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(72) Inventeurs/Inventors:
SHIBA, TOSHIKAZU, JP;
NOGUCHI, TOSHITADA, JP

(73) Propriétaire/Owner:
YAMASA CORPORATION, JP

(74) Agent: GOWLING LAFLEUR HENDERSON LLP

(54) Titre : NOUVELLE ENZYME POLYPHOSPHATE:AMP PHOSPHOTRANSFERASE
(54) Title: NOVEL POLYPHOSPHATE:AMP PHOSPHOTRANSFERASE

(57) Abrégé/Abstract:
This invention relates to a novel polyphosphate:AMP phosphotransferase (PAP), a gene coding this PAP, and their use. The PAP has the following properties: (A) action: catalyzing of the following two reactions: NMP + $PolyP_{(n)}$ → NDP + $PolyP_{(n-1)}$ dNMP + $PolyP_{(n)}$ → dNDP + $PolyP_{(n-1)}$ (wherein NMP represents nucleoside monophosphate, NDP represents nucleoside diphosphate, dNMP represents deoxynucleoside monophosphate, dNDP represents deoxynucleoside diphosphate, n represents degree of polymerization of the polyphosphate which is an integer of up to 100); (B) substrate specificity: specific to AMP, GMP, IMP, dAMP, and dGMP, also acting with CMP, UMP, dCMP, and TMP; (C) molecular weight: about 55 to 56 Kd (kilodalton); and (D) specific activity: at least 70 units per 1 mg of enzyme protein,

ABSTRACT

This invention relates to a novel polyphosphate:AMP phosphotransferase (PAP), a gene coding this PAP, and their use. The PAP has the following properties:

(A) action: catalyzing of the following two reactions:

$$NMP + PolyP_{(n)} \rightarrow NDP + PolyP_{(n-1)}$$

$$dNMP + PolyP_{(n)} \rightarrow dNDP + PolyP_{(n-1)}$$

(wherein NMP represents nucleoside monophosphate, NDP represents nucleoside diphosphate, dNMP represents deoxynucleoside monophosphate, dNDP represents deoxynucleoside diphosphate, n represents degree of polymerization of the polyphosphate which is an integer of up to 100);

(B) substrate specificity: specific to AMP, GMP, IMP, dAMP, and dGMP, also acting with CMP, UMP, dCMP, and TMP;

(C) molecular weight: about 55 to 56 Kd (kilodalton); and

(D) specific activity: at least 70 units per 1 mg of enzyme protein,

SPECIFICATION

Novel Polyphosphate:AMP Phosphotransferase

TECHNICAL FIELD

This invention relates to a novel polyphosphate:AMP phosphotransferase, a gene coding for this phosphotransferase, and their use.

BACKGROUND ART

Recent progress in genetic engineering has enabled large scale production of various enzymes at a low cost, and economical process using an enzymatic reaction has also been enabled for the production of physiologically active substances of value that has traditionally been produced by means of bioconversion using a live bacterium, fermentation, or chemical synthesis.

In the meanwhile, an enzymatic reaction requiring energy as in the case of phosphorylation and amination needs adenosine 5′-triphosphate (ATP) for its energy donor or phosphate donor. In the conventional microbial transformation and fermentation, ATP has been supplied by the microorganism employed, whereas, in the case of the enzymatic process, addition of ATP to the reaction system and development of efficient ATP regeneration system are required.

However, process of inexpensive ATP synthesis has not yet been established and commercially available ATP is still very expensive. In addition, both the substrate and

the enzyme used in the common ATP regeneration system, namely, the combination of phosphocreatine and phosphocreatine kinase, or acetyl phosphate and acetate kinase are very expensive, and their use has been unpractical and limited to the laboratory level.

In contrast to such high price of the ATP, adenosine 5′-monophosphate (AMP) can be produced at a relatively low cost. ATP is currently produced either by chemical synthesis or by using microorganism or yeast from AMP or adenine. Accordingly, development of an efficient ATP regeneration process has been highly awaited that can be used in the enzymatic reaction system using the ATP wherein the ATP is enzymatically produced from the relatively inexpensive AMP and the consumed ATP is efficiently regenerated instead of adding the expensive ATP.

In constructing a practical ATP generation/ regeneration system, selection of the phosphate donor used is also important, and polyphosphate, which is inexpensive and stable, has been considered the most promising candidate of the phosphate donor. Enzymes which are known to be involved in the metabolism of the polyphosphate and which also act with adenosine nucleotide include polyphosphate kinase and polyphosphate:AMP phosphotransferase (hereinafter abbreviated as "PAP").

PAP is an enzyme which phosphorylates AMP to produce ADP by using polyphosphate as the phosphate donor (J.Bacteriol., 173, 6484-6488(1991)). Zenhder et al. has reported that an ATP generation/regeneration system wherein

AMP and polyphosphate are the substrates functions, when PAP obtained from *Acinetobacter johnsonii* is partially purified, and the partially purified PAP is used in combination with adenylate kinase (Appl. Environ. Microbiol., 66, 2045-2051(2000)). Kameda et al. has reported that, in the enzymatic reaction system wherein ATP is consumed to generate AMP, a system wherein ATP is generated from AMP using polyphosphate as the phosphate donor functions efficiently when the combination of the PAP from *Myxococcus xanthus* and *E. coli* polyphosphate kinase is used.

However, PAP is present in the *Acinetobacter johnsonii* cell in an extremely small amount, and with regard to the use of the crude PAP such as cell extract, a problem has been pointed out that contamination of the enzymes which decompose the substance involved in the reaction (AMP, ADP, ATP, reaction substrate and/or reaction product) may invite loss of reaction efficiency. Such problem can be obviated by the use of highly purified PAP instead of the crude enzyme. PAP, however, is very unstable, and the purification procedure of PAP is far too complicated to adopt such purified PAP into practical use.

In coping with such problem, the inventors of the present invention estimated that such problem can be challenged by producing the PAP of *Acinetobacter johnsonii* in a large amount using a recombinant DNA technique. However, the amino acid sequence of the enzyme and the gene for such enzyme have not been reported at all.

DISCLOSURE OF THE INVENTION

The inventors of the present invention have succeeded in the mass-production of the PAP in E. coli by constructing a screening system adapted for the gene cloning by PAP activity, and cloning the gene coding for the PAP by using the thus constructed screening system. In the analysis of the thus produced recombinant PAP, the inventors also found that the enzyme has an extremely high specific activity, and combination of such enzyme with adenylate kinase enables construction of an efficient ATP generation/regeneration system.

Unexpectedly, the inventors also found that the recombinant PAP has the activity of phosphate-transfer using polyphosphate for the phosphate donor to generate nucleoside diphosphate even if the nucleoside monophosphate were not AMP or GMP, and this is a difference from the conventional PAP obtained from *Acinetobacter johnsonii*.

While nucleoside diphosphate has been generally found useful as a starting material for enzymatically synthesizing polynucleotide used in medical or chemical commodities, its synthesis has been far from easy. In the case of bioconversion, the phosphorylation can not be terminated at the diphosphate stage, and chemical phosphorylation has been the only usable way for the nucleoside diphosphate production. However, chemical phosphorylation is associated with the problem of simultaneous occurrence of the side reaction that results

in the formation of byproducts, and isolation and purification of the target nucleoside diphosphate from the reaction solution has been extremely complicated. In view of such situation, development of an efficient process for synthesizing the nucleoside diphosphate by enzymatic phosphorylation of the nucleoside monophosphate is highly awaited, and PAP has been conceived as one candidate for the enzyme used in the enzymatic synthesis.

However, Zehnder et al. has reported that, PAP from *Acinetobacter johnsonii* is AMP-specific, and while some phosphate-transfer is found for GMP, no phosphotransfer at all was found for other nucleotides (CMP, UMP, and IMP) (Appl. Environ. Microbiol., 66, 2045-2051 (2000)), and it has been conceived that, PAP can not be used in the synthesis of nucleoside diphosphate by enzymatic phosphorylation of the nucleoside monophosphate.

The inventors of the present invention have made an intensive study based on the novel findings as described above, and completed the present invention. This invention relates to the PAP (the PAP of the present invention) having the physical and chemical properties as described below:

(A) action: catalyzing the following two reactions:

$$NMP + PolyP_{(n)} \rightarrow NDP + PolyP_{(n-1)}$$

$$dNMP + PolyP_{(n)} \rightarrow dNDP + PolyP_{(n-1)}$$

(wherein NMP represents nucleoside monophosphate, NDP represents nucleoside diphosphate, dNMP represents deoxynucleoside monophosphate, dNDP represents

deoxynucleoside diphosphate, n represents degree of polymerization of the polyphosphate which is an integer of up to 100);

(B) substrate specificity: specific to AMP, GMP, IMP, dAMP, and dGMP, also acting with CMP, UMP, dCMP, and TMP;

(C) molecular weight: about 55 to 56 Kd (kilodalton); and

(D) specific activity: at least 70 units per 1 mg of enzyme protein.

This invention also relates to a PAP having the amino acid sequence of SEQ ID NO: 1 or the amino acid sequence of SEQ ID NO: 1 wherein deletion, substitution, or addition of one to several amino acids has occurred.

This invention also relates to a PAP gene encoding the amino acid sequence of SEQ ID NO: 1 or the amino acid sequence of SEQ ID NO: 1 wherein deletion, substitution, or addition of one to several amino acids has occurred.

This invention also relates to a PAP gene having the nucleotide sequence of SEQ ID NO: 2 or the nucleotide sequence of SEQ ID NO: 2 wherein deletion, substitution, or addition of one to several nucleotides has occurred.

