, salt concentration and/or the presence/absence and the concentration of organic solvents. Typically, water activity is inversely proportional to the concentrations of salts and/or organic solvents. Therefore, a reaction mixture containing

30 higher concentrations of salts and/or organic solvents has lower water activity and *vice versa*.

For the purpose of this invention, the phrase “high salt concentration” refers to salt concentration which is typically unfavorable for non-halophilic enzymes. Halophilic enzymes are the enzymes that are active in the presence of high salt concentration, for example, about 1M to about 5M. Accordingly, “high salt concentration” refers to a salt concentration of about 1M to about 5M, about 1.5 M to about 4M, about 2M to about 3M, or about 2.5 M.

For the purpose of this invention, the phrase “high organic solvent concentration” refers to organic solvent concentration of about 10% to about 50%, about 15% to about 40%, about 20% to about 30%, or about 25%. The organic solvents typically used in a reaction mixture include dimethylsulfoxide (DMSO), N,N-dimethylformamide (DMF), ethanol or methanol. Additional examples of organic solvents used in a reaction are well known to a person of ordinary skill in the art and such embodiments are within the purview of the current invention. Accordingly, “the conditions of low water activity” refer to a salt concentration of about 1M to about 5M, about 1.5 M to about 4M, about 2M to about 3M, or about 2.5 M and/or an organic solvent concentration of about 10% to about 50%, about 15% to about 40%, about 20% to about 30%, or about 25%. In certain embodiments, the reaction which produces PPi is performed at moderately high temperature, for example, about 40°C to about 100°C, about 40°C to about 80°C, about 40°C to about 60°C, about 40°C to about 50°C or about 42°C.. In a further embodiment, the reaction which produces PPi is performed under low water activity. In an even further embodiment, the reaction which produces PPi is performed under moderately high temperature and/or low water activity.

An HPPA, for example, HvPPA, is active under conditions of low water activity, thermostable at high temperatures (such as about 50°C to about 100°C, about 60°C to about 90°C, about 70°C to about 80°C, or about 75°C) and enzymatically active at moderately high temperatures (temperatures of about 40°C to about 100°C, about 40°C to about 80°C, about 40°C to about 60°C, about 40°C to about 50°C or about 42°C.). Accordingly, the invention provides a method of driving a reaction which produces PPi in the direction of the production of PPi, the method comprising adding an HPPA to the reaction mixture for the reaction which produces PPi, wherein the reaction which produces PPi is carried out under the conditions of low water activity, moderately high temperatures and/or after enzyme exposure to high temperatures.

In an embodiment, the HPPA is HvPPA having the amino acid sequence of SEQ ID NO: 2. In another embodiment, the HPPA has the amino acid sequence selected from SEQ ID NOs: 3 to 108.

In one embodiment, the enzymatic reaction which produces PPi is the biosynthesis of DNA, RNA, protein, peptidoglycan, lipids, cellulose, starch and other biopolymers, post-translational modification of proteins including adenylation, uridylation and ubiquitylation, SNP genotyping, RNA synthesis by viral RNA-dependent RNA polymerases and aminoacyl-tRNA synthetase activity. Typically, any enzymatic reaction which produces PPi comprises hydrolysis of nucleoside triphosphate, for example, adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP), 5-methyluridine triphosphate (m⁵UTP), and uridine triphosphate (UTP). A non-limiting list of enzymatic reactions that produce PPi are provided in Jukka K. Heinonen, Biological Role of Inorganic Pyrophosphate, New York, Spring Science+Business media, 2001, particularly, in Chapter 1, Tables 1.1 and 1.2 on pages 1-9, the contents of which are herein incorporated by reference in their entirety. Additional examples of reactions which produce PPi, particularly, enzymatic reactions which produce PPi, are well known to a person of ordinary skill in the art and such embodiments are within the purview of the invention. The reactions will be conducted, typically, under conditions that include moderately high temperatures (such as temperatures of about 40°C to about 100°C, about 40°C to about 80°C, about 40°C to about 60°C, about 40°C to about 50°C or about 42°C).

A further embodiment of the invention provides a composition comprising a reaction mixture for the reaction which produces PPi, the composition further comprising an HPPA. In an embodiment, the reaction which produces PPi is an enzymatic reaction.

In certain embodiments, the enzymatic reaction mixture for the enzymatic reaction which produces PPi comprises one or more substrates, an enzyme which catalyzes the production of PPi and/or one or more co-factors for the enzyme. Non-limiting examples of substrates include proteins, single stranded polynucleotide, double stranded polynucleotide, carbohydrates, lipids, ATP, GTP, CTP, UTP or m⁵UTP. Non-limiting examples of cofactors include Mg²⁺, Mn²⁺, Zn²⁺ and Co²⁺. Additional examples of substrates and co-factors are well known to a person of ordinary skill in the art and such embodiments are within the purview of the invention.

In one embodiment, the invention provides a composition comprising a reaction mixture for a reaction which produces PPi and HvPPA which has the amino acid sequence of

SEQ ID NO: 2. In another embodiment, the invention provides a composition comprising a reaction mixture for the reaction which produces PPi and an HPPA having the amino acid sequence selected from SEQ ID NOs: 3 to 108.

A further embodiment of the invention provides an assay of determining the reaction kinetics for a reaction which produces PPi, the method comprising the steps of:

- a. conducting the reaction which produces PPi,
- b. periodically separating an aliquot of the reaction mixture from the enzymatic reaction which produces PPi and immediately subjecting the aliquots to a condition which stops the reaction which produces PPi to obtain a plurality of aliquots corresponding to various time points for the reaction which produces PPi,
- c. introducing sufficient amount of an HPPA to each of the plurality of the aliquots,
- d. incubating the HPPA with each of the plurality of aliquots under suitable conditions and for sufficient amount of time to allow the HPPA to convert all of the PPi present in the aliquot in to Pi,
- e. determining the amount of Pi present in each of the plurality of aliquots, and
- f. determining the reaction kinetics of the reaction which produces PPi based on the amount of Pi in each of the plurality of aliquots. The reaction that produces PPi is conducted, typically, under moderately high temperatures (temperatures of about 40°C to about 100°C, about 40°C to about 80°C, about 40°C to about 60°C, about 40°C to about 50°C or about 42°C).

In one embodiment, the HPPA is HvPPA having the amino acid sequence of SEQ ID NO: 2. In another embodiment the HPPA has the amino acid sequence selected from SEQ ID NOs: 3 to 108.

In certain embodiments, the condition which stops the reaction which produces PPi includes exposure to a temperature at which the enzyme producing PPi is inactivated (temperatures such as about 50°C to about 100°C, about 60°C to about 90°C, about 70°C to about 80°C, or about 75°C or about 100°C for a period of 2 to 24 hours), addition of an inhibitor of the enzyme producing PPi or addition of a reagent which stops the reaction producing PPi. Additional methods of stopping a reaction are well known in the art and such embodiments are within the purview of the current invention.

In a certain embodiment, the assay used to detect the concentration of Pi present in each of the plurality of aliquots is a colorimetric assay, for example, malachite green based assay for detecting Pi. Additional examples of assays to detect the concentration of Pi are

well known to a person of ordinary skill in the art and such embodiments are within the purview of the current invention.

A further embodiment of the invention provides an assay for monitoring the reaction and/or determining the reaction kinetics for a reaction which produces PPi in the presence and/or the absence of an HPPA, the method comprising the steps of:

a. conducting the reaction which produces PPi in the presence of the HPPA at moderately high temperatures,

b. conducting the reaction which produces PPi in the absence of the HPPA at moderately high temperatures,

b. periodically separating aliquots from the reaction mixtures in the presence or the absence of the HPPA and immediately subjecting the aliquots to a condition which stops the reaction which produces PPi to obtain a plurality of aliquots corresponding to various time points for the reactions which produces PPi in the presence or the absence of the HPPA,

c. determining the amount of Pi present in each of the plurality of aliquots, and

d. based on the amount of Pi present in each of the plurality of aliquots, determining the reaction kinetics for the reaction which produces PPi in the presence or the absence of the HPPA, and

e. determining the effect of the presence of the HPPA on the reaction producing PPi.

In one embodiment, the HPPA is HvPPA having the amino acid sequence of SEQ ID NO: 2. In another embodiment the HPPA has the amino acid sequence selected from SEQ ID NOs: 3 to 108.

In certain embodiments, the condition which stops the reaction which produces PPi includes exposure to the temperature at which the enzyme producing PPi is inactivated (temperatures such as about 50°C to about 100°C, about 60°C to about 90°C, about 70°C to about 80°C, or about 75°C or about 100°C for a period of 2 to 24 hours), addition of an inhibitor of the enzyme producing PPi or addition of a reagent which stops the reaction producing PPi. Additional methods of stopping a reaction are well known in the art and such embodiments are within the purview of the current invention.

In a certain embodiment, the amount of Pi present in each of the plurality of aliquots is determined using a colorimetric assay, for example, malachite green based assay. Additional examples of determining the amount of Pi are well known to a person of ordinary skill in the art and such embodiments are within the purview of the current invention.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Atlanta, GA). Phusion and Taq DNA polymerases, restriction endonucleases and T4 DNA
 5 ligase were purchased from New England Biolabs (Ipswich, MA). Desalted oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Agarose used for routine analysis of DNA was purchased from Bio-Rad Laboratories (Hercules, CA).

Strains and media

10 Strains used in this study are listed in Table 1. *E. coli* TOP10 was used for routine recombinant DNA analysis. *E. coli* GM2163 was used for preparation of plasmid DNA prior to transformation of *Hfx. volcanii* H26 by standard methods. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium supplemented with ampicillin (Amp at 0.1 mg/ml) as needed. *Hfx. volcanii* strains were grown at 42 °C in ATCC 974 medium supplemented with
 15 novobiocin (+Nv, 0.1 µg/ml⁻¹) as needed. Cells were grown in liquid cultures with rotary shaking at 200 rpm and on solid medium (1.5 % [w/v] agar plates). Growth was monitored by measuring OD₆₀₀ [where 1 OD₆₀₀ unit equals approximately 1 × 10⁹ colony forming units (CFU)/ml⁻¹].

Table 1. Purification of HvPPA from *Hfx. volcanii* (H26-pJAM2920).

