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Enzymatic production of 5′-inosinic acid by a newly synthesised acid phosphatase/phosphotransferase

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ABSTRACT

5′-Nucleotides including 5′-inosinic acid have characteristic taste and important application in various foods as flavour potentiators. The selective nucleoside acid phosphatase/phosphotransferase (AP/PTase) can catalyse the synthesis of 5′-nucleotides by transfer of phosphate groups. In this study, a 747-bp gene encoding AP/PTase from *Escherichia blattae* was synthesised. After expression, the recombinant AP/PTase was purified using nickel–NTA. The optimal temperature and pH of this enzyme were 30 °C and 5.0, respectively. The activity was partially inhibited by metal ions such as Hg^{2+}, Ag^{+} and Cu^{2+}, but not by chelating reagents such as EDTA. The values of K_m and V_{max} for inosine were 40 mM and 3.5 U/mg, respectively. Using this purified enzyme, 16.83 mM of 5′-IMP was synthesised from 37 mM of inosine and the molar yield reached 45.5%. Homology modelling and docking simulation were discussed.

1. Introduction

Nucleotides have been widely used as food additives and pharmaceutical intermediates (Asano, Mihara, & Yamada, 1999a). Among the nucleotides, 5′-inosinic acid (5′-IMP) and 5′-guanylic acid (5′-GMP) have received increased attention, because of their characteristic taste and important application in various foods as flavour potentiators (Mihara, Ishikawa, Suzuki, & Asano, 2004). Recently, the demand for nucleotides in the food additives market is increasing, and the production of nucleotides has been well studied. Nucleotides can be produced by both chemical and biological methods, including enzymatic reaction and fermentation (Hiroshi, Katsuaki, & Hitoshi, 1982). The enzymatic phosphorylation process using acid phosphatase/phosphotransferase (AP/PTase) as biocatalyst requires mild reaction conditions (Mori, Iida, Fujio, & Teshiba, 1997; Mori, Iida, Teshiba, & Fujio, 1995), and has potential application in industrial production of nucleotides. Currently, screening and exploration of AP/PTase and its industrial application for the preparation of 5′-nucleotides are hot topics (Mihara et al., 2004).

AP/PTs are classified into two major categories, including nonspecific and specific AP/PTase, according to the substrate specificity (Mihara, Utagawa, Yamada, & Asano, 2001; Tanaka, Hasan, Hartog, van Herk, & Wever, 2003). As opposed to the specific AP/PTase, nonspecific enzymes have a board substrate spectrum, which can catalyse nucleotide (Asano, Mihara, & Yamada, 1999b), glucose 6-phosphate (Tanaka et al., 2003), and dihydroxyacetone (Mihara et al., 2004) synthesis. It is reported that several microorganisms including *Salmonella typhimurium* (Kasahara, Nakata, & Shinagawa, 1991), *Zymomonas mobilis*, *Morganella morganii* (Thaller, Berlutti, Schippa, Lombardi, & Rossolini, 1994), *Shigella flexneri* (Uchiya, Tohsuji, Nikai, Sugihara, & Sasakawa, 1996) and *Prevotella intermedia* (Chen et al., 1999) could produce AP/PTase. Mihara et al. purified an acid phosphatase with regioselective pyrophosphate nucleoside phosphotransferase activity from *M. morganii* NCIMB 10466, and cloned its gene, subsequently improving its phosphotransferase activity by random mutation (Mihara, Utagawa, Yamada, & Asano, 2000).