This invention also relates to a DNA fragment which hybridizes with the gene as described above under stringent conditions, and which codes for the polypeptide having PAP activity.

This invention also relates to a method for producing nucleoside diphosphate wherein the nucleoside diphosphate is enzymatically produced from nucleoside monophosphate by

using the PAP of the present invention as the enzyme and polyphosphate as a phosphate donor.

This invention also relates to a method for producing ATP wherein ATP is enzymatically produced from AMP by using two enzymes, namely, the PAP of the present invention and adenylate kinase as the enzyme and polyphosphate as a phosphate donor.

This invention also relates to an ATP generation/regeneration system comprising AMP, polyphosphate, PAP, and adenylate kinase wherein the PAP used is the PAP of the present invention.

Finally, this invention also relates to a method for producing a compound by using an ATP-consuming enzymatic reaction, wherein ATP is regenerated from the AMP simultaneously with the enzymatic reaction by using an ATP regeneration system comprising polyphosphate, PAP, and adenylate kinase, and wherein the PAP used is the PAP of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a restriction map of the DNA fragment of about 10 kb including the PAP gene obtained from *Acinetobacter johnsonii* strain 210A. The PAP gene is included in SacI-HpaI DNA fragment.

FIG. 2 shows the first half of the nucleotide sequence of the 2.5 kb DNA fragment including the PAP gene, and the first half of the amino acid sequence of the PAP.

FIG. 3 shows the second half of the nucleotide sequence of the 2.5 kb DNA fragment including the PAP gene, and the second half of the amino acid sequence of the PAP.

FIG. 4 shows the results of the pH stability evaluation according to the PAP of the present invention.

FIG. 5 shows the results of the optimal pH evaluation according to the PAP of the present invention.

FIG. 6 shows the results of the thermal stability evaluation according to the PAP of the present invention.

FIG. 7 shows the results of the optimal temperature evaluation according to the PAP of the present invention.

FIG. 8 shows synthesis of ADP and ATP from AMP using polyphosphate for the phosphate donor and using the PAP and adenylate kinase.

BEST MODE FOR CARRYING OUT THE INVENTION

(1) PAP of the present invention

The PAP of the present invention has the physical and chemical properties as described below. (Also, see the Examples as will be presented later.)

(A) Action: PAP catalyzes the following two reactions:

$$NMP + PolyP_{(n)} \rightarrow NDP + PolyP_{(n-1)}$$

$$dNMP + PolyP_{(n)} \rightarrow dNDP + PolyP_{(n-1)}$$

(wherein NMP represents nucleoside monophosphate, NDP represents nucleoside diphosphate, dNMP represents deoxynucleoside monophosphate, dNDP represents deoxynucleoside diphosphate, n represents degree of

polymerization of the polyphosphate which is an integer of up to 100).

(B) Substrate specificity: PAP acts specifically to AMP, GMP, IMP, dAMP, and dGMP, and it also acts with CMP, UMP, dCMP, and TMP.

(C) Molecular weight: PAP has a molecular weight of about 55 to 56 Kd (kilodalton).

(D) Specific activity: PAP has a specific activity of at least 70 units per 1 mg of enzyme protein.

(E) Optimal pH: around pH 8.5.

(F) Optimal temperature: around 50°C.

(G) pH stability: around pH 7 to 9.

(H) Thermal stability: PAP is stable up to about 50°C.

The "unit" used herein is the unit measured under the following conditions, and 1 unit corresponds to the activity of producing 1 μmole of ADP at 37°C in 1 minute.

<Measurement conditions>

Sample enzyme is added to 50mM Tris HCl buffer solution (pH 7.8) containing 20mM magnesium chloride, 10mM AMP, and polyphosphate (30mM calculated in terms of inorganic phosphate) and the reaction is allowed to proceed by incubating the temperature at 37°C, and the reaction is thereafter terminated by a heat treatment at 100°C for 1 minute, and amount of ADP in the reaction solution is measured by high performance liquid chromatography (HPLC).

The PAP of the present invention has the amino acid sequence represented by SEQ ID NO: 1, and in particular, the recombinant PAP produced by recombinant DNA process is

substantially pure in terms of enzymatic activity, and such recombinant PAP is free from the AMP-degrading activity which is disadvantageous for phosphorylation of the AMP.

The amino acid sequence may also be the one wherein deletion, substitution, modification, or addition of one to several amino acids has occurred as long as the activity of catalyzing the reactions as described above is retained. The deletion, substitution, modification, or addition of the amino acid sequence may be induced by site specific mutagenesis (for example, Proc. Natl. Acad. Sci. USA, 81, 4662-5666(1984); Nucleic Acid Res. 10, 6487-6500(1982); Nature 316, 601-605(1985)) or the like which were known in the art before the filing of the present invention. The PAP of the present invention also includes an enzyme which has a homology of at least 90%, and more preferably at least 95% with the amino acid sequence of SEQ ID NO: 1 as long as the activity of catalyzing the reactions as described above is retained.

The PAP of the present invention is prepared by cloning the gene coding for the enzyme having the amino acid sequence shown in SEQ ID NO: 1, typically, the PAP gene having the nucleotide sequence shown in SEQ ID NO: 2 from *Acinetobacter johnsonii*, and using this gene. When explained by referring to an exemplary case of the gene from *Acinetobacter johnsonii*, FIGS. 2 and 3 show the sequencing result of the nucleotide sequence of the DNA fragment cleaved by SacI and HpaI in the restriction map of FIG. 1, and the sequence of nucleotide numbers 604 to 2031

in FIGS. 2 and 3 corresponds to the structural gene of PAP, and this sequence is the same as the nucleotide sequence shown in SEQ ID NO: 2.

In the present invention, a gene having the nucleotide sequence of SEQ ID NO: 2 wherein deletion, substitution, insertion, or addition of one or plural nucleotides has occurred in such sequence; a gene which hybridizes with such gene under stringent conditions; and a gene which has a homology of at least 90%, and more preferably at least 95% with the nucleotide sequence shown in SEQ ID NO: 2 may also be employed as long as the gene is capable of producing the PAP of the present invention.

As in the case of the amino acid sequence as described above, the gene wherein deletion, substitution, insertion, or addition of single or plural nucleotides has occurred means the gene wherein nucleotides of the number that can be deleted, substituted, inserted, or added by the technique known in the art such as site specific mutagenesis has been deleted, substituted, inserted, or added. The hybridization under stringent conditions means the hybridization using a solution containing 5× SSC (1× SSC corresponds to 8.76 g of sodium chloride and 4.41 g of sodium citrate dissolved in 1 liter of water), 0.1% w/v N-lauroylsalcosine sodium salt, 0.02% w/v SDS, and 0.5% w/v blocking agent, under the reaction temperature conditions of about 60°C for about 20 hours.

In the present invention, use of a gene further comprising SD sequence (Shine-Dalgarno Sequence) in the

upstream the gene coding for the PAP is also favorable, since the yield of the enzyme can be markedly improved by the use of such gene.

Cloning of such gene, preparation of the expression vector using the thus cloned DNA fragment, preparation of the PAP using such expression vector, and the like are well known to those skilled in the field of molecular biology, and such process may be done, for example, by using the procedure described in "Molecular Cloning" (Maniatis et al. ed., Cold Spring Harbor Laboratories, Cold Spring Harbor, New York (1982)).

In an exemplary method, amino acid sequence on N-terminal, C-terminal, or the like of the PAP purified from a microorganism belonging to genus *Acinetobacter* may be partly sequenced by a known method, and the oligonucleotide corresponding to the thus determined sequence is synthesized. Then, the DNA fragment containing the gene coding for the PAP may be cloned from the chromosomal DNA of the bacterium belonging to genus *Acinetobacter* by using the thus synthesized oligonucleotide as the probe. Alternatively, chromosomal DNA may be cleaved by using appropriate restriction enzymes, and a genomic library may be produced by the method commonly used in the art. The resulting genomic library may be screened based on the PAP activity to thereby clone the target gene.

There is, however, a fair risk that the PAP is inactivated when the PAP is purified to a high degree, and therefore, use of a screening system using the PAP activity

is preferable. The PAP activity used in the screening is preferably the activity of generating ATP from AMP by the combination of the PAP with polyphosphate kinase (PPK). More specifically, PAP and PPK may be used for generation of the ATP using AMP for the substrate and isotope-labeled polyphosphate for the phosphate donor to thereby detect the generation of the isotope-labeled ATP.

The host used for the cloning is not particularly limited. Use of *E. coli*, however, is preferable in view of the working and handling convenience.

Expression system with high expression rate of the cloned gene may be constructed by preparing a recombinant expression vector wherein expression regulatory signals (transcription initiation signal and translation initiation signal) are ligated in the upstream of the gene, after identification of the coding region of the gene through the method to sequence the nucleotide sequence of the cloned DNA fragment,for example, Maxam-Gilbert (Methods in Enzymology, 65, 499(1980)), dideoxy chain terminator method (Methods in Enzymology, 101, 20(1983)), or the like.

In order to produce the PAP in a large amount in the heterologous microorganism, the expression regulatory signals used are desirable transcription and translation initiation signals which can be regulated by an artificial means, and which are strong signals enabling a dramatic increase in the PAP yield. Examples of transcription initiation signal which can be used when the host is *E. coli* include lac promoter, trp promoter, and tac promoter

(Proc. Natl. Acad. Sci. USA., 80, 21(1983), and Gene, 20, 231(1982)), and trc promoter (J. Biol. Chem., 260, 3539(1985)).