Fraction	Protein (mg)	Total Activity (U ± SD)	SP act U·Mg ⁻¹ ± SD)	Yield (%)	Purification (fold enrichment)
Lysate	1680	356 ± 30	0.21 ± 0.02	100	1
HisTrap HP	22.5	180 ± 14	7.98 ± 0.61	51	38
Superdes 200	0.56	42 ± 0.26	75.3 ± 0.47	12	357

20 PPA activity monitored in Tris-salt buffer at 37°C and pH 8.5 with 0.1 mM PPi for 10 min. U, units defined as µmol product/min. SD, standard deviation of three experiments.

DNA manipulations

25 Plasmids and primers used in this study are listed in Table 2. Primers 1 and 2 were used for PCR-based amplification of the HvPPA gene with *Hfx. volcanii* H26 genomic DNA as template. The 0.55 kb PCR product was ligated into the NdeI to BlnI sites of pJAM503 to

generate plasmid pJAM2920 for expression of HvPPA with an N-terminal poly-histidine tag (His₆-HvPPA) linked with a thrombin-cleavage site. Plasmid DNA was isolated by QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). PCRs were according to standard methods using an iCycler (BioRad Laboratories). Genomic DNA was extracted from *Hfx. volcanii* cells by boiling colonies resuspended in ddH₂O or by DNA spooling. Phusion DNA polymerase was used for high-fidelity PCR-based cloning. Taq DNA polymerase was used for colony screening. DNA fragments were separated by 0.8-2% (w/v) agarose gel electrophoresis (90 V, 30-45 min) in TAE buffer [40 mM Tris, 20 mM acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0]. Gels were stained with ethidium bromide at 0.25 µg/ml and visualized with a Mini visionary imaging system (FOTODYNE, Hartland, WI). Hi-Lo DNA molecular weight markers (Minnesota Molecular, Minneapolis, MN) were used for comparison. DNA fragments were isolated directly from PCR by MinElute PCR purification (Qiagen) or from 0.8 % (w/v) SeaKem GTG agarose (FMC Bioproducts, Rockland, ME) gels in TAE buffer at pH 8.0 using the QIAquick gel extraction kit (Qiagen) as needed. The fidelity of DNA plasmid constructs was verified by DNA Sequencing.

Table 2. Strains, plasmids and primers used in this study^a.

Strain, plasmid or primer	Description	Source or reference
<i>E. coli</i> TOP10	F ⁻ <i>recA1 endA1 hsdR17(rK⁻ mK⁺) supE44 thi-1 gyrA relA1</i>	Invitrogen
<i>E. coli</i> GM2163	F ⁻ <i>ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 rpsL136 dam13::Tn9 xylA5 mtl-1 thi-1 mcrB1 hsdR2</i>	New England Biolabs
<i>Hfx. volcanii</i> DS70	wild-type isolate DS2 cured of plasmid pHV2	[60]
<i>Hfx. volcanii</i> H26	DS70 Δ <i>pyrE2</i>	[61]
Plasmid pJAM503	Ap ^r ; Nv ^r ; <i>Hfx. volcanii</i> - <i>E. coli</i> shuttle vector with coding sequence for N-terminal His ₆ tag	[38]
Plasmid pJAM292	Ap ^r ; Nv ^r ; pJAM503-derived, His ₆ -HvPPA	This study
Primer 1: PPA NdeI FW	5'-taCATATGGTGAACCTCTGGGAAGATATGGAG-3' (SEQ ID NO: 110)	This study
Primer 2: PPA BlnI RV	5'-CTACGAAGAGAACTTCGCGTAAgcgaGCTGAGCta-3' (SEQ ID NO: 111)	This study

^aAp^r, ampicillin resistance; Nv^r, novobiocin resistance.

Purification of HvPPA

HvPPA (HVO_0729, UniProt: D4GT97) was expressed with an N-terminal poly-histidine tag (His₆) in *Hfx. volcanii* H26-pJAM2920 (Table 1). *Hfx. volcanii* cells were grown to stationary phase (OD₆₀₀ of 3-3.5) (4 x 1-liter cultures in 2.8-liter Fernbach flasks) and harvested by centrifugation (10-15 min at 9,200 × *g* and 25°C). Cell pellets were resuspended at 5 ml per g wet weight cells in Tris-salt buffer (20 mM Tris-HCl, pH 7.5, 2 M NaCl and 2.5 mM MgCl₂) supplemented with 40 mM imidazole and 1 mini-tablet protease inhibitor cocktail (Roche product no. 05892791001) per 10 ml buffer. Cells were lysed by passing through a French Press (three times at 20,000 psi). Whole cell lysate was clarified by centrifugation (twice for 30 min at 9,200 × *g* and 4°C) and sequential filtration using 0.8 µm and 0.2 µm cellulose acetate filters (Thermo Scientific Nalgene). Clarified cell lysate was applied to a HisTrap HP column (5 ml, 17-5248-01, GE Healthcare) pre-equilibrated and washed in 100 ml of Tris-salt buffer with 40 mM imidazole. Fractions containing HvPPA were eluted in Tris-salt buffer with a 25 ml gradient from 40 mM to 500 mM imidazole. Fractions were tested for activity and dialyzed overnight with a buffer change after four hours against Tris-salt buffer containing 2.5 mM MgCl₂ and 1 mM DTT, and concentrated by centrifugal filtration using an Amicon Ultra-x mL 10K device (EMD Millipore). HvPPA was further purified by size exclusion chromatography (SEC) in which protein (500 µl at 14.5 mg/ml) was applied at a flow rate of 0.3 ml/min to a Superdex 200 10/300 GL column (GE Healthcare) equilibrated in Tris-salt buffer supplemented with fresh 1 mM DTT. HvPPA fractions eluting at 14.8 ml (hexamer) and 15.9 ml (trimer) were further purified using a similar SEC strategy. Purity of HvPPA was assessed by Coomassie Blue stained SDS-PAGE gels and PPi activity assay. Molecular mass standards used for analytic SEC were blue dextran (for void volume), β-amylase, cytochrome C, bovine serum albumin, and alcohol dehydrogenase (Sigma Aldrich, #MWGF200-1KT). HvPPA fractions were pooled and stored at 4°C.

PPi assay

HvPPA-mediated hydrolysis of PPi to Pi was determined spectrophotometrically. Reagents were in nanopure water (Barnstead/Thermolyne Nanopure lab water system). Sodium pyrophosphate tetrabasic decahydrate (Sigma Aldrich) was used as a substrate. For kinetic measurements, reaction mixtures (500 µl total) contained 0.5 - 1 µg HvPPA and 1

mM PPi in high-salt buffer (3 M NaCl and 20 mM Tris-HCl, pH 8.5). Reactions were incubated at 42°C for 1-3 min. Orthophosphate levels were determined by malachite green assay. Briefly, 2.5 ml of 14% (w/v) $(\text{NH}_4)_2\text{MoO}_4$ and 0.2 ml of 11% (v/v) Tween 20 were added into a 10-ml color reagent I (containing 1.67 ml conc. sulfuric acid, 8.33 ml nanopure H_2O , and 12.22 mg malachite green). In triplicate, 50 μl of the color reagent was mixed with 200 μl of the reaction mixture and incubated at room temperature for 10 min. The formation of $(\text{MG}^+)(\text{H}_2\text{PMo}_{12}\text{O}_{40})$ (where MG^+ represents ionized malachite green) was monitored at A_{630} . A less sensitive malachite green assay was also used in which reaction aliquots (50 μl) were mixed with 250 μl color reagent II and 10 μl 1.5% (v/v) Tween 20 in triplicate; product formation was immediately monitored at A_{650} . Color reagent II was generated by mixing 1 volume of 4.2% (w/v) $(\text{NH}_4)_2\text{MoO}_4$ in 5 N HCl with 3 volumes of 0.2% (w/v) malachite green; after 30 minutes, the solution was filtered with a 0.45 μm filter and stored at room temperature. Freeze-dried KH_2PO_4 was used as the standard. Assay mixtures with PPi minus HvPPA were used for individual background subtraction. All proteins used in this assay were buffer-exchanged with Tris-high-salt buffer in nanopure H_2O prior to use. All experiments were performed in triplicate and the mean $\pm$ standard deviation (S.D.) was calculated.

Coupled assay of ubiquitin-like protein adenylation

UbaA-mediated hydrolysis of ATP to AMP and PPi in the presence of the ubiquitin-like SAMP1 was monitored by coupled assay with HvPPA. Proteins were buffer-exchanged with HEPES-salt buffer in Nanopure H_2O prior to use. Reaction mixtures (500 μl total) containing 20 μM UbaA, 20 μM SAMP1, 0.5 μM HvPPA, 2.5 mM nucleotide, 2.5 mM MgCl_2 , 50 μM ZnCl_2 in high-salt buffer (2 M NaCl and 50 mM HEPES in Nanopure H_2O , pH 7.5) were incubated at 37-42°C for 1 h. Nucleotides were ATP, AMP, ADP, AMP-PNP, CTP, GTP, TTP and UTP. Proteins were removed by Ultracel-3 centrifugal filtration prior to determining orthophosphate levels by malachite green assay modified as described above. Assay mixtures with nucleotide alone were used for individual background subtraction.

Protein concentration assay

The molar protein concentration was calculated using absorption at 280 nm and extinction coefficient 26,025 /M.cm (with the assumption that all cysteines were cystines).

These values were comparable to protein concentration calculated by the Bradford method using bovine serum albumin (BSA; ThermoScientific) as the standard.

SDS-PAGE and immunoblotting

5 Proteins were separated by reducing SDS-PAGE according to the Laemmli system. His-tagged HvPPA was analyzed by immunoblotting using a monoclonal unconjugated α -his IgG₂ antibody from mouse (27-4710-01 GE Healthcare) and alkaline phosphatase-linked goat anti-mouse IgG antibody (A5153 Sigma Aldrich). Immunoreactive antigens were detected by chemiluminescence using CDP-Star (Applied Biosystems), as the alkaline phosphatase
10 substrate, and X-ray film (Research Products Intl. Corp.).

Dendrogram analysis

Evolutionary analyses were conducted in MEGA6. The evolutionary history of
15 archaeal PPAs was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the p-distance method and were in the units of the number of amino acid differences per site. The analysis involved 225 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 263 positions in the final dataset.

20 All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. Percentages are by total activity, by
25 amino acid sequence and/or by weight. All solvent mixture proportions are by volume unless otherwise noted.