In the present study, to explore the production of 5′-IMP by enzymatic methods, the gene encoding AP/PTase from *Escherichia blattae* was synthesised and expressed. After purification, the detailed characteristics of this recombinant acid phosphotransferase were investigated. Nucleoside phosphorylation reaction was studied as well, using the pyrophosphate as the phosphate donor. This study showed that this recombinant AP/PTase could possess potential for the large scale production of nucleotides.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

Escherichia coli BL21 (DE3) (Invitrogen, Karlsruhe, Germany) was used as host for expression. The plasmid pET28b(+) (Novagen, Darmstadt, Germany) was used for expression of AP/PTase. Luria–Bertani (LB) (yeast extract 5 g/L, Tryptone 10 g/L, NaCl 5 g/L) was used for the culture of *E. coli* and recombinant *E. coli* strains grown

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aerobically at 37 °C. To maintain the presence of the plasmids, the corresponding antibiotics were added to the media for *E. coli* harbouring plasmids. All the chemicals used were of analytical grade and commercially available.

2.2. Construction of expression plasmid for AP/PTase gene

A 747-bp nucleotide sequence of AP/PTase from *E. blattae* (Genbank Accession No.: E12610.1) (Ishikawa, Mihara, Gondoh, Suzuki, & Asano, 2000) with mutations of S72F/G74D/I153T was synthesised using PCR assembly method (Liu, Zhang, Cheng, Ruan, & Zheng, 2011; Rydzanicz, Zhao, & Johnson, 2005) after optimisation of the codons by gene designer software against *E. coli* as host (Villalobos, Ness, Gustafsson, Minshull, & Govindarajan, 2006). The synthesised gene with 6× His-tag was inserted into expression vector pET28b(+) between *Nco*I and *Xho*I restriction endonuclease sites. The ligated plasmid pET28b(+)-AP/PT was transformed into *E. coli* BL21 (DE3) by heat shock method (Chung, Niemela, & Miller, 1989). For the selection of *E. coli* transformants, Kanamycin (Kan) with appropriate concentration was added to the medium. DNA manipulation, plasmid isolation, and agarose gel electrophoresis were operated according to standard protocol (Sambrook & Russell, 2001) unless additionally stated.

2.3. Expression and purification of recombinant AP/PTase

E. coli BL21 (DE3) harbouring the recombinant plasmid was grown at 37 °C in 50 mL LB media containing 50 μg/mL of Kan until the absorbance of the culture suspension reached an optimal density of 0.6–0.8 at 600 nm. The expression of recombinant AP/PTase was induced by 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) and the growth was incubated at 28 °C for 10 h. The bacterial cells were harvested by centrifugation at 10,000*g* for 10 min at 4 °C and washed twice with physiological saline solution (NaCl 0.9%). Then the cells were resuspended in 30 mL 100 mM Tris–HCl (pH 7.6) and destroyed by sonication with a Vibra-Cell VC 505 ultrasonic processor (Sonics and Materials Inc., Newtown, CT) at 300 W for 30 min. Cell debris were removed by centrifugation at 12,000*g* for 20 min at 4 °C. The supernatant was used as the crude extract.

The recombinant proteins were purified under the native condition using a nickel–NTA resin, and the detailed enzyme purification procedure was carried out according to a previous report (Liu et al., 2011). The purity of the preparations was confirmed by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and the protein concentration (Tripathi, Tomar, & Jagannadham, 2011) was determined by the Bradford method with bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.4. SDS–PAGE analysis

Fifteen microlitres of crude extracts and elution fractions were analysed by SDS–PAGE performed using a Mini-gel system (Bio-Rad, Hercules, CA). The gels were cast with 0.75-mm spacers (Bio-Rad). SDS–PAGE was performed as described by Laemmli (1970). Polyacrylamide gels were 5% acrylamide stacking gel (pH 6.8) and 12% separating gel (pH 8.8). Proteins in the gel were stained with Coomassie Brilliant Blue R-250.