Exemplary vectors which can be used include plasmid vector, phage vector, and other vectors, and use of a plasmid vector is desirable since it can be amplified in the microorganism, it includes an adequate drug-resistant marker and cleavage site for the predetermined restriction enzymes, and it is copied in a large number in the microorganism. Examples of the plasmid vectors which can be used when the host is *E. coli* include pBR322 (Gene, 2, 95(1975)), pUC18, and pUC19 (Gene, 33, 103(1985)).

The microorganism is transformed by using the thus produced recombinant vector. The microorganism used for the host is not particularly limited as long as it has high safety and handling convenience, and *E. coli*, yeast, and other microorganisms commonly used in the DNA recombinant techniques can be used as desired. Use of *E. coli*, however, is favorable, and exemplary strains include K12, C600, JM105, JM109 (Gene, 33, 103-119(1985)), and other strains normally used in the DNA recombinant experiments.

Various methods have been reported for use in the transformation of a microorganism, and an adequate method may be selected depending on the type of the microorganism used for the host. When *E. coli* is used for the host, *E. coli* may be transformed by the treatment with calcium chloride followed by the introduction of the plasmid to the

interior of the cell at low temperature (J. Mol. Biol., 53, 159 (1970)).

The resulting transformant is cultivated in a culture medium wherein the microorganism is propagatable, and the cultivation is continued and the expression of the cloned PAP gene is induced until a large amount of the PAP is accumulated in the microorganism. The transformant may be cultivated by a method commonly used in the art using a culture medium containing a carbon source, a nitrogen source, and other nutrient sources necessary for the propagation of the microorganism cultivated. For example, when *E. coli* is used for the host, the culture medium used may be those commonly used for the cultivation of *E. coli* such as 2xYT medium (Methods in Enzymology, 100, 20 (1983)), LB medium, M9CA medium (Molecular Cloning, supra), and the cultivation may be conducted at an incubation temperature of 20 to 40°C with aeration shaking. When a plasmid is used for the vector, an appropriate antibiotic agent (ampicillin, kanamycin, or the like corresponding to the drug-resistant marker) of an appropriate amount is added to the culture medium to thereby prevent loss of the plasmid in the course of the cultivation.

When the expression of the PAP gene should be induced during the cultivation, the gene expression may be induced by the method commonly used with the promoter used. For example, when the promoter used is lac promoter or tac promoter, an expression inducing agent such as isopropyl-β-D-thiogalactopyranoside (hereinafter abbreviated to as

IPTG) may be added at an appropriate amount in the intermediate period of the cultivation.

The cells are collected from the thus produced culture by membrane separation, centrifugation, or the like. While the collected cells may be used as PAP with no further treatment, the PAP used is preferably a cell-free extract prepared by suspending the cells collected in an appropriate buffer solution, lyzing the cell by a physical treatment using ultrasonication, French press, or the like, or by an enzymatic treatment using lysozyme or the like, and removing the bacterial residue to obtain the extract. While cell-free extract may be used for the enzyme source with no further treatment since the cell-free extract contains an abundant amount of PAP, the PAP used may also be a partially purified product or a purified product prepared by either one or combination of two or more of heat treatment, ammonium sulfate precipitation, dialysis, treatment using a solvent such as ethanol, various chromatographic treatments, and other treatments commonly used in the enzyme purification.

(2) Use of PAP in the present invention

The thus prepared PAP of the present invention can be used in the synthesis of nucleoside diphosphate or deoxynucleoside diphosphate, and in the synthesis or regeneration of the ATP.

First, the nucleoside 5′-monophosphate (NMP) or the deoxynucleoside 5′-monophosphate (dNMP) used in the synthesis of nucleoside 5′-diphosphate (NDP) or

deoxynucleoside 5′-diphosphate (dNDP) may be a commercially available product, and it may be used at a concentration in the range of, for example, 1 to 200mM, and preferably 10 to 100mM.

The polyphosphate used may also be a commercially available product, and it may be used at a concentration in the range of 1 to 1000mM, and preferably 10 to 200mM calculated in terms of inorganic phosphate. The degree of polymerization (n) of the polyphosphate may be up to 100, and preferably about 10 to 50.

The synthesis of the NDP or dNDP may be accomplished by the reaction wherein NMP or dNDP and polyphosphate are added to a buffer solution at a pH in the range of 4 to 9, and at least 0.001 unit/ml, and preferably 0.001 to 10 units/ml of the PAP of the present invention is added to the solution at 20°C or higher, and preferably at 30 to 40°C for about 1 to 50 hours with optional stirring.

The thus produced NDP or dNDP may be isolated and purified by a chromatographic process or other process known in the art.

The ATP synthesis may be accomplished by converting AMP to ADP, and then, to ATP by using the PAP of the present invention and adenylate kinase in the presence of polyphosphate.

The AMP added to the reaction solution may be a commercially available product, and it may be used at an adequate concentration selected from the range of, for example, 1 to 200mM, and preferably 10 to 100mM.

The polyphosphate added may also be a commercially available product, and it may be used at a concentration selected from the range of 1 to 1000mM, and preferably 10 to 200mM calculated in terms of inorganic phosphate. The degree of polymerization (n) of the polyphosphate may be up to 100, and preferably about 10 to 50.

The synthesis of the ATP may be accomplished by adding AMP and polyphosphate in an appropriate buffer solution at pH in the range of 4 to 9, and further adding at least 0.001 unit/ml, and preferably 0.001 to 10 units/ml of the PAP of the present invention and at least 0.01 unit/ml, and preferably 0.01 to 100 units/ml or more of adenylate kinase to allow the reaction to proceed at 20°C or higher, and preferably at 30 to 40°C for about 1 to 50 hours with optional stirring.

The thus generated ATP may be isolated and purified by a chromatographic method or other method known in the art.

The unit of the adenylate kinase activity is the one measured and calculated by the procedure as described below. Sample enzyme is added the 50mM Tris HCl buffer solution (pH 7.8) containing 10mM magnesium chloride, 10mM AMP, and 10mM ATP, and the solution is incubated at 37°C to promote the reaction, and the reaction is terminated by a 1 minute heating at 100°C. Amount of the ADP in the reaction solution is measured by using HPLC, and the activity of producing 2 μmole of ADP at 37°C in 1 minute is designated 1 unit.

The ATP regeneration/regeneration system comprising the AMP, the polyphosphate, the PAP of the present invention, and the adenylate kinase may be used in detecting a minute amount of ATP in the course of detecting invisible microorganisms to check the cleanliness of the food in the food plant, or in the detection of adenine nucleotide by bioluminescence, which is applicable in the measurement of freshness of meat, fish, vegetable, and other foods (see, for example, WO01/53513).

The ATP regeneration system comprising polyphosphate, the PAP of the present invention, and adenylate kinase is also applicable to the production of a compound using an ATP-consuming enzymatic reaction for allowing the ATP regeneration from the AMP to take place simultaneously with the enzymatic synthesis of the target compound thereby improving the efficiency of the synthesis.

Exemplary non-limited enzymatic reactions which can be combined with such ATP regeneration system include galactose-1-phosphate synthesis system using galactokinase, UDP synthesis system using UMP kinase, and phosphocholine synthesis system using choline kinase, and the ATP regeneration system may be applied to any ATP-consuming enzymatic reaction.

The reaction conditions of the ATP synthesis system and the enzymatic reaction may be adequately determined by conducting tests in smaller scale, and the isolation and the purification of the target compound may be accomplished by a method known in the art.

EXAMPLES

Next, the present invention is described in further detail by referring to the Examples, which by no means limit the scope of the invention. In the Examples, preparation of the DNA, cleavage with the restriction enzyme, DNA ligation by using T4 DNA ligase, and transformation with the DNA were all conducted in accordance with "Molecular cloning II" (Sambrook et al. ed., Cold spring Harbor Laboratory, Cold Spring Harbor, New York (1989)). The restriction enzyme, AmpliTaq*DNA polymerase, T4 DNA ligase, and other DNA related enzymes were all obtained from Takara Shuzo K.K. Quantitative measurement of the nucleotides were conducted by HPLC, and more specifically, ODS-AQ312 column manufactured by YMC was used for separation purpose with the eluent of 0.5M monopotassium phosphate solution.

Example 1: Preparation of the PAP of the present invention

(1) Cloning of the PAP gene of *Acinetobacter johnsonii* strain 210

(1-1) *E. coli* polyphosphate kinase and isotope-labeled polyphosphate

E. coli polyphosphate kinase was prepared by the method described in the document (J. Biol. Chem., 268, 633-639 (1993). Isotope-labeled polyphosphate was prepared using the thus prepared *E. coli* polyphosphate kinase

* Trade-mark

according to the method of Akiyama et al. (J. **Biosci. Bioeng.**, **91, 557-563 (2001)**.

(1-2) Production of genomic library of *Acinetobacter johnsonii* and its screening

Acinetobacter johnsonii strain 210A was inoculated in LB medium, and the cultivated overnight at 30°C with stirring. The cells were collected by centrifugation, and chromosomal DNA was purified. The chromosomal DNA of *Acinetobacter johnsonii* was partially decomposed with restriction enzyme Sau3AI, and the fragments were fractionated by sucrose density gradient centrifugation to recover the fraction of about 7 to 10 Kb. This DNA fragment and the plasmid vector pBlueScript SK(+) (purchased from Toyobo) which had been cleaved with BamHI were ligated by using T4 DNA ligase, and *E. coli* strain JM109 (purchased from Takara Shuzo) was transformed with the solution of this DNA. The thus produced 6000 ampicillin-resistant transformants were divided into groups of 50 transformants.