EXAMPLE 1 – HALOARCHAEAL PPA HOMOLOGS ARE DISTINCT FROM OTHER PPA

30 The evolutionary relationships of archaeal PPAs of the Class A type (IPR008162 family) were analyzed by hierarchical clustering to identify new PPAs with distinct features in amino acid sequence that may correlate with novel biochemical properties. In contrast to PPAs of other families/domains (*i.e.*, IPR004131, IPR022934, IPR004097 and IPR023733),

the Class A type PPAs are soluble and the most widespread among extreme organisms such as Archaea. By this approach, haloarchaeal PPA homologs of the Class A type were found to cluster to a single node suggesting a close evolutionary relationship among these proteins that was distinct from other PPAs (Fig. 1). Included in the tight cluster of haloarchaeal PPA homologs was HvPPA.

HvPPA was further analyzed by multiple amino acid sequence alignment and Phyre2-based homology modeling. By this analysis, HvPPA was found to have 42-55% identity and 60-71% similarity in amino acid sequence to biochemically characterized archaeal PPAs of the Class A type (IPR008162 family) (Fig. 2A). HvPPA was predicted to have an OB-fold with a central β -barrel structure and α -helices in a $\beta_{1-8}-\alpha_1-\beta_9-\alpha_2$ topology (Fig. 2A) that was analogous to related archaeal PPAs and compatible with their quaternary structure, *i.e.*, dimer of trimers and trimer) (Fig. 2B). The active site residues of *Saccharomyces cerevisiae* ScPPA, including those that bind substrate and product were found conserved in HvPPA (Fig. 2B). In particular, HvPPA D69 was found analogous to ScPPA D117, a key residue in pyrophosphate hydrolysis in which the nucleophile is generated by coordinating a water molecule to two metal ions and further strengthened by donating a hydrogen bond to D117. Interestingly, HvPPA had two cysteine residues (C24 at the C-terminus of the β_1 strain and C85 at the N-terminus of the β_6 strain) in a Cys-X₆₃-Cys motif highly conserved among haloarchaeal PPAs, yet unusual for this group of enzymes (Fig. 2A-B). These conserved cysteine residues were at a significant distance from the predicted active site, with C85 residing notably near the intrasubunit interfaces suggesting redox status could play a role in quaternary structure configuration. HvPPA was also found to have a highly negative surface charge compared to other PPAs that have been characterized (Fig. 2C).

Based on the hierarchical clustering and 3D-structural modeling described above, the HPPAs were found to be phylogenetically distinct members of the Class A type (IPR008162 family) with unique features in primary amino acid sequence and 3D-structure. Thus, PPAs of the haloarchaea were provided for identifying new enzymes with novel biochemical properties.

EXAMPLE 2 – HVPPA PURIFIED TO HOMOGENEITY AS A TRIMER AND HEXAMER

HvPPA was chosen as a representative of the haloarchaeal PPAs for purification and biochemical characterization. To accomplish this, HvPPA was constitutively expressed from an rRNA P2 promoter and purified to homogeneity at high yield from an engineered strain of *Hfx. volcanii* (H26-pJAM2920) that expressed HvPPA with an N-terminal His₆-tag *in trans*. Thus, HvPPA was purified 357-fold by a tandem approach that relied upon Ni²⁺-based immobilized metal ion affinity chromatography (IMAC) and size exclusion chromatography (SEC) (Table 1; Fig. 3A). Based on SEC, HvPPA was purified as two distinct homooligomeric fractions including hexamers of ~134 kDa and trimers of ~64 kDa (Fig. 3B). The HvPPA trimers were only 78% as active as the hexamer and, thus, were not further pursued for biochemical characterization. The hexameric HvPPA consisted of His₆-HvPPA as well as minor fraction of genome-encoded HvPPA (Fig. 3A). The gel-based estimations for the wild type and His₆-HvPPA of 25.6 and 36.6 kDa that were observed by SDS-PAGE were found to be 5-8 kDa larger than the theoretical M_r of 20.4 and 22.5 kDa, respectively. This finding is explained by the prediction that HvPPA has a pI of 3.98 (with the pI of His₆-HvPPA also low at 4.28). This overall negative charge would likely alter the SDS-coating and retard the migration of HvPPA in SDS-PAGE gels, as is commonly observed for proteins with highly acidic regions. The finding that HvPPA associates as a homo-trimer and homo-hexamer is consistent with the configuration of Class A type PPAs of thermophilic and hyperthermophilic archaea (Table 3). Thus, HvPPA is a dimer of trimers as has been observed in x-ray crystal structures of PPAs (*e.g.*, PDB: 1QEZ and 3I98).

Table 3. Archaeal inorganic pyrophosphatases (PPAs) [E.C. 3.6.1.1] of the Class A type (IPR008162 family). ¹						
Organism	<i>Thermoplasma acidophilum</i>	<i>Pyrococcus horikoshii</i>	<i>Sulfolobus sp.</i>	<i>Methano-thermobacter thermoauto-trophicus</i>	<i>Thermococcus thioeducens</i>	<i>Haloferax volcanii</i>
Gene locus_tag	Ta0399	PH1907	STK_05240 Saci_0955	MTH_263 (presumed)	H0USY5_9EURY	HVO_0729
Amino acid number (aa)	179 aa	178 aa	172-173 aa	176 aa	178 aa	177 aa
pI (theoretical)	5.33	4.97	4.83 – 4.92 (4.8 obs)	4.69	4.76	3.98
M _r (theoretical)	20.5 kDa	20.8 kDa	19.4 kDa	20.1 kDa	20.9 kDa	20.4 kDa
M _r (observed)	6 × 22 kDa	6 × 24.5 kDa	4-6 × 17-21 kDa	2-4 × 25 kDa	—	3-6 × 27 kDa
Cation-dependence	Mg ²⁺	Mg ²⁺ , Co ²⁺ , Zn ²⁺ , Mn ²⁺ (partial)	Mg ²⁺ , Co ²⁺ , Zn ²⁺ , Mn ²⁺ (partial)	Mg ²⁺ , Co ²⁺ (partial)	—	Mg ²⁺ , Mn ²⁺
Inhibitors	Ca ²⁺ , phenylglyoxal	NaF	NaF, Ca ²⁺ , phenylglyoxal	NaF	—	NaF
Substrate(s) hydrolyzed	PPi	PPi [P ₃ (2.7%); ATP (5.9%); ADP (2.9%)]	PPi [pNP, PEP (1-2%); ATP, P ₃ , ADP (3-6%); TTP, ITP (10%)]	PPi	—	PPi
K _m	7 μM PPi 1.7 mM Mg ²⁺	14-11 μM PPi 0.3 mM Mg ²⁺	5 μM PPi 0.9 mM Mg ²⁺	0.16 mM PPi 4.9 mM Mg ²⁺	—	0.55 mM PPi (42 °C) 0.26 mM PPi (25 °C) 13.4 mM Mg ²⁺ (25 °C)

V_{max}	1100 U·mg ⁻¹ (56°C)	930 U·mg ⁻¹ (60°C)	860 U·mg ⁻¹ (75°C)	570 U·mg ⁻¹	—	465 U·mg ⁻¹ (42 °C) 53 U·mg ⁻¹ (25 °C)
k_{cat}	2200 s ⁻¹	744-3436 s ⁻¹	1700 s ⁻¹	—	—	1050 s ⁻¹
Hill coefficient(s)	1.8 (Mg ²⁺)	Cooperative binding	1.9 (Mg ²⁺)	3.3 (PPi) ₂ 2.0 (Mg ²⁺)	—	2.1 (PPi, 42 °C) 1.4 (PPi, 25 °C) 2.6 (Mg ²⁺ , 25 °C)
Temp., optimum	85 °C	70-88 °C	75 °C	70 °C	—	42 °C
pH, optimum		pH 7.5-10	pH 6.5-7.0	pH 7.7 (60°C)	—	pH 8.5 (25 °C)
Thermal inactivation (half-life)		50 min (105 °C)	2.5 h (95 °C) >24 h (75 °C)	—	—	2 h (65 °C)
Soluble, cytoplasmic	Yes	Yes	Yes	Yes	—	Yes
Heterologous system	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>	—	<i>E. coli</i>	—
Crystal structure (PDB number)	—	1UDE	1QEZ	—	3Q4W, 3I98, 3R5U, 3R5V, 3R6E, 3Q9M	—
Example of Coupled assay	DNA polymerase (PCR, sequencing)	DNA polymerase (PCR)	—	—	—	Ub/Ubl adenylation
Ref.	[4; 18; 19]	[5; 20; 21; 22]	[23; 24; 25; 26; 27]	[28]	[29], unpublished	This study

[†]Abbreviations: U/mg is μmol PPi hydrolyzed per min (mg protein)⁻¹; —, not reported. HVO_0729 (sp:D4GT97); PH1907 (sp:O59570); MJ0608 (sp:Q58025); Ta0399 (sp:P37981); MTH_263 (sp:O26363); STK_05240 (sp:Q974Y8); Saci_0955 (sp:P50308); H0USY5_9EURY (sp:H0USY5); *Sulfolobus* sp. (*S. acidocaldarius* ATCC 33909 and *S. tokodaii* str. 7); V_{max} is highest reported.

EXAMPLE 3 – CATALYTIC ACTIVITY OF HVPPA

HvPPA hexamers readily hydrolyzed PPi to orthophosphate with optimal activity detected at 42°C and basic pH (pH 8-9) (Figs. 4A-B). Supplementation of reactions with NaCl (within a 120-fold range of 0.025 to 3 M NaCl) had little, if any, effect on the relative activity of HvPPA (Fig. 4C). HvPPA was inactivated when divalent cations were removed from the reaction by dialysis against the metal chelator EDTA. The PPi hydrolyzing activity of the EDTA-treated HvPPA could be partially restored by supplementation of the enzyme with Mn^{2+} or Mg^{2+} ions, but not by addition of Zn^{2+} , Ca^{2+} , Co^{2+} or Ni^{2+} (Fig. 4D) (with the restored enzyme at only half the activity of the untreated control when similarly assayed at 2.5 mM $MgCl_2$). HvPPA was found to be significantly stimulated by addition of Mg^{2+} to the reaction buffer with optimal PPi hydrolyzing activity at 20-40 mM Mg^{2+} (Fig. 4E). Addition of other divalent cations such as Mn^{2+} did not stimulate the activity of HvPPA, when it was not treated with EDTA. Thus, HvPPA is most likely coordinated to Mg^{2+} (and not Mn^{2+}) ions upon purification from *Hfx. volcanii* and requires relatively high concentrations of Mg^{2+} for full activity. Intracellular Mg^{2+} ions are quite high in the haloarchaea with the intracellular concentration of Mg^{2+} reported at 120 mM for *Halobacterium salinarum*.