2.5. Enzymatic activity assay

Acid phosphotransferase activity was assayed in a standard mixture containing 100 mM of sodium acetate buffer (pH 5.0), 10 mg/ml of inosine, 250 mg/ml of disodium hydrogen pyrophosphate, and 2.12 mg of the enzyme in a total volume of 10 ml. The reaction mixture was incubated for 10 min at 30 °C. One millilitre of sample was taken from the reaction mixture and stopped by adding 0.2 ml of 2 M HCl, then centrifuged at 12,000*g* for 5 min at 4 °C. Quantitative determination of inosine and 5′-IMP was carried out by an LC-20AD prominence liquid chromatography instrument (Shimadzu, Kyoto, Japan) equipped with an SPD-2A prominence UV/vis detector using a C18 column (5 μm × 250 nm × 4.6 nm, Elite Analytical Instruments Co., Ltd., Dalian, China) with detection at 245 nm. The mobile phase was 10 mM potassium phosphate buffer (pH 2.8):methanol = 90:10 (v/v) and the flow rate was 1 ml/min. The retention times of 5′-IMP and inosine were 3.7 and 8.3 min, respectively.

One unit of acid phosphotransferase activity was defined as the amount of enzyme that produces 1 μmol of 5′-IMP per min under the assay conditions. Accordingly, one unit of acid phosphatase activity was defined as the amount of enzyme that produces 1 μmol of inosine per min under the assay conditions. Protein quantitative analysis was determined by the Bradford method (Bradford, 1976) with BSA as a standard. Specific enzyme activities were defined as units per mg enzyme.

2.6. Characterisation of acid phosphotransferase

2.6.1. Effects of temperature and pH

The effect of temperature on acid phosphotransferase activity was determined in a range from 20 to 60 °C under the standard assay conditions mentioned above. Thermal stability was investigated by incubating enzymes at temperatures of 30, 40, 50 and 60 °C for the indicated time intervals from 5 min to 7 h in 10 ml of 100 mM sodium acetate buffer (pH 5.0) in water bath. The half-life of acid phosphotransferase at each temperature was calculated by plotting the natural logarithm of residual activity (ln RA) at each temperature against time. The effect of pH on acid phosphotransferase activity was measured in a range from 3.6 to 9.0. The following buffers with a final concentration of 100 mM were used in testing the effects of pH on enzymatic activity: sodium acetate buffer (pH 3.6–5.8), potassium phosphate buffer (pH 5.8–8.0) and Tris–HCl buffer (pH 8.0–9.0). The pH stability of the enzyme was examined under various pH conditions. After the enzyme was incubated at 30 °C for 1 h in different buffers ranging from 3.6 to 9.0, the relative acid phosphotransferase activity was assayed under standard reaction conditions (Balqis & Rosma, 2011). All experiments were performed in triplicate.

2.6.2. Effects of metal ions and chemicals on enzymatic activity

The effects of metal ions on acid phosphotransferase activity were determined by the addition of metal ions, including $CuCl_2$, $CoCl_2$, $Al_2(SO_4)_3$, $NiCl_2$, $MnCl_2$, $CaCl_2$, LiCl, $MgCl_2$, $BaCl_2$, $ZnSO_4$, $AgNO_3$, $HgCl_2$, $FeCl_3$ and $FeSO_4$ to the reaction mixture at a final concentration of 2 mM. The effects of chemicals, including ethylenediaminetetraacetie acid (EDTA), dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), Tween-20, Tween-80, dithiothreitol (DTT) and Triton X-100, on the enzyme activity were also investigated (Ye & Ng, 2010). Activities were measured as described above and were presented as a percentage of the activity obtained.

2.6.3. Circular dichroism (CD) measurements

CD spectra of acid phosphotransferase were recorded on a JASCO J-815 Spectropolarimeter (JASCO Inc., Easton, MD) using Spectra Manager 228 software. Temperature was controlled using a Fisher Isotemp Model 3016S water bath (Fisher Scientific, Waltham, MA). A lyophilised powder (purity >95% w/w) was prepared. Far-UV scans were performed at 0.10 mg/ml protein in a 10-mm cuvette. The spectra were recorded from 200 to 250 nm with a scan speed of 20 nm/min. Results are expressed in terms of mean residue ellipticity ($[\theta]_{mrw,\lambda}$) (in deg cm^2 d mol^{-1}) determined according to Eq. (2) (Fasman, 1996, chap. 16):

$$[\theta]_{mrw,\lambda} = MRW(\theta_\lambda)/10lc,$$

where MRW is the mean residue weight of the acid phosphotransferase, θ_λ is the observed ellipticity (mdeg), l is the path length (cm), and c is the protein concentration (μM).