After cultivating each group overnight at 37°C in LB medium, the cells were collected by centrifugation and washed with 20mM Tris-HCl (pH 8.0), and again suspended in the same buffer solution. BugBuster (purchased from Takara Shuzo) was added to the cell suspension at the equal amount, and the suspension was allowed to stand at room temperature for 30 minutes for cell lysis. Three volumes of 20mM Tris-HCl (pH 8.0) was then added to prepare the cell extract.

To 20 μl of the activity detecting solution (50mM Tris HCl (pH 8.0), 40mM $(NH4)_2SO_4$, 4mM $MgCl_2$, and 1mM AMP)

containing the isotope-labeled polyphosphate (0.24mM calculated in terms of phosphate) prepared above was added 1 μl of the cell extract, and the reaction was allowed to proceed at 37°C for 1 hour. The reaction solution was then subjected to thin layer chromatography (using developer: 0.75M KH_2PO_4 , pH 3.5), and the ADP formation was detected on phospho-image analyzer BASS2000 (manufactured by Fujix) for screening of the transformants. PAP activity was detected for 1 clone in the 6000 strains.

Plasmid pPAP2 having the PAP gene of Acinetobacter johnsonii strain 210A inserted therein was obtained from the resulting clone (FIG. 1). It is to be noted that the plasmid pPAP2 had inserted therein the chromosomal DNA of Acinetobacter johnsonii strain 210A which is about 10 kb, and it was internationally deposited on the basis of Budapest treaty on May 22, 2002 with the designation of plasmid DNA (pPAP2) to The National Institute of Advanced Industrial Science and Technology (an Independent Administrative Institution), International Patent Organism Depositary (Chuo-6, 1, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan (Post code, 305-8566)) with the accession number of FERM BP-8047.

(1-3) Analysis of PAP gene of *Acinetobacter johnsonii* strain 210

The DNA of *Acinetobacter johnsonii* strain 210A of 10 kb inserted in the pPAP2 was subcloned in various plasmids to determine the PAP activity of these transformants as described above, and it was then found that the PAP gene is

located in SacI-HpaI DNA fragment of about 2.5 kb (FIG. 1). The nucleotide sequence of this DNA fragment was determined by dideoxy chain terminator method (Science, 214, 1295(1981)), and it was then found that the PAP gene codes for the polypeptide (molecular weight: 55.8 kd) comprising 475 amino acids (FIGS. 2 and 3).

(2) Preparation of *Acinetobacter johnsonii* PAP

The *E. coli* JM109 carrying the plasmid pPAP2 was cultivated overnight at 28°C in 2xYT medium containing ampicillin at 100 μg/ml. The cells were collected by centrifugation, and suspended in the buffer solution comprising 50mM Tris HCl (pH 7.8) and 1mM EDTA. After ultrasonication, cell extract was collected by centrifugation. In the measurement of the extract for the PAP activity, it was found that PAP was produced at a rate of 18.1 unit per 1 ml of the culture solution, and that the activity was about 9000 folds higher than that of the contrast (the E. coli JM109 carrying no the plasmid).

It is to be noted that this productivity corresponds to about 150 times the productivity of the *Acinetobacter Johnsonii*. PAP was partially purified by fractionating the extract by ion exchange chromatography using DEAE TOYOPEARL 650M (TOSO) (eluent: 50mM Tris HCl (pH 7.8), concentration gradient of 0 to 0.5M NaCl), and the collected fraction was used for the enzyme sample. The specific activity of the PAP in the collected fraction was 80.5 units/mg protein.

(3) Analysis of various properties of PAP

(3-1) Synthesis of various NDP (analysis of substrate specificity)

To 50mM Tris HCl buffer solution (pH 8.0) containing 100mM $MgCl_2$, polyphosphate (10mM calculated in terms of inorganic phosphate), and 5mM of various NMP or dNMP was added PAP at various concentration, and the solution was incubate at 37°C for 10 minutes. The reaction was terminated by heat treatment at 100°C for 1 minute, and the solution after the reaction was subjected to HPLC to measure the amount of the NDP or the dNDP produced. The specific activity of each NMP or dNMP is shown in Table 1 as a relative value in relation to the specific activity of the PAP in the phosphorylation of the AMP, which is assumed 100%.

Table 1

Substrate	Specific activity (relative value)
AMP	100 %
GMP	10
CMP	0.09
UMP	0.13
IMP	2.2
dAMP	18
dGMP	2.6
dCMP	0.008
TMP	0.012

(3-2) pH stability

PAP was incubated in either 50mM maleate or 50mM Tris HCl buffer solution of various pH at 37°C for 10 minutes in the presence of 100mM magnesium chloride to measure the residual activity. The residual activity was measured by allowing the reaction to take place at 37°C for 10 minutes in the presence of 50mM Tris buffer solution (pH 8.0), 100mM magnesium chloride, 5mM AMP, and polyphosphate (10mM calculated in terms of inorganic phosphate), and measuring the resulting ADP by HPLC.

As shown in FIG. 4, the enzyme of the present invention was found to exhibit an enzymatic activity of 80%

or more at a pH in the range of 7 to 9 when the enzymatic activity at pH 8 was 100%.

(3-3) Optimal pH

The reaction was allowed to proceed in either 50 mM mateate or 50mM Tris HCl buffer solution of various pH at 37°C for 10 minutes in the presence of 100mM magnesium chloride, polyphosphate (10mM calculated in terms of inorganic phosphate), and 5mM AMP to measure the amount of the ADP produced by HPLC.

As shown in FIG. 4, the optimal pH of the enzyme of the present invention was approximately 8.5.

(3-4) Thermal stability

PAP was incubated for 10 minutes in 50mM Tris HCl buffer solution (pH 8.0) containing 100mM magnesium chloride in the presence or absence of polyphosphate (10mM calculated in terms of inorganic phosphate) in the bath at various temperatures to measure the residual activity by the procedure as described above.

As shown in FIG. 6, the enzyme of the present invention was found to be stable up to approximately 50°C in the presence of the polyphosphate.

(3-5) Optimal temperature

PAP was incubated for 10 minutes in 50mM Tris HCl buffer solution (pH 8.0) containing 100mM magnesium chloride in the presence of polyphosphate (10mM calculated in terms of inorganic phosphate) and 5mM AMP in the bath at various temperatures, and the enzymatic activity was evaluated by measuring the amount of ADP produced by HPLC.

As shown in FIG. 7, optimal reaction temperature of the enzyme of the present invention was found to be approximately 50°C.

Example 2: Synthesis of ATP using PAP and adenylate kinase

(1) Preparation of *E. coli* adenylate kinase

E. coli adenylate kinase was prepared by the procedure described in the document (Proc. Natl. Acad. Sci. USA, 97, 14168-14171 (2000)). However, the cell extract produced by ultrasonication was used for the enzyme solution, and the specific activity of the adenylate kinase in the enzyme solution was 12.5 units/mg protein.

(2) Synthesis of ATP

To 50mM Tris HCl buffer solution (pH 7.8) containing 20mM $MgCl_2$, polyphosphate (30mM calculated in terms of inorganic phosphate), and 10mM AMP were added 1.5 units/ml of PAP and 0.4 unit/ml of adenylate kinase, and the solution was incubated at 37°C for 60 minutes. After the completion of the reaction, amount of the nucleotide in the reaction solution was measured by HPLC.

As shown in FIG. 8, it was confirmed that AMP is promptly phosphorylated by the PAP to produce ADP, and the thus produced ADP is promptly converted to ATP and AMP by the adenylate kinase that is also present in the system, the repetition of this cycle resulting in the ATP accumulation.

Example 3: Synthesis of galactose-1-phosphate using ATP regeneration system comprising the combination of PAP and adenylate kinase

(1) Preparation of *E. coli* galactokinase

E. coli strain JM109 having the plasmid pDR540 containing *E. coli* galactokinase gene (Gene, 20, 231(1982), obtained from Pharmacia) was inoculated in 2xYT medium containing 100 μg/ml ampicillin, and the *E. coli* was cultivated at 37°C with shaking. When the cell density reached $4x10^8$/ml, IPTG was added to the culture medium to a final concentration of 1mM, and the incubation was continued at 30°C for another 5 hours. After completion of the cultivation, the cells were collected by centrifugation and suspended in 30ml buffer solution (50mM Tris HCl (pH 7.8), 1mM EDTA). The cells were lyzed by ultrasonication, and bacterial residue was removed by further centrifugation. The solution collected was subjected to ion exchange chromatography using DEAE Toyopearl*650M (Toso) (eluent: 50mM Tris HCl (pH 7.8), concentration gradient of 0 to 0.5M NaCl) for fractionation and partial purification of the galactokinase. The thus recovered fraction was used for the enzyme solution of galactokinase. The specific activity of the galactokinase in the enzyme solution was 6. 5 units/mg protein.

The unit of the glactokinase activity was the one measured and calculated by the procedure as described below. Sample enzyme was added the 100mM Tris HCl buffer solution (pH 7.8) containing 5mM $MgCl_2$, 10mM ATP, and 10mM galactose,

* Trade-mark

and the solution was incubated at 37°C to promote the reaction, and the reaction was terminated by a 1 minute heat treatment at 100°C. Amount of the galactose-1-phosphate in the reaction solution was measured by using a sugar analyzer (Dionex), and the activity of producing 1 μmole of galactose-1-phosphate at 37°C in 1 minute was designated 1 unit.