Similar to the other Class A type PPAs, HvPPA was inhibited by sodium fluoride (NaF) with K_i values of 1.8 mM NaF at pH 8.5 and 0.2 mM NaF at pH 7.5 (Fig. 5A). Increased sensitivity to fluoride inhibition at more neutral pH is commonly observed for Class A type PPAs and other enzymes including catalases and peroxidases. Consistent with the NaF inhibition of HvPPA, amino acid residues interacting with the F^- , PPi, H_2O and Mg^{2+} bound molecules in the X-ray crystal structure of *E. coli* PPA (PDB: 2AUU) are conserved in the haloarchaeal enzyme (Fig. 5B). F^- ions inhibit the activity of Class A type PPAs by substituting the attacking nucleophile in the PPi hydrolysis reaction.

HvPPA displayed non-Michaelis-Menten kinetics for PPi hydrolysis. When assayed at 42°C, HvPPA has the V_{max} of 465 U/mg and K_m of 0.55 mM for the PPi substrate. In contrast, HvPPA had the reduced V_{max} of 53 U/mg and K_m of 0.26 mM for PPi at 25°C. Sigmoidal kinetic profiles indicative of positive cooperative binding were detected for Mg^{2+} with the degree of cooperativity represented by a Hill coefficient of 2.62 at 25°C (a K_m of 13 mM was also determined for Mg^{2+} at these conditions). HvPPA did not hydrolyze detectable levels of nucleoside triphosphate (ATP, TTP, GTP or CTP) or nucleoside diphosphate (ADP) hydrolysis, a property useful for coupling PPAs with nucleotide-dependent enzymes in

assays. Based on these results, HvPPA catalyzes the hydrolysis of PPi with kinetic properties that are most closely related to *M. thermautotrophicus* PPA among the HPPAs of thermophilic and hyperthermophilic archaea (Table 3). The low affinity of HvPPA for Mg^{2+} and PPi based on K_m values is consistent with the unusually high levels of these types of ions within the cytosol of haloarchaea.

EXAMPLE 4 – HVPPA TOLERANCE TO HIGH TEMPERATURE AND ORGANIC SOLVENTS

HvPPA is thermostable with a thermal inactivation half-life of 2 h at 65°C; 82% activity remained after 2h at 42°C (Fig. 6A). HvPPA is not only thermostable but also stable in organic solvent with little if any inactivation of enzyme after 2 h incubation in buffer supplemented with 50% [v/v] DMSO, DMF, ethanol or methanol (Fig. 6B). HvPPA-mediated hydrolysis of PPi was also found to be robust in organic solvents, with the enzyme displaying 110-150% activity in buffers supplemented with 25% (v/v) methanol or ethanol and 63-94% activity in buffers with 25% (v/v) DMSO or DMF, compared to no solvent controls (Fig. 6C). HvPPA is more stable when stored in buffers supplemented with 2-3 M NaCl compared to 1.5 M NaCl or less (Fig. 6D). However, the enzyme was fully active over a wide range of salts (as noted earlier in Fig. 4C).

EXAMPLE 5 – HVPPA FOR DETECTION OF PPI BY-PRODUCT IN COUPLED ASSAY AT LOW WATER ACTIVITY

PPAs are not yet available for use in conditions of high salt or organic solvent to drive the activity of enzymes that generate PPi as a by-product. The use of HvPPA with a PPi-generating enzyme that functions in low water activity (high salt) by coupled assay is provided. In particular, HvPPA was used to monitor the PPi by-product of the ‘salt loving’ enzyme UbaA of *Hfx. volcanii* at 42°C in a buffer system with 2 M NaCl. UbaA has a NAD/FAD-binding fold domain common to ubiquitin activating E1 family enzymes and is required for the formation of ubiquitin-like bonds in archaea. UbaA is presumed to adenylate the C-terminal α -carboxylate group of ubiquitin-like proteins (named SAMPs in archaea) and release PPi as a by-product (Fig. 7A). To monitor this activity, HvPPA was used in a coupled assay to drive UbaA-mediated adenylation of SAMP1 and hydrolyze the PPi by-product to 2 Pi for detection by colorimetric assay. Significant levels of Pi were detected when UbaA and HvPPA were coupled with ATP and SAMP1 in the reaction (Fig. 7B). Pi was not detected

when ATP, UbaA, HvPPA or SAMP1 were omitted from the adenylation assay (Fig. 7B). Deletion of the C-terminal diglycine residues of SAMP1 (Δ GG) were found to significantly reduce the level of Pi detected by this assay. Likewise, the reaction was found highly specific for ATP, with little if any Pi generated enzymatically when ATP was replaced by other nucleotides (AMP, ADP, AMP-PNP, CTP, GTP, TTP and UTP) (Fig. 7B). Based on these results, HvPPA is useful for hydrolysis of PPi in coupled assays that require conditions of low water activity, moderately high temperatures and HvPPA can withstand high temperature pretreatments.

EXAMPLE 6 – HVPPA IS EVOLUTIONARILY, STRUCTURALLY AND BIOCHEMICALLY DISTINCT

PPAs are inactivated in dose dependent manner by organic solvents. In contrast, HvPPA provided in the instant invention displays thermostable and solvent tolerant properties and catalytic activities. HvPPA is useful for coupled assay with enzymes that generate PPi as a by-product and can perform this activity in conditions of high temperature and low water activity. In contrast, HvPPA opens new possibilities for the hydrolysis of PPi and related compounds in high salt and organic solvent systems to increase the solubility of hydrophobic substrates (such as lipids, phospholipids and fatty acids), and allows for novel synthetic chemistry, altering substrate specificity, easy of product recovery, and reducing microbial contamination.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims. In addition, any elements or limitations of any invention or embodiment thereof disclosed herein can be combined with any and/or all other elements or limitations (individually or in any combination) or any other invention or embodiment thereof disclosed herein, and all such combinations are contemplated within the scope of the invention without limitation thereto.

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CLAIMS

We claim:

1. An isolated inorganic pyrophosphatase from a microorganism belonging to family *Halobacteriaceae* (HPPA).
2. The isolated HPPA of claim 1, wherein the HPPA is encoded by a nucleotide having the sequence of SEQ ID NO: 1 and has the sequence of SEQ ID NO: 2.
3. The HPPA of claim 1, wherein the HPPA has the sequence of SEQ ID NO: 3 to 108.
4. An isolated nucleotide encoding the HPPA of claim 1.
5. A DNA construct comprising the nucleotide of claim 4.
6. A cell comprising the nucleotide of claim 4 or the DNA construct of claim 5.
7. The cell of claim 6, wherein the cell is a bacterial cell, archaeal cell, fungal cell, animal cell or plant cell.
8. A method of driving a reaction which produces PPi towards the production of PPi, the method comprising: adding the HPPA of claim 1 to the reaction mixture of the reaction which produces PPi.
9. The method of claim 8, wherein the reaction is an enzymatic reaction.
10. The method of claim 9, wherein the enzymatic reaction is performed under high temperature.
11. The method of claim 9, wherein the enzymatic reaction is performed under low water activity.

12. The method of claim 9, wherein the enzymatic reaction is performed under moderately high temperature, such as about 40°C to about 100°C, about 40°C to about 80°C, about 40°C to about 60°C, about 40°C to about 50°C or about 42°C and/or low water activity.

13. The method of claim 12, wherein low water activity comprises high salt concentration and/or high organic solvent concentration.

14. The method of claim 13, wherein the high salt concentration comprises the concentration of about 1M to about 5M, about 1.5 M to about 4M, about 2M to about 3M, or about 2.5 M.

15. The method of claim 13, wherein the high organic solvent concentration comprises the concentration of about 10% to about 50%, about 15% to about 40%, about 20% to about 30%, or about 25%.

16. The method of claim 15, wherein the organic solvent is dimethylsulfoxide, N,N-dimethylformamide, ethanol or methanol.

17. The method of claim 9, wherein the HPPA is thermostable at high temperature of about 50°C to about 100°C, about 60°C to about 90°C, about 70°C to about 80°C, or about 75°C.

18. The method of claim 9, wherein the enzymatic reaction which produces PPi is the biosynthesis of DNA, RNA, protein, peptidoglycan, lipids, cellulose, starch or the post-translational modification of proteins.

19. The method of claim 18, wherein the biosynthesis of DNA or RNA comprises DNA polymerization in a polymerase chain reaction, SNP genotyping or RNA synthesis by RNA polymerases.

20. The method of claim 18, wherein the synthesis of protein comprises aminoacyl-tRNA synthesis.

21. The method of claim 18, wherein the post-translational modification of proteins is adenylation, uridylation or ubiquitylation.

22. A composition comprising a reaction mixture for a reaction which produces PPi and the HPPA of claim 1.

23. The composition of claim 22, wherein the reaction is an enzymatic reaction.

24. The composition of claim 23, wherein the enzymatic reaction mixture comprises one or more substrates for the reaction, an enzyme which catalyzes the production of PPi and one or more co-factors.

25. The composition of claim 22, wherein the HPPA has the amino acid sequence of SEQ ID NO: 2.

26. The composition of claim 22, wherein the HPPA has the amino acid sequence of SEQ ID NOs: 3 to 108.

27. The composition according to any one of claims 22-26, wherein said reaction mixture contains high salt concentration(s) and/or high organic solvent concentration(s) and/or is at a moderately high temperature.

28. The composition of claim 27, wherein the high salt concentration comprises the concentration of about 1M to about 5M, about 1.5 M to about 4M, about 2M to about 3M, or about 2.5 M.

29. The composition of claim 27, wherein the high organic solvent concentration comprises the concentration of about 10% to about 50%, about 15% to about 40%, about 20% to about 30%, or about 25%.

30. The composition of claim 29, wherein the organic solvent is dimethylsulfoxide, N,N-dimethylformamide, ethanol or methanol.

31. The composition of claim 27, wherein the moderately high temperature comprises a temperature of about 40°C to about 100°C, about 40°C to about 80°C, about 40°C to about 60°C, about 40°C to about 50°C or about 42°C.