2.7. Kinetic parameters

Because the solubility of inosine was limited, the steady-state kinetic parameters of the recombinant acid phosphotransferase were measured for inosine using concentrations ranging from 3 to 45 mM. The initial velocities were measured under the standard reaction conditions described above and kinetic parameters were calculated from Lineweaver–Burk plots.

Acid phosphatase activity was assayed in a standard mixture containing 100 mM of sodium acetate buffer (pH 5.0), 0.1 to 1 mM 5′-IMP, and the enzyme solution, in a total volume of 10 ml. After incubation at 30 °C for 10 min, 1 ml of sample was taken from the reaction mixture and stopped by adding 0.2 ml of 2 M HCl. Quantitative determination of inosine was carried out by HPLC. Kinetic parameters were calculated from Lineweaver–Burk plots.

2.8. Production and isolation of 5′-IMP

2.8.1. Time course of 5′-IMP synthesis

The time course of 5′-IMP synthesis was examined using 100, 150 and 200 mg of inosine, 250 mg of pyrophosphate and 2.12 mg of the purified enzyme in 10 ml sodium acetate buffer. The reaction was carried out at 30 °C with moderate shaking and terminated by adding 1 ml of 2 M HCl. Quantitative determination of 5′-IMP was carried out by HPLC as mentioned above.

2.8.2. Isolation and identification of 5′-IMP

Reaction mixture (50 ml) containing 5′-IMP was centrifuged at 12,000*g* for 10 min at 4 °C to remove the cells for purification of 5′-IMP. Later, the supernatant was applied onto a column of 201 × 7 Strong-Base Anion-Exchange Resin (Cl^- form). The elution procedure was used as follows. The supernatant was equilibrated with water and then the impurities were eluted from the column using a solution containing 0.03 M HCl and 0.1 M NaCl; subsequently, the expected product was washed out with an elution buffer (0.1 M HCl and 0.1 M NaCl). Additionally, the fractions containing 5′-IMP were collected, neutralised with NaOH, and then distilled and dried under vacuum. The crystals of 5′-IMP obtained were studied further.

The ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectra of 5′-IMP were obtained on an NMR spectrometer (AVANCE 500 MHz, Bruker, Fällanden, Switzerland) with D_2O as the solvent, using 500 and 125 MHz for carbon and proton determinations, respectively. The FTIR spectrum for the 5′-IMP was recorded on a Fourier transform infrared spectrometer (Nicolet 6700; Thermo, Waltham, MA) with Contimuμm FTIR Micro system in the region of 4000–400 cm^{-1} using KBr pellets.

2.9. Homology modelling and docking

The three-dimensional homology models of wild-type and mutant AP/PTase were generated using Build Homology Models (MODELER) in Discovery Studio 2.1 (Accelrys Software, San Diego, CA) (Morris et al., 1998), using crystal structures of AP/PTase from *E. blattae* at 2.4 Å resolution (PDB Accession Code 1EOI) as the template (Ishikawa et al., 2000). The generated structures were improved by subsequent refinement of the loop conformations by assessing the compatibility of an amino acid sequence to known PDB structures using the Protein Health module in Discovery Studio 2.1. The geometry of loop regions was corrected using Refine Loop/MODELER. Finally, the best quality model was chosen for further calculations, molecular modelling, and docking studies by Autodock 4.0 (Morris et al., 1998).