(2) Synthesis of galactose-1-phosphate

To 50mM Tris HCl buffer solution (pH 7.8) containing 20mM $MgCl_2$, polyphosphate (30mM calculated in terms of inorganic phosphate), 5mM AMP, and 50mM (d) galactose were added 0.2 unit/ml PAP and 0.2 unit/ml adenylate kinase, and then, galactokinase to 0.5 unit/ml, and the solution was incubated at 37°C for 8 hours. During the reaction, polyphosphate was also added respectively at 2 hours and 4 hours after the start of the reaction to 20mM calculated in terms of inorganic phosphate. The reaction solution was analyzed with a sugar analyzer (Dionex), and production of 37.8mM galactose-1-phosphate was confirmed.

Example 4: Synthesis of nucleotide diphosphate

(1) Synthesis of various NDP

To 50mM Tris HCl buffer solution containing 100mM $MgCl_2$, polyphosphate (30mM calculated in terms of inorganic phosphate), and 10mM of an NMP (AMP, GMP, CMP, UMP, or IMP) was added PAP at 16 units/ml, and the solution was incubated at 37°C for 30 minutes. The results of the HPLC analysis of the reaction solution are shown in Table 2. It

is to be noted that no NDP generation was found when cell extract of E. coli JM109 was used for the contrast.

Table 2

Substrate	NDP generated
AMP	6.76mM ADP
GMP	7.16mM GDP
CMP	0.98mM CDP
UMP	0.85mM UDP
IMP	6.75mM IDP

(2) Enzymatic synthesis of IDP

To 50mM Tris HCl buffer solution containing 100mM $MgCl_2$, polyphosphate (65mM calculated in terms of inorganic phosphate), and 40mM IMP was added PAP at 16 units/ml, and the solution was incubated at 37°C for 19 hours. The reaction solution was analyzed by HPLC, and formation of 21.2mM IDP was confirmed.

INDUSTRIAL APPLICABILITY

This invention provides a novel PAP and its gene. This invention has also enabled a large scale production of the PAP with no difficulty, which could not have been done by the conventional technology. An efficient synthesis and regeneration of ATP from AMP at a low cost has been realized, and combination of this with an ATP-consuming enzymatic reaction system enables regeneration of the ATP

consumed in the reaction system to facilitate efficient synthesis of the target compound.

In contrast to the conventional PAP, the PAP of the present invention exhibits phosphorylation activity for various types of nucleoside 5′-monophosphate other than AMP and deoxynucleoside 5′-monophosphate, and this enables smooth production of various types of nucleoside 5′-diphosphate and deoxynucleoside 5′-diphosphate by an enzymatic process.

SEQUENCE LISTING

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<120> Novel Polyphosphate:AMP Phosphotransferase

<130> YS0007

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Gln Ser Asn Ala Lys Ser Leu Val Ile Leu Val Ser Gly Ile Glu Leu
        35                  40                  45
Ala Gly Lys Gly Glu Ala Val Lys Gln Leu Arg Glu Trp Val Asp Pro
    50                  55                  60
Arg Phe Leu Tyr Val Lys Ala Asp Pro Pro His Leu Phe Asn Leu Lys
65                  70                  75                  80
Gln Pro Phe Trp Gln Pro Tyr Thr Arg Phe Val Pro Ala Glu Gly Gln
                85                  90                  95
Ile Met Val Trp Phe Gly Asn Trp Tyr Gly Asp Leu Leu Ala Thr Ala
            100                 105                 110
Met His Ala Ser Lys Pro Leu Asp Asp Thr Leu Phe Asp Glu Tyr Val
        115                 120                 125
Ser Asn Met Arg Ala Phe Glu Gln Asp Leu Lys Asn Asn Asn Val Asp
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Thr Arg Phe Thr Asp Asp Trp Gln Ile Ile Asp Gly Glu Asp Glu Asp
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225                 230                 235                 240
Ile Pro Asp Ile Leu Thr Gln Phe Glu Val Pro Gln Ala Glu Asp Ala
                245                 250                 255
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            260                 265                 270
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    290                 295                 300
Pro Arg Glu Tyr Glu Ile His Thr Ile Ala Ala Pro Glu Lys Tyr Glu
305                 310                 315                 320
Leu Arg Arg Pro Tyr Leu Trp Arg Phe Trp Ser Lys Leu Gln Ser Asp
                325                 330                 335
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Glu Ile Asn Arg Phe Glu Lys Asn Leu Ser Ser Ser Gln Thr Val Leu
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Ile Lys Phe Trp Leu Ala Ile Asp Lys Asp Glu Gln Ala Ala Arg Phe
385 390 395 400
Lys Ala Arg Glu Ser Thr Pro His Lys Arg Phe Lys Ile Thr Glu Glu
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420 425 430
Asp Met Phe Ala His Thr Asp Thr Ser Tyr Ala Pro Trp Tyr Ile Ile
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CLAIMS:

1. A polyphosphate:AMP phosphotransferase comprising the amino acid sequence of SEQ ID NO:1 and having the physical and chemical properties as described below:

(A) action: catalyzing of the following two reactions:

$$NMP + PolyP_{(n)} \rightarrow NDP + PolyP_{(n-1)}$$

$$dNMP + PolyP_{(n)} \rightarrow dNDP + PolyP_{(n-1)}$$

(wherein NMP represents nucleoside monophosphate, NDP represents nucleoside diphosphate, dNMP represents deoxynucleoside monophosphate, dNDP represents deoxynucleoside diphosphate, n represents degree of polymerization of the polyphosphate which is an integer of up to 100);

(B) substrate specificity: specific to AMP, GMP, IMP, dAMP, and dGMP, also acting with CMP, UMP, dCMP, and TMP;

(C) molecular weight: about 55 to 56 Kd (kilodalton); and

(D) specific activity: at least 70 units per 1 mg of enzyme protein, with the proviso that 1 unit corresponds to the activity capable of forming 1 μmole of ADP in 1 minute at 37°C measured under the following conditions:

<measurement conditions>

enzyme sample is added to 50mM Tris HCl buffer solution (pH 7.8) containing 20mM magnesium chloride, 10mM AMP, and polyphosphate (30mM calculated in terms of inorganic phosphate) and the reaction is allowed to proceed by maintaining the temperature at 37°C, and the reaction is thereafter terminated by a heat treatment at 100°C for 1

minute, and amount of ADP in the reaction solution is measured by high performance liquid chromatography (HPLC).

2. A polyphosphate:AMP phosphotransferase gene encoding the amino acid sequence of SEQ ID NO: 1.

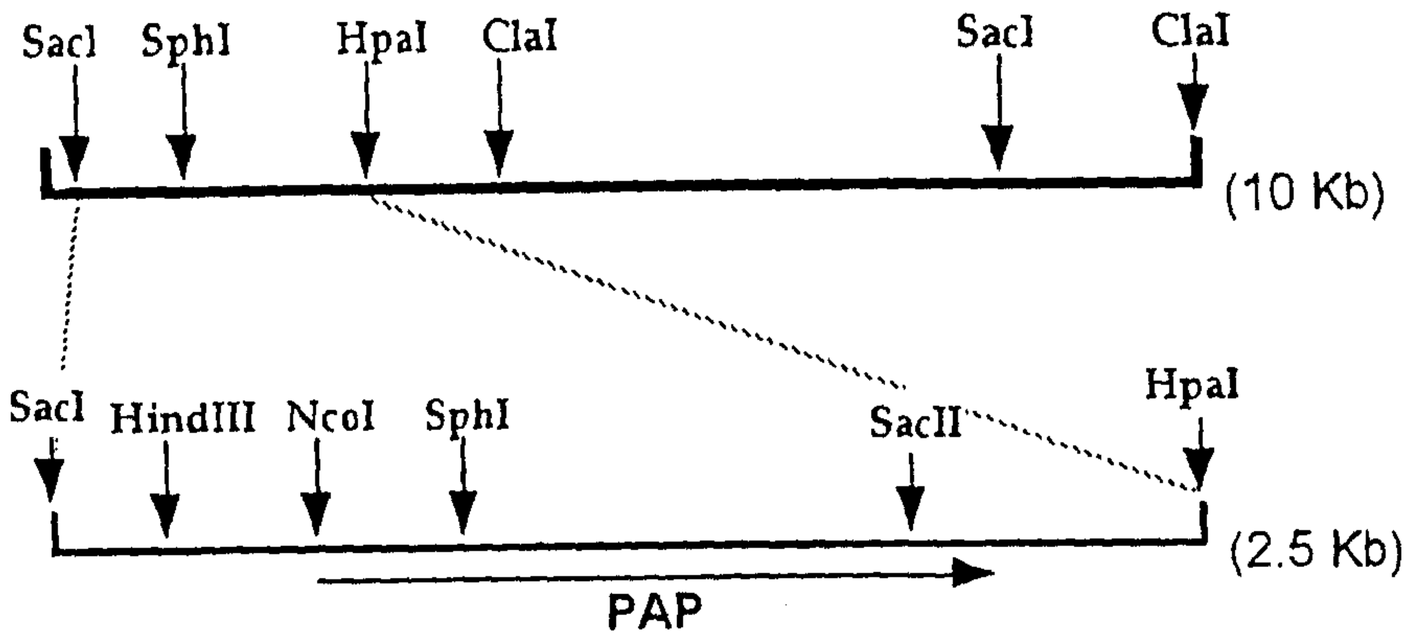
3. A gene according to claim 2 wherein the polyphosphate: AMP phosphotransferase gene **comprises** the nucleotide sequence of SEQ ID NO: 2 or a nucleotide sequence **which is at least 95% identical to** SEQ ID NO: 2.