32. An assay of determining the reaction kinetics of a reaction which produces P_{Pi}, the method comprising the steps of:

- a) conducting the reaction which produces P_{Pi},
- b) periodically separating an aliquot of the reaction mixture from the enzymatic reaction which produces P_{Pi} and immediately subjecting the aliquots to a condition which stops the reaction which produces P_{Pi} to obtain a plurality of aliquots corresponding to various time points for the reaction which produces P_{Pi},
- c) introducing sufficient amount of an HPPA to each of the plurality of the aliquots,
- d. incubating the HPPA with each of the plurality of aliquots under suitable conditions and for sufficient amount of time to allow the HPPA to convert all of the P_{Pi} present in the aliquot in to P_i,
- e) determining the amount of P_i present in each of the plurality of aliquots, and
- f) determining the reaction kinetics of the reaction which produces P_{Pi} based on the amount of P_i in each of the plurality of aliquots.

33. The method of claim 32, wherein the HPPA has the amino acid sequence of SEQ ID NO: 2.

34. The method of claim 32, wherein the HPPA has the amino acid sequence of SEQ ID NOs: 3 to 108.

35. The method of claim 32, wherein the condition which stops the reaction which produces P_{Pi} comprises exposing the aliquots to the temperature at which the enzyme producing P_{Pi} is inactivated, adding an inhibitor of the enzyme producing P_{Pi} or adding a reagent which stops the reaction producing P_{Pi}.

36. The method of claim 32, wherein determining the amount of P_i present in each of the plurality of aliquots is performed in a colorimetric assay.

37. The method of claim 36, wherein the colorimetric assay is a malachite green based assay.

38. An assay for monitoring the reaction and/or determining the reaction kinetics for a reaction which produces PPi in the presence and/or the absence of a HPPA from a microorganism belonging to the family *Halobacteriaceae*, the method comprising the steps of:

- a) conducting the reaction which produces PPi in the presence of the HPPA,
- b) conducting the reaction which produces PPi in the absence of the HPPA,
- b) periodically separating aliquots from the reaction mixtures in the presence or the absence of the HPPA and immediately subjecting the aliquots to a condition which stops the reaction which produces PPi to obtain a plurality of aliquots corresponding to various time points for the reactions which produces PPi in the presence or the absence of the HPPA,
- c) determining the amount of Pi present in each of the plurality of aliquots, and
- d) based on the amount of Pi present in each of the plurality of aliquots, determining the reaction kinetics for the reaction which produces PPi in the presence or the absence of the HPPA, and
- e) determining the effect of the presence of the HPPA on the reaction producing PPi.

39. The method of claim 38, wherein the HPPA has the amino acid sequence of SEQ ID NO: 2.

40. The method of claim 38, wherein the HPPA has the amino acid sequence of SEQ ID NOs: 3 to 108.

41. The method of claim 38, wherein the condition which stops the reaction which produces PPi comprises exposing the aliquots to the temperature at which the enzyme producing PPi is inactivated, adding an inhibitor of the enzyme producing PPi or adding a reagent which stops the reaction producing PPi.

42. The method of claim 38, wherein determining the amount of Pi present in each of the plurality of aliquots is performed in a colorimetric assay.

43. The method of claim 42, wherein the colorimetric assay is malachite green based assay.

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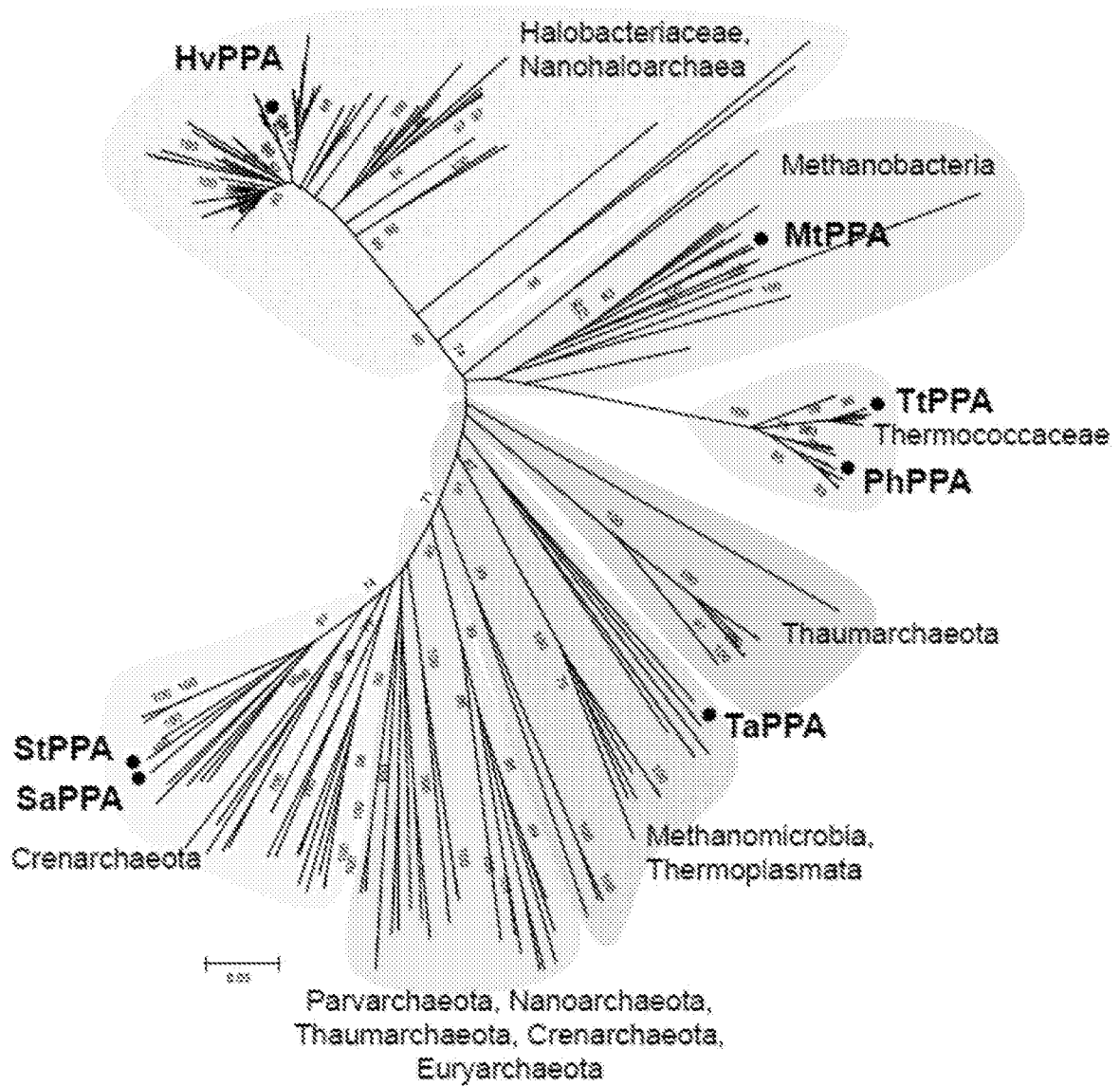


Figure 1

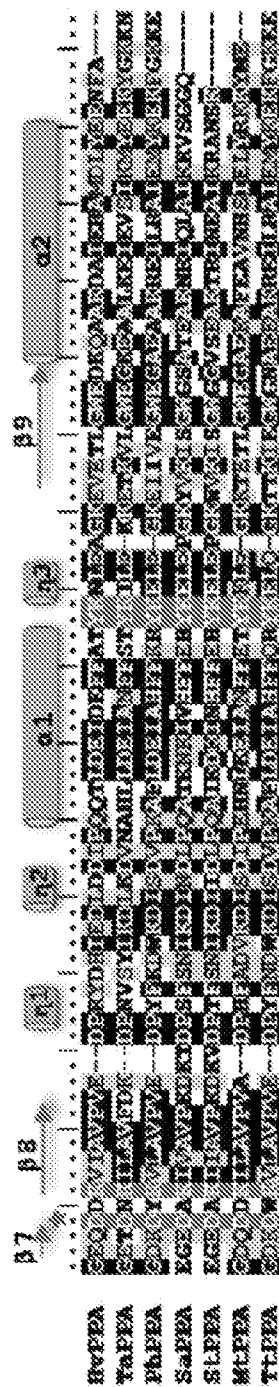
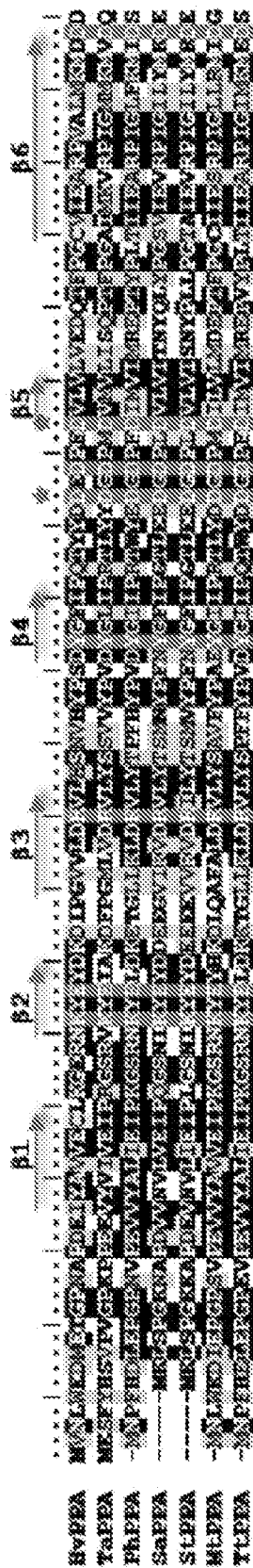