3. Results and discussion

3.1. Expression of acid AP/PTase gene

Based on the reported nucleotide sequence, the *E. blattae* AP/PTase gene was synthesised after optimisation of the codons. The synthesised DNA fragment was subcloned into an expression vector pET28b(+), to construct the recombinant plasmid pET28b(+)-AP/PT. Subsequently, the recombinant plasmids were introduced into competent cells of *E. coli* BL21 (DE3) and several clones were picked up. The positive transformant containing recombinant pET28b(+)-AP/PT was identified by colony PCR and double enzymatic digestion. The recombinant *E. coli* harbouring pET28b(+)-AP/PT was induced by 0.1 mM of IPTG at 28 °C for 10 h when the OD_{600} reached the value of 0.6. SDS–PAGE showed protein bands were visualised in gels by staining with Coomassie Brilliant Blue. The SDS–PAGE analysis (Fig. 1) shows a band with molecular mass of about 25 kDa in Lane 5 is obtained, which is in agreement with the predicted value (24.8 kDa) based on the amino acid sequence of AP/PTase. The purified protein was observed as a single band on an SDS–PAGE, indicating that the protein was quite pure.

3.2. Enzyme characterisation

3.2.1. Optimal temperature and thermostability

To test the optimal temperature for the enzymatic activity of the recombinant acid phosphotransferase, 10 mg/ml of inosine was used as the substrate and the reaction mixture was incubated at 20, 25, 30, 35, 40, 45, 50, 55 and 60 °C. The enzyme remains active at temperatures ranging from 20 to 50 °C and had maximal activity at around 30 °C (Fig. 2a). Mihara et al. (2001) reported that 30 °C was the optimal temperature of this enzyme from *Providencia stuartii* and *M. morganii*, whereas acid phosphotransferase from *Klebsiella planticola* had maximal activity at approximately 35 °C. After this apex point, the activity decreased and negligible acid phosphotransferase activity was observed at 60 °C. To test the

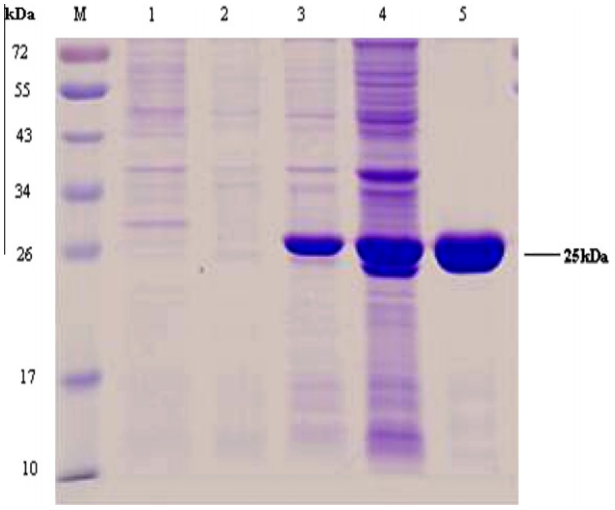

Fig. 1. SDS–polyacrylamide gel electrophoresis of the purified enzyme. *Lane M*: Marker proteins; *lane 1*: *E. coli* BL21 (DE3); *lane 2*: uninduced *E. coli* BL21 (DE3)/pET28b(+)-AP/PT; *lane 3*: *E. coli* BL21 (DE3)/pET28b(+)-AP/PT induced by IPTG; *lane 4*: crude extract; *lane 5*: the purified enzyme.