4. A method for producing nucleoside diphosphate wherein the nucleoside diphosphate is enzymatically produced from nucleoside monophosphate by using polyphosphate:AMP phosphotransferase of claim 1 and polyphosphate as a phosphate donor.

5. A method for producing ATP wherein ATP is enzymatically produced from AMP by using two enzymes, namely, the polyphosphate:AMP phosphotransferase of claim 1 and adenylate kinase and polyphosphate as a phosphate donor.

6. A method for producing a compound by using an ATP-consuming enzymatic reaction, wherein ATP is regenerated from the AMP simultaneously with the enzymatic reaction by using an ATP regeneration **complex** comprising polyphosphate, polyphosphate:AMP phosphotransferase, and adenylate kinase, and wherein the polyphosphate:AMP phosphotransferase used is the enzyme of claim 1.

Fig. 1


SacI
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HpaI
ClaI
SacI
ClaI
(10 Kb)
SacI
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NcoI
SphI
SacII
HpaI
(2.5 Kb)
PAP

Fig. 2

```
        10         20         30         40         50         60         70         80         90
GAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCTTTCAGCAATTGCATTGCCACTTGTTCATCCAGTCGCACCGCCAAAATC

       100        110        120        130        140        150        160        170        180
GCCAACTCAACCCCAGAGGTACCGGTTTTAATGGCATTTTCAGGAATTAATTTCTCTGCTTTGTGCGCATATTTGCTCAAGCGCGCAATT

       190        200        210        220        230        240        250        260        270
TGTCCTGCCAAAGCGTCAAAATCTGGGCCACCATATAAACGGTTGATAAAATACTCGGCCATCAACAGATTTTGCTTTTCTGCGAAAGCT

       280        290        300        310        320        330        340        350        360
TTGTTGTGGGTACGCTGCATTCGCTGTTTTTGCCAGACTTGCACATCTTGTAAACGTTGGAAGAGCACCGAATCTTGATGATAATGCAAT

       370        380        390        400        410        420        430        440        450
TGGTAATACTGTTCTAAAAGCTCATCGAGGGCGGCGAGTTTGGACATTTGCTGTCTCAGTTCTATTTAAAGATAGCTAAATCTTAGTGCG

       460        470        480        490        500        510        520        530        540
TGATGACGGAACTCGCTATGACAAAGTGACAAAAAAACTATTTATTTGTGGTCAGTTTGATGTAGAAGTAGTCAAAAGATACATATGAAA

       550        560        570        580        590        600        610        620        630
ATCATCTAGACCATCATTTTTTAGGATCTAGTGATATGATTAAATAGAAAAAAGGAGGGGGCCATGGATACAGAAACGATCGCCAGTGCA
                                                                  M  D  T  E  T  I  A  S  A

       640        650        660        670        680        690        700        710        720
GTGCTGAATGAAGAACAGCTTTCACTGGACTTAATTGAAGCGCAATATGCGTTGATGAATACCCGTGATCAGAGCAATGCAAAAAGTTTA
V  L  N  E  E  Q  L  S  L  D  L  I  E  A  Q  Y  A  L  M  N  T  R  D  Q  S  N  A  K  S  L

       730        740        750        760        770        780        790        800        810
GTGATTTTGGTCAGTGGAATCGAACTTGCGGGTAAAGGCGAAGCGGTGAAACAGCTCCGCGAATGGGTCGATCCTCGTTTTTTATATGTC
V  I  L  V  S  G  I  E  L  A  G  K  G  E  A  V  K  Q  L  R  E  W  V  D  P  R  F  L  Y  V

       820        830        840        850        860        870        880        890        900
AAAGCCGATCCACCGCATCTGTTTAATCTAAAACAGCCTTTTTGGCAGCCCTATACCCGATTTGTGCCTGCCGAAGGGCAAATTATGGTG
K  A  D  P  P  H  L  F  N  L  K  Q  P  F  W  Q  P  Y  T  R  F  V  P  A  E  G  Q  I  M  V

       910        920        930        940        950        960        970        980        990
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W  F  G  N  W  Y  G  D  L  L  A  T  A  M  H  A  S  K  P  L  D  D  T  L  F  D  E  Y  V  S

      1000       1010       1020       1030       1040       1050       1060       1070       1080
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N  M  R  A  F  E  Q  D  L  K  N  N  N  V  D  V  L  K  V  W  F  D  L  S  W  K  S  L  Q  K

      1090       1100       1110       1120       1130       1140       1150       1160       1170
CGTCTAGATGATATGGACCCGAGCGAAGTGCATTGGCATAAGTTGCATGGGCTAGACTGGCGCAATAAAAAACAATATGACACCTTACAA
R  L  D  D  M  D  P  S  E  V  H  W  H  K  L  H  G  L  D  W  R  N  K  K  Q  Y  D  T  L  Q

      1180       1190       1200       1210       1220       1230       1240       1250       1260
AAGCTACGTACGCGCTTCACCGATGACTGGCAAATCATTGATGGTGAAGATGAGGATTTGCGTAATCACAATTTTGCACAAGCAATTTTA
K  L  R  T  R  F  T  D  D  W  Q  I  I  D  G  E  D  E  D  L  R  N  H  N  F  A  Q  A  I  L

      1270       1280       1290       1300       1310       1320       1330       1340       1350
ACGGCACTACGACACTGCCCAGAGCATGAAAAAAAGGCCGCGCTAAAATGGCAGCAAGCACCAATACCAGATATTCTGACTCAGTTTGAA
T  A  L  R  H  C  P  E  H  E  K  K  A  A  L  K  W  Q  Q  A  P  I  P  D  I  L  T  Q  F  E
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First part

CA 02499885 2004-11-26

Fig. 3

```
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V  P  Q  A  E  D  A  N  Y  K  S  E  L  K  K  L  T  K  Q  V  A  D  A  M  R  C  D  D  R  K

      1450      1460      1470      1480      1490      1500      1510      1520      1530
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V  V  I  A  F  E  G  M  D  A  A  G  K  G  G  A  I  K  R  I  V  K  K  L  D  P  R  E  Y  E

      1540      1550      1560      1570      1580      1590      1600      1610      1620
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I  H  T  I  A  A  P  E  K  Y  E  L  R  R  P  Y  L  W  R  F  W  S  K  L  Q  S  D  D  I  T

      1630      1640      1650      1660      1670      1680      1690      1700      1710
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I  F  D  R  T  W  Y  G  R  V  L  V  E  R  V  E  G  F  A  T  E  V  E  W  Q  R  A  Y  A  E

      1720      1730      1740      1750      1760      1770      1780      1790      1800
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      1810      1820      1830      1840      1850      1860      1870      1880      1890
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F  K  A  R  E  S  T  P  H  K  R  F  K  I  T  E  E  D  W  R  N  R  D  K  W  D  D  Y  L  K

      1900      1910      1920      1930      1940      1950      1960      1970      1980
GCAGCCGCGGATATGTTTGCGCATACCGACACCAGCTATGCGCCTTGGTATATTATTTCCACCAATGATAAACAACAGGCCCGCATTGAA
A  A  A  D  M  F  A  H  T  D  T  S  Y  A  P  W  Y  I  I  S  T  N  D  K  Q  Q  A  R  I  E

      1990      2000      2010      2020      2030      2040      2050      2060      2070
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V  L  R  A  I  L  K  Q  L  K  A  D  R  D  T  D  *

      2080      2090      2100      2110      2120      2130      2140      2150      2160
GAGGCAGATTTAGTTTTTTAACTTAAGGGAATTTGGGTCACTGGCGCTGCAACAGGAACACCTTGTTCAGCGGCTTGTTTTAGTTGAATG

      2170      2180      2190      2200      2210      2220      2230      2240      2250
CCTTTGGCGAGCTTATACGACTCTTCCACATGGGTTTCCGCAATTTTTTTCATCACGACATAACCCAAGCTGGCCGCAATCATTTGTCCA

      2260      2270      2280      2290      2300      2310      2320      2330      2340
CCTAAGGGAATAAATTTAGTGACTTGTTTGGTAATGAATTTGGCTGCCATGTTATTGATGGATTTTTTCACGGCTGTACGTGCGACCACC

      2350      2360      2370      2380      2390      2400      2410      2420      2430
AGTCCAGAGAACTCCACACCACGTTTACGCAGTTCTGACCAATGTATTTGCTTAGTTTCTGGGTCGTAGACACTGACTTGCTCAGGGGTT