Figure 2A

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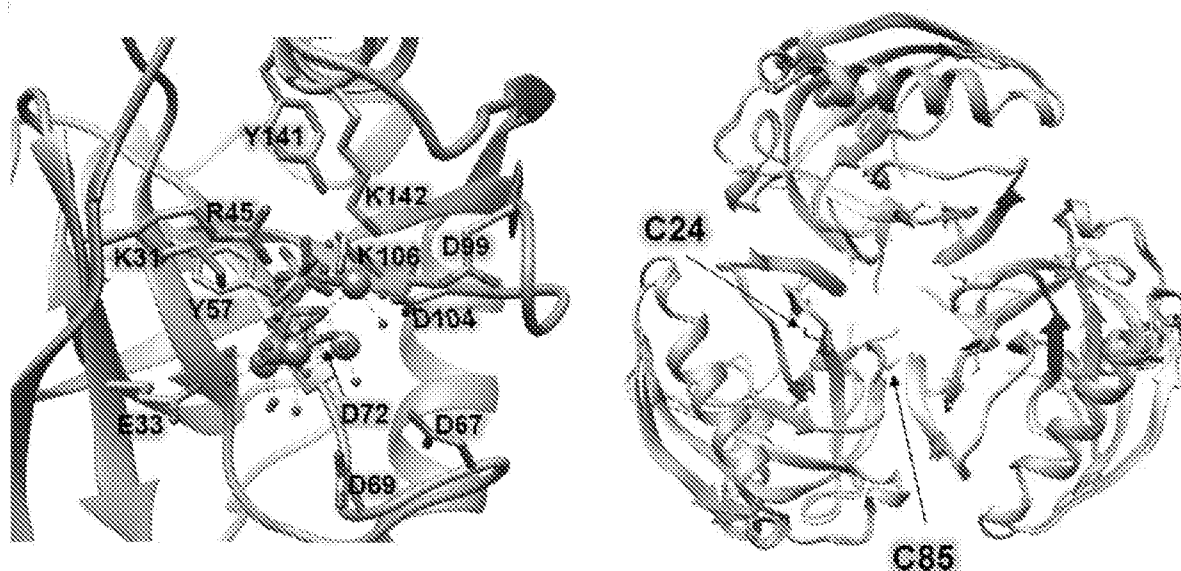


Figure 2B

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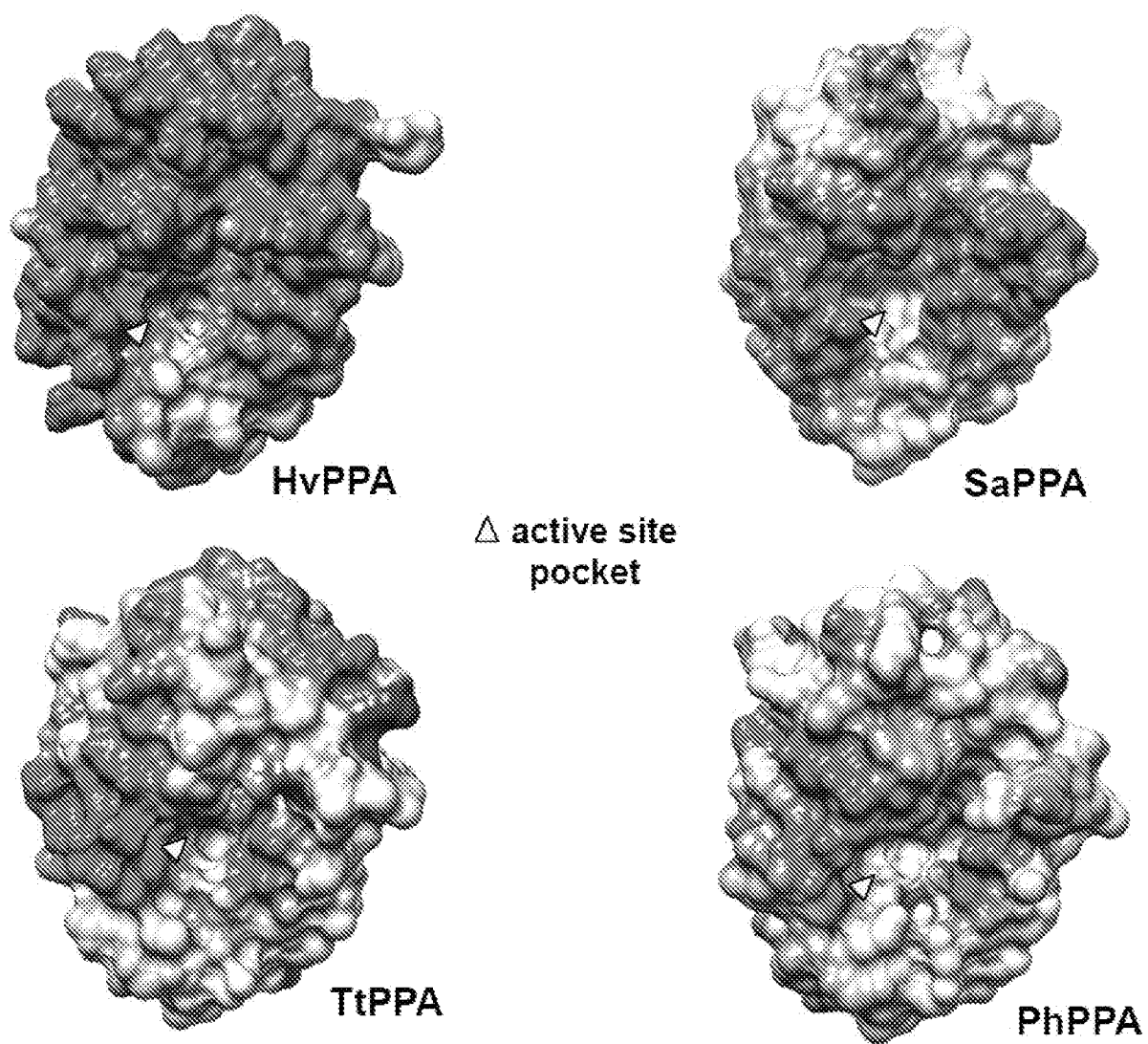


Figure 2C

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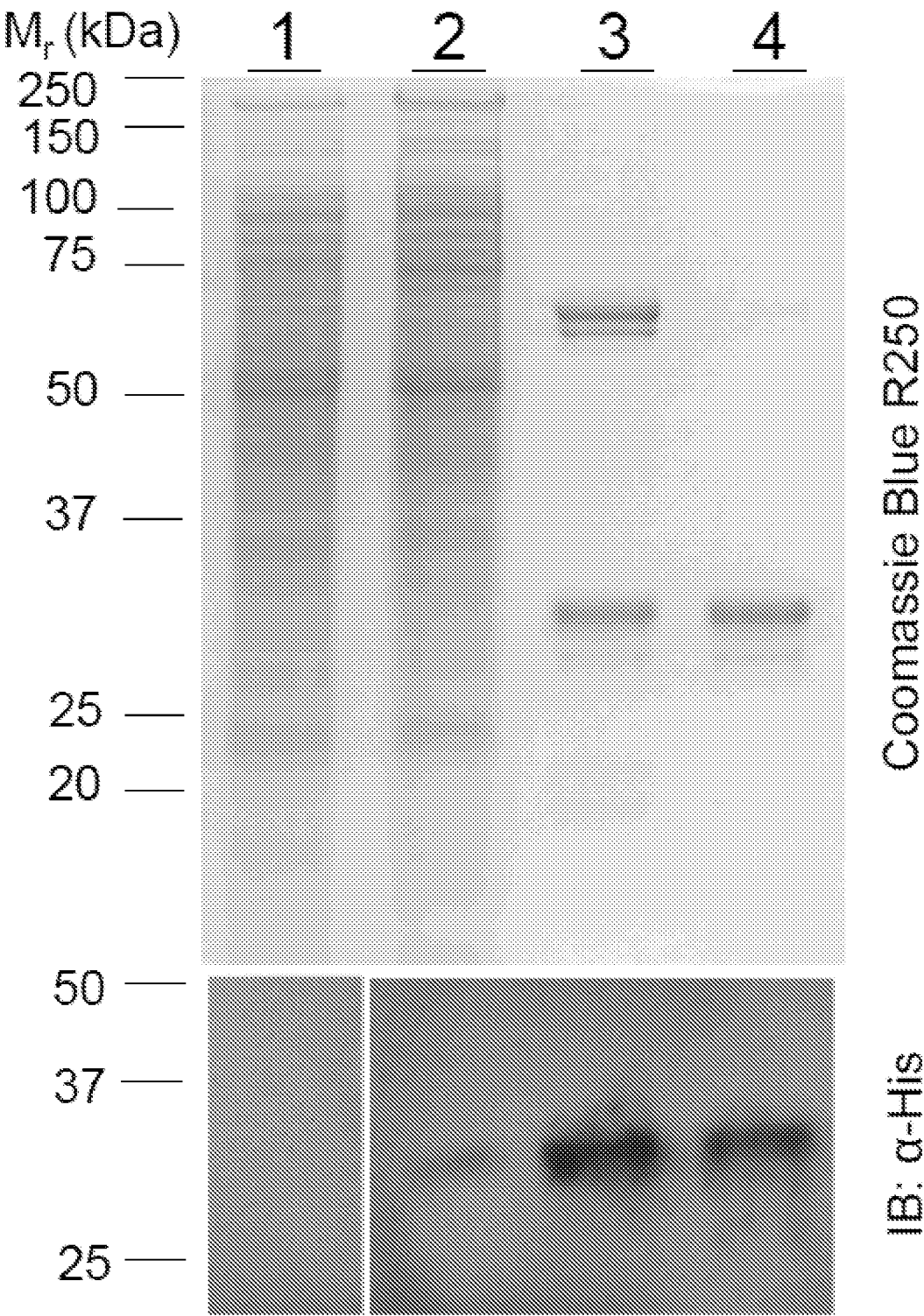