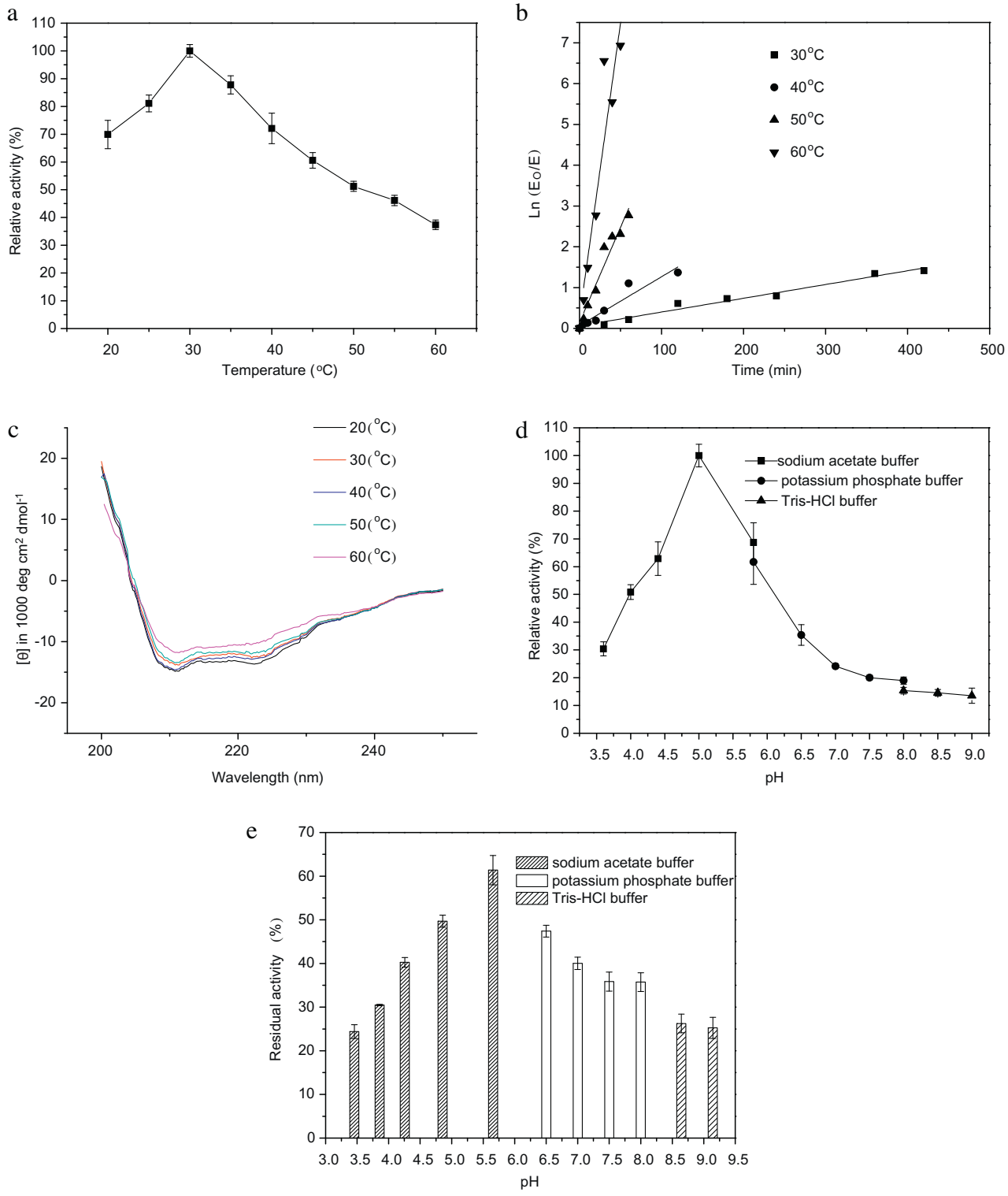

Fig. 2. Enzyme characterisation. (a) Effect of temperature on the acid phosphotransferase activity. Reactions were performed at 150*g* for 10 min at various temperatures (20–60 °C) with 100 mM sodium acetate buffer. (b) The thermostability of the purified enzyme. By incubating enzymes at temperature settings of 30, 40, 50 and 60 °C for the indicated time intervals from 5 min to 7 h. ■ 30 °C; ● 40 °C; ▲ 50 °C; ▼ 60 °C. (c) Spectroscopic structural analysis. (d) Effect of pH on the acid phosphotransferase activity. The enzyme activity was measured at different pH values with 100 mM sodium acetate buffer, pH 3.6–5.8 (■), potassium phosphate buffer, pH 5.8–8.0 (●), Tris–HCl buffer, pH 8.0–9.0 (▲). (e) pH