      2440      2450
AAACCAAAGCGGGCGTTAAC
```

Second part

CA 02499885 2004-11-26

Fig. 4

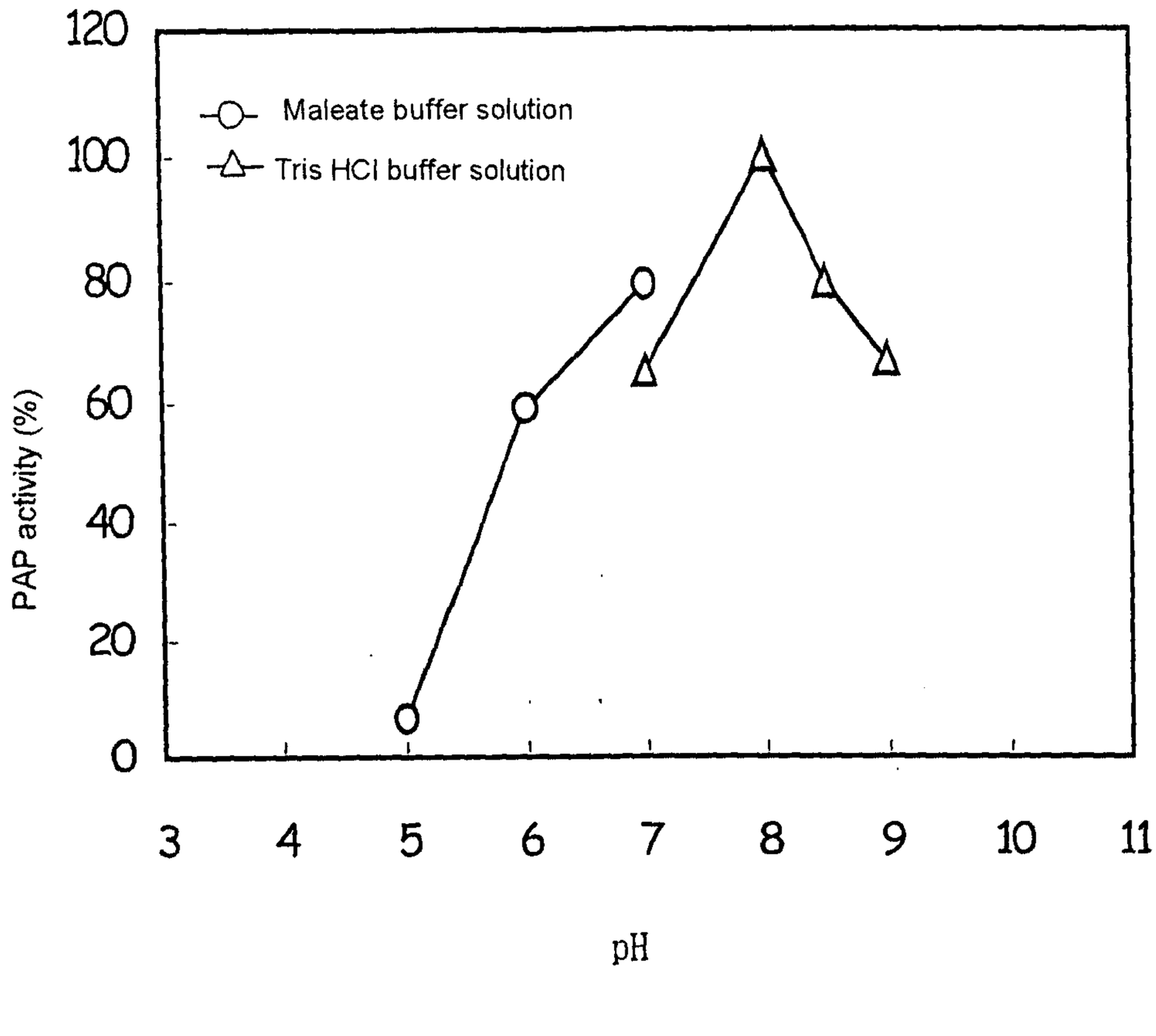

120
100
80
60
40
20
0
PAP activity (%)
Maleate buffer solution
Tris HCl buffer solution
3
4
5
6
7
8
9
10
11
pH

Fig. 5

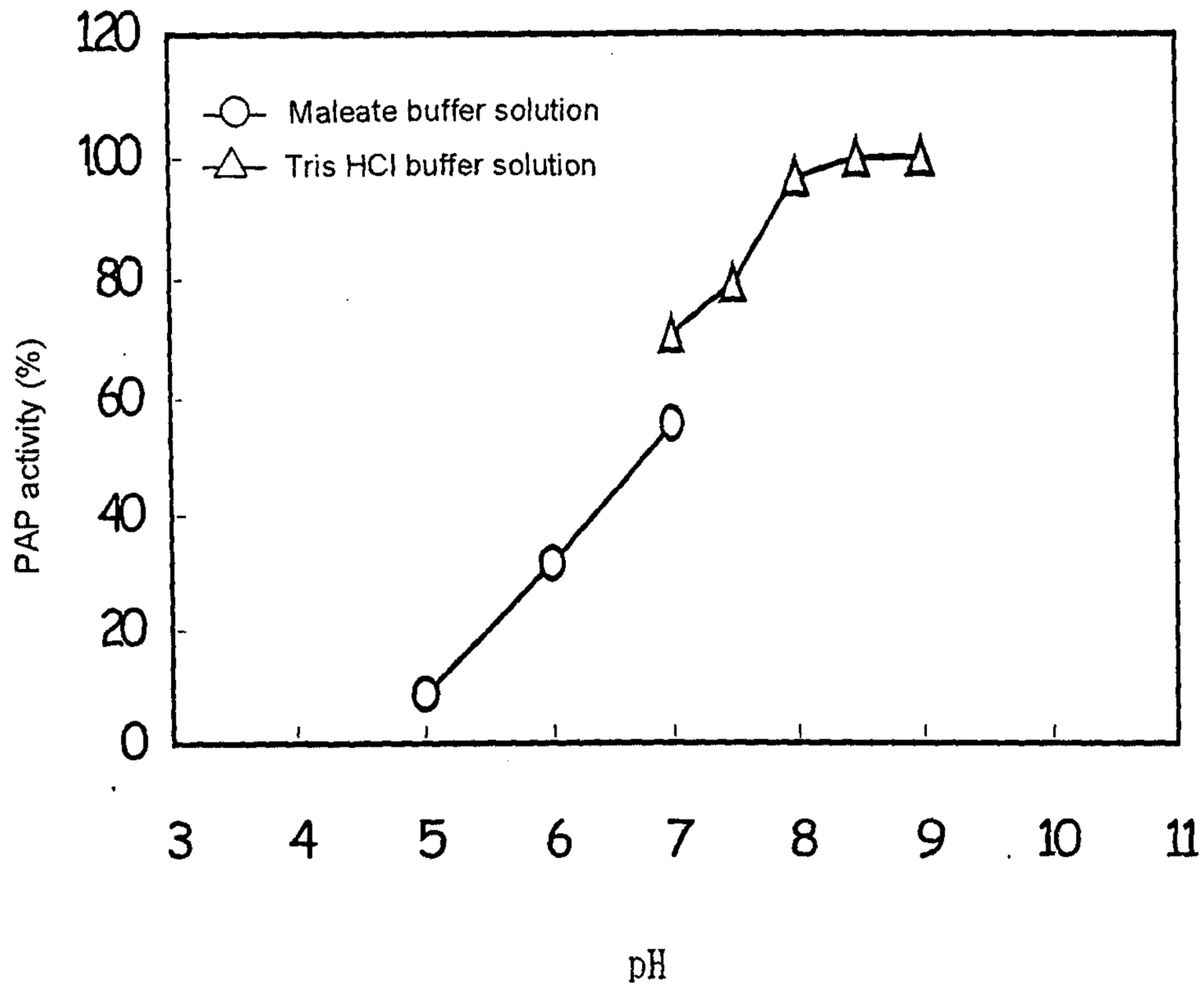

Maleate buffer solution
Tris HCl buffer solution
PAP activity (%)
120
100
80
60
40
20
0
3
4
5
6
7
8
9
10
11