Figure 3A

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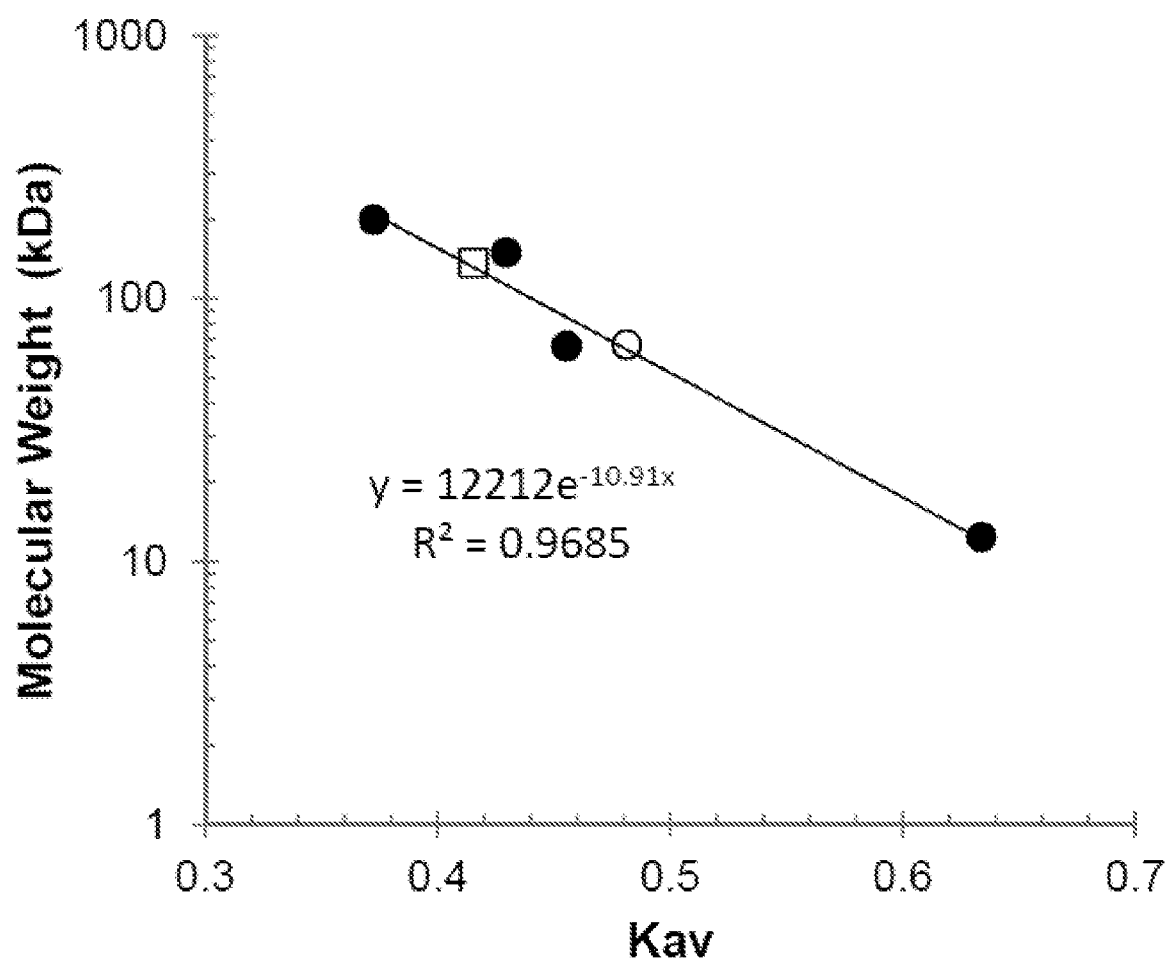


Figure 3B

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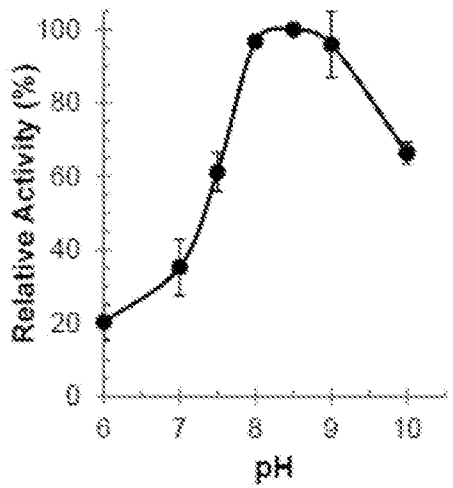


Figure 4A

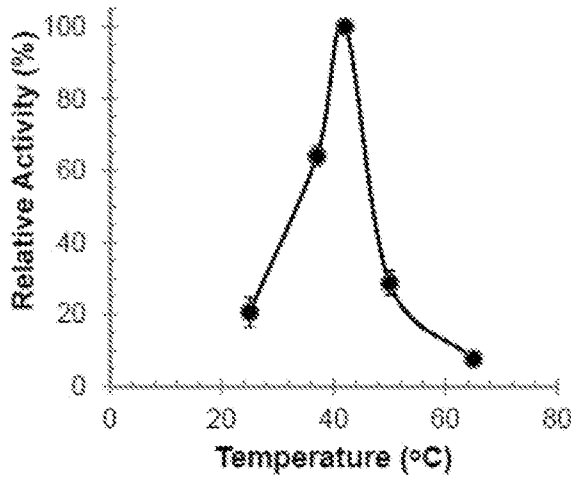


Figure 4B

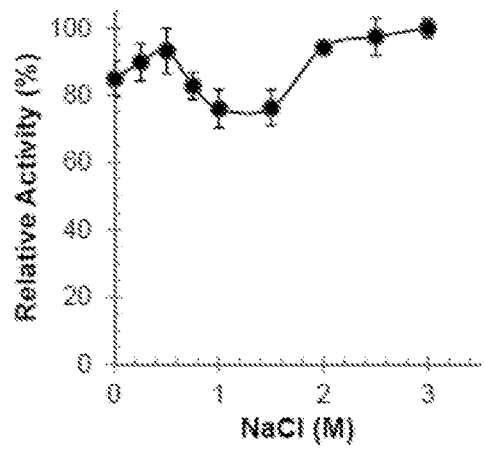


Figure 4C

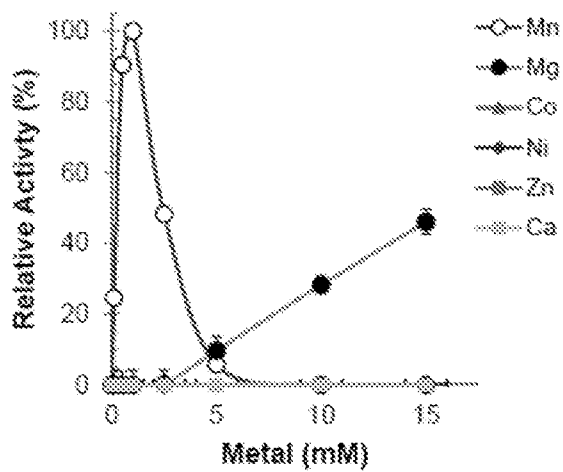


Figure 4D

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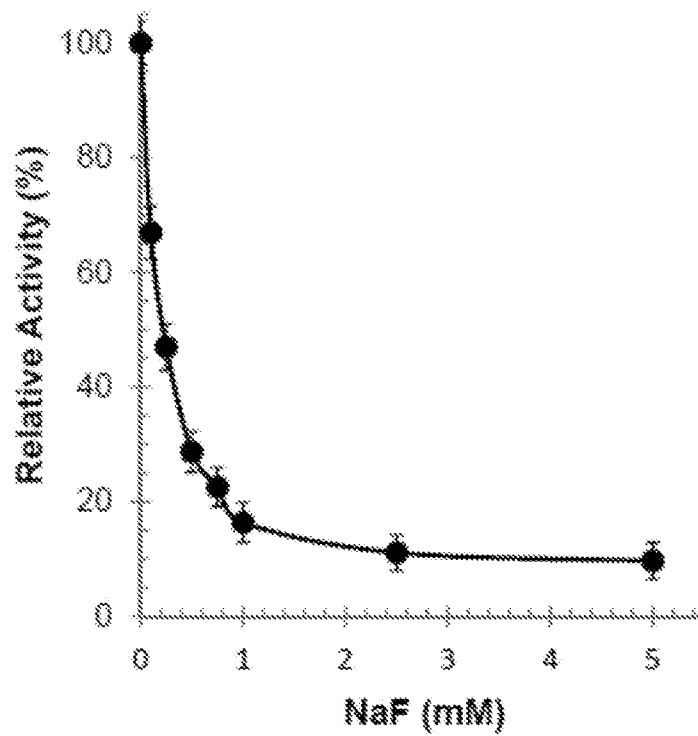


Figure 5A

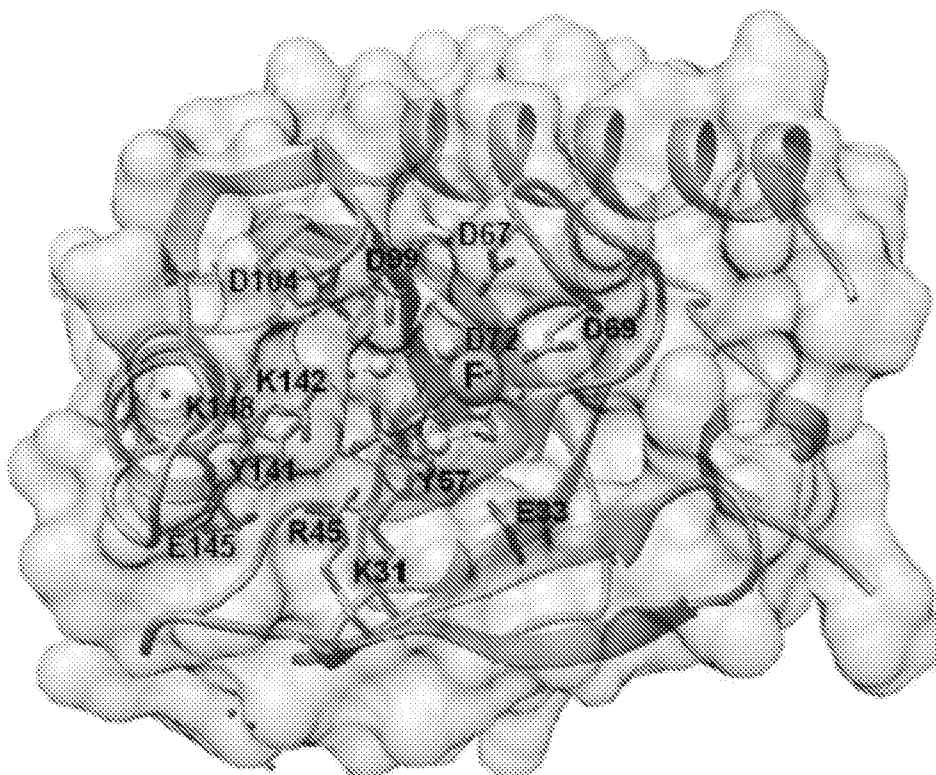


Figure 5B

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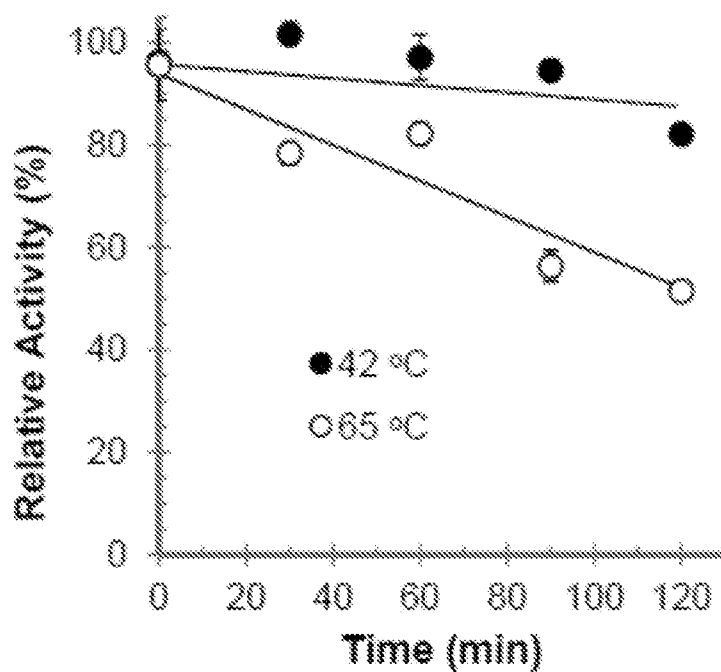