pH

Fig. 6

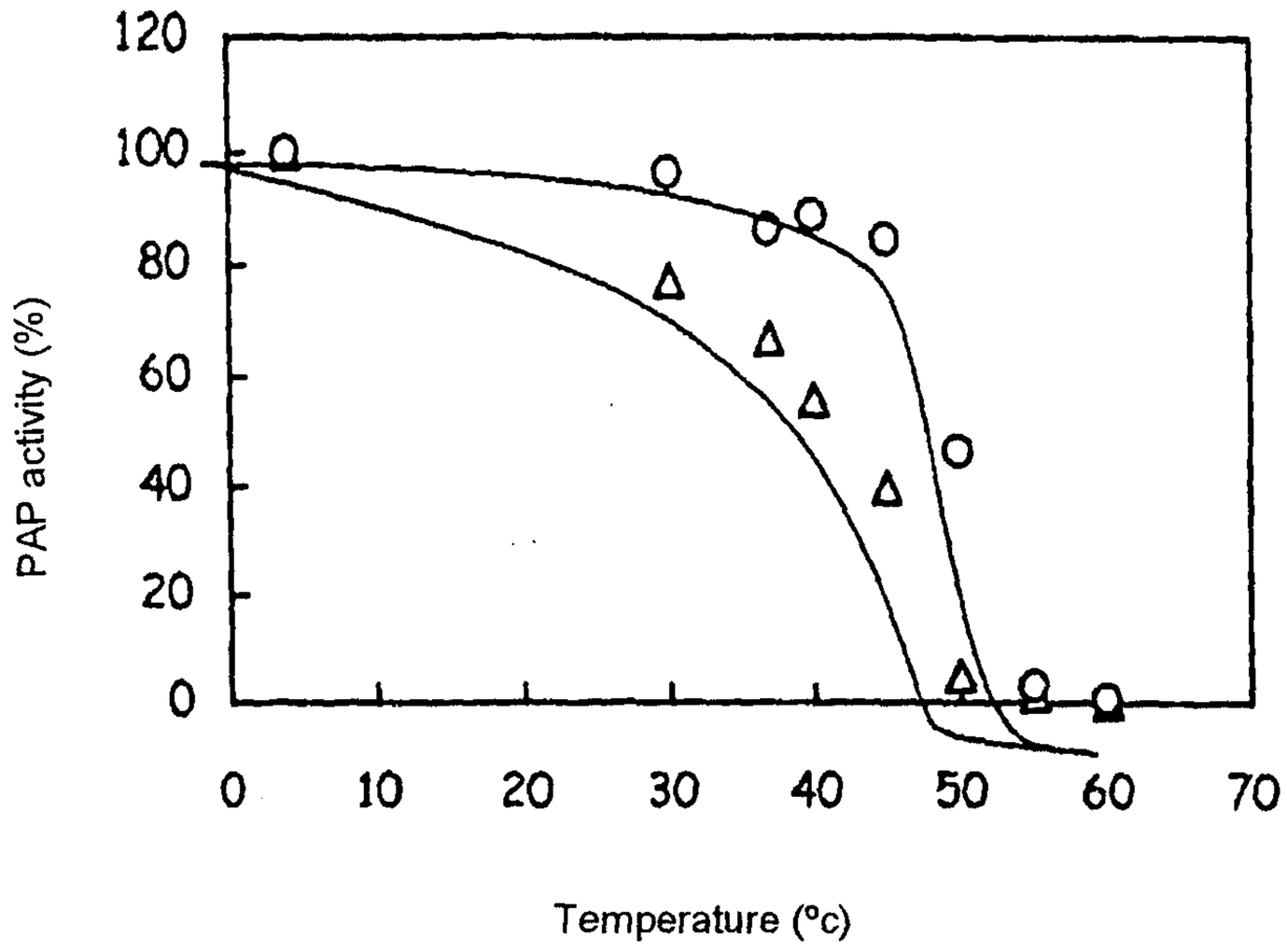

120
100
80
60
40
20
0
PAP activity (%)
0
10
20
30
40
50
60
70
Temperature (°c)

Fig. 7

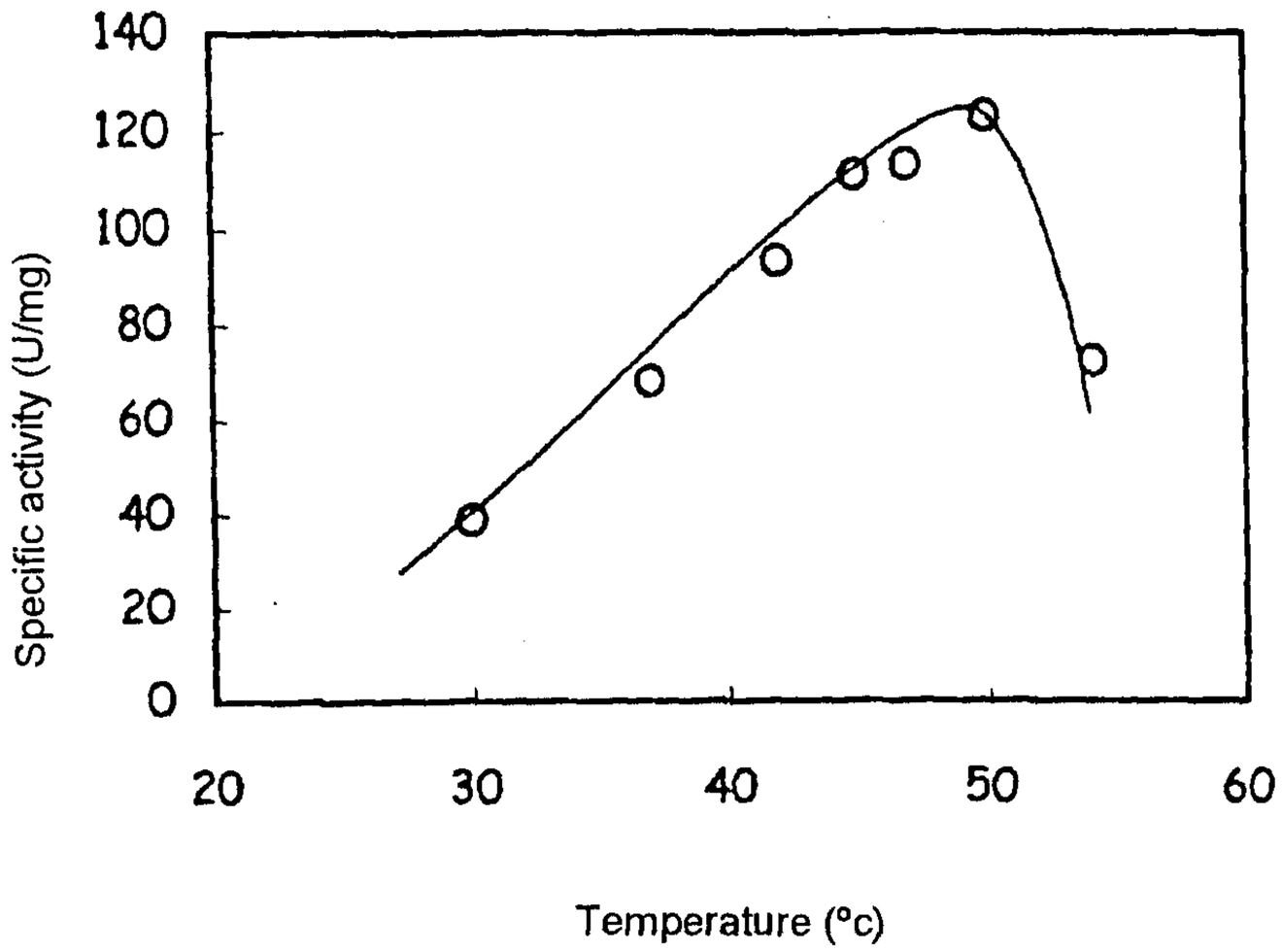
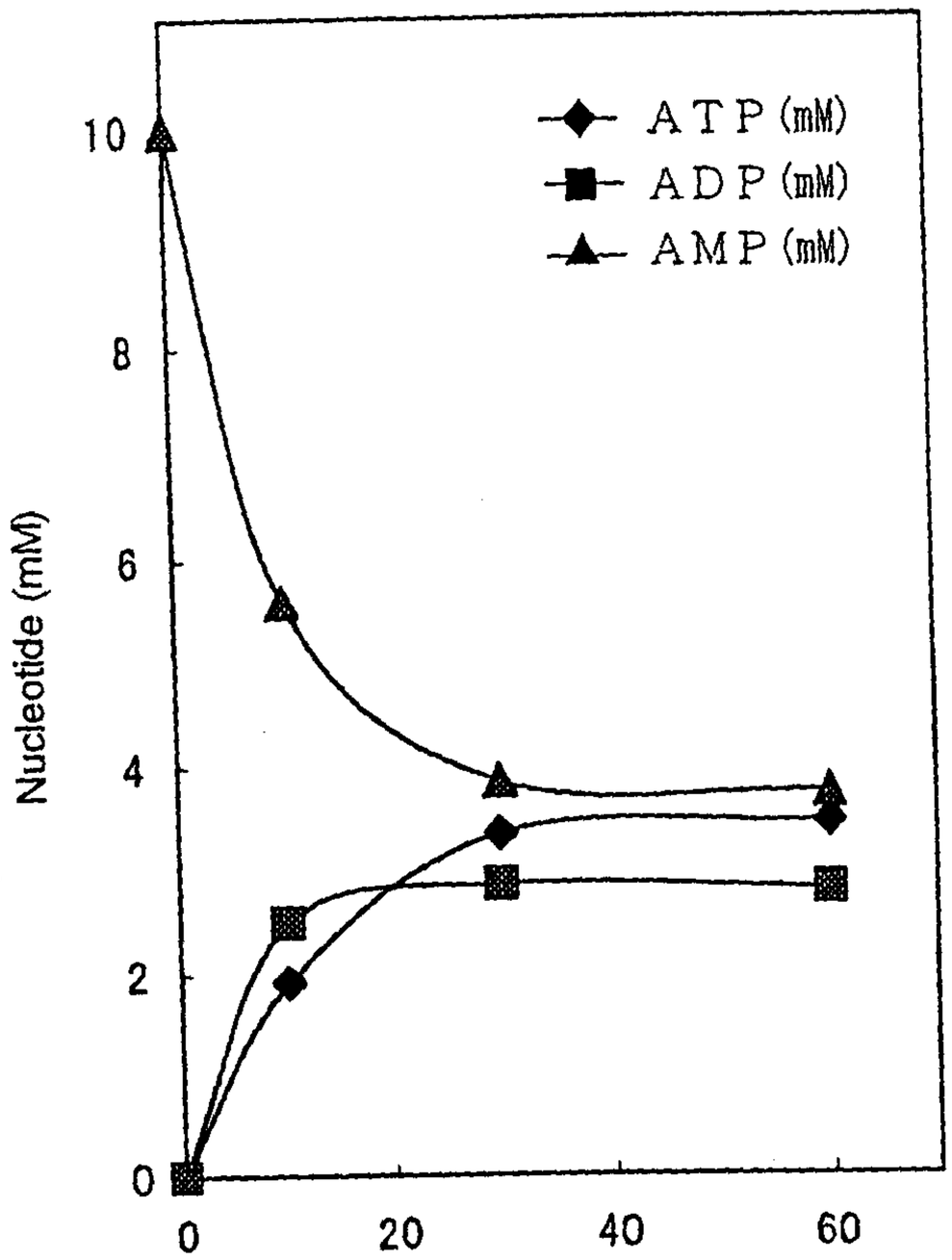

140
120
100
80
60
40
20
0
Specific activity (U/mg)
20
30
40
50
60
Temperature (°c)

Fig. 8


ATP (mM)
ADP (mM)
AMP (mM)
Nucleotide (mM)
10
8
6
4
2
0
0
20
40
60
Incubation period (min)