Figure 6A

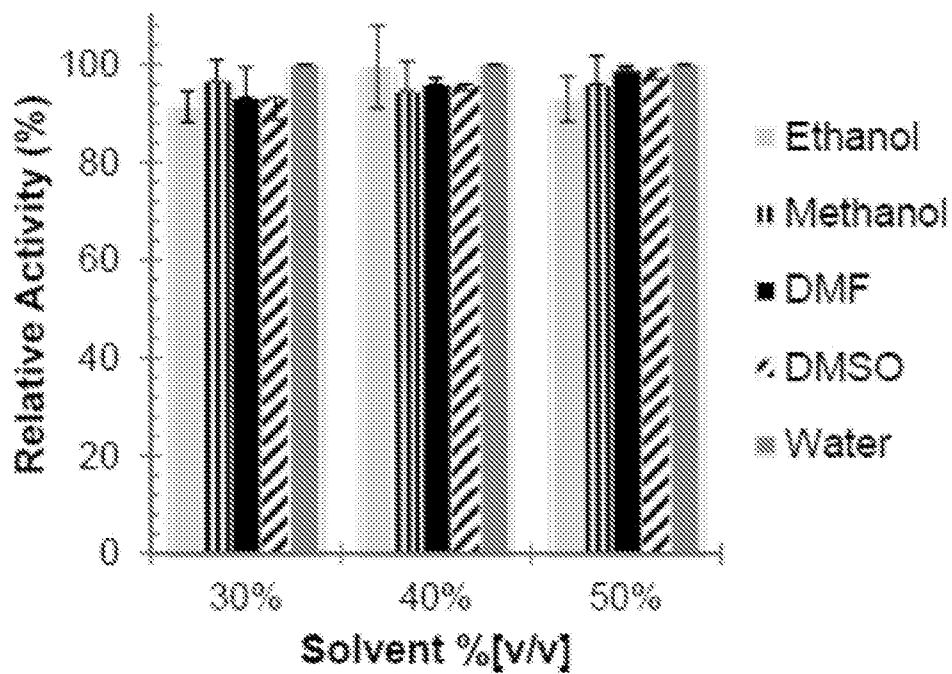


Figure 6B

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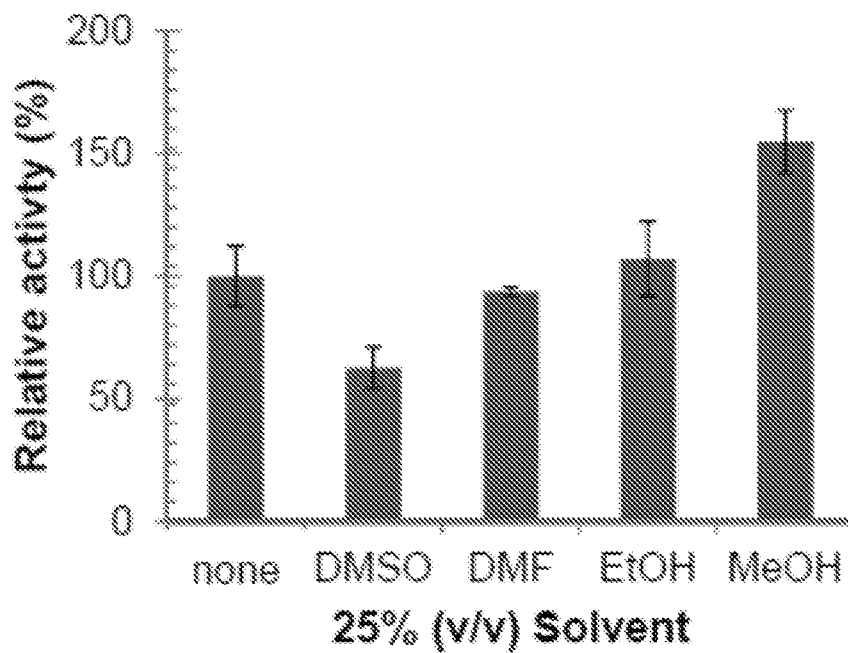


Figure 6C

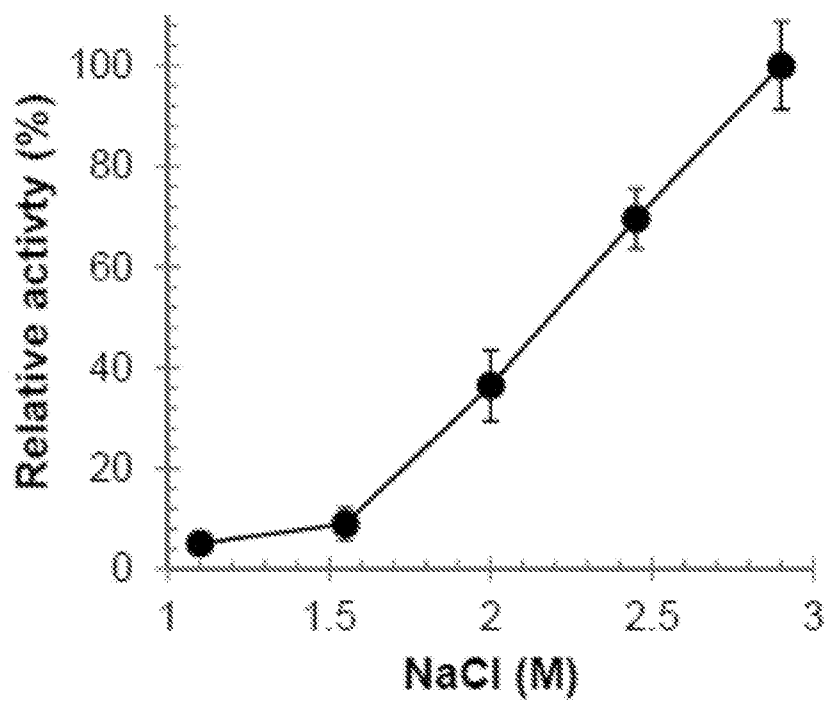


Figure 6D

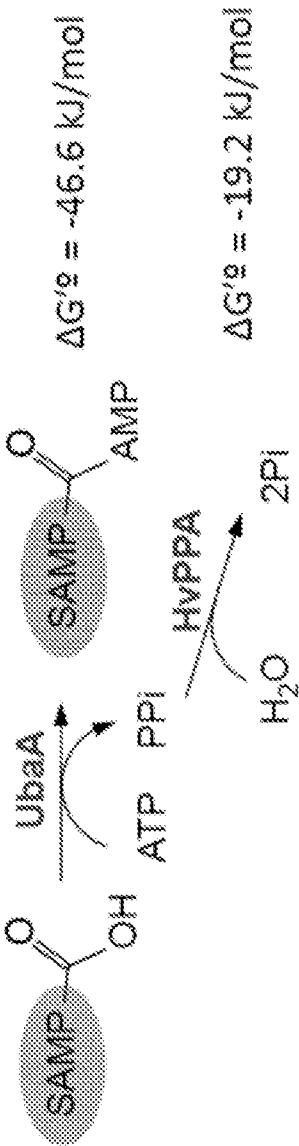


Figure 7A

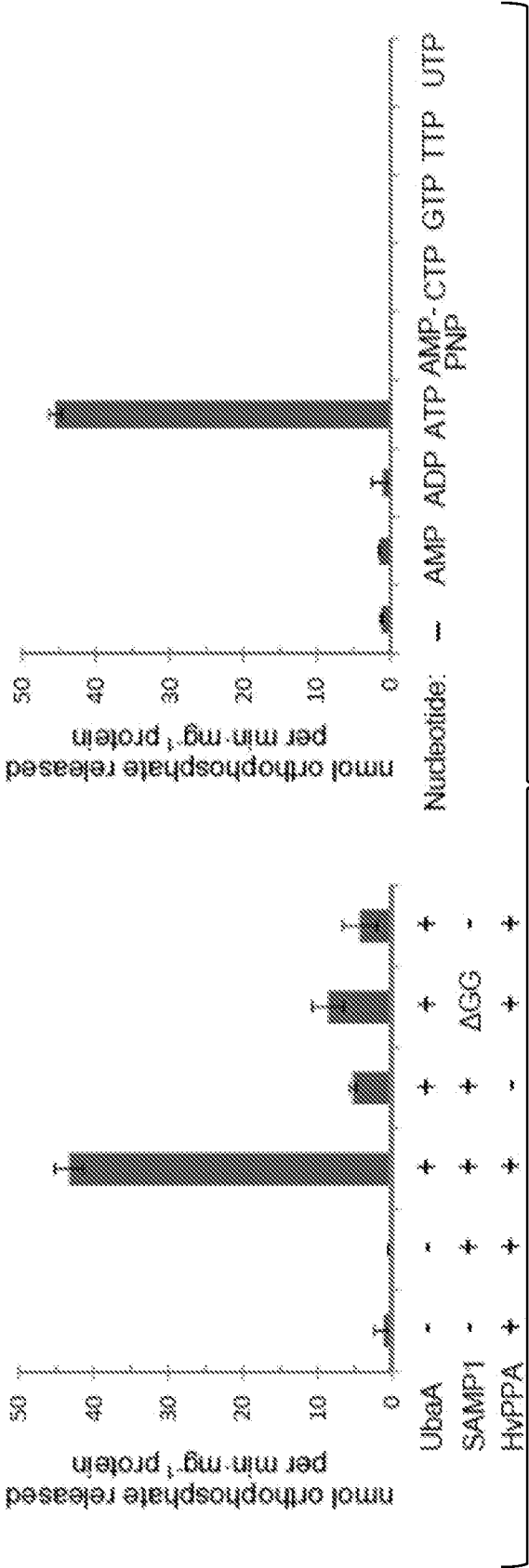


Figure 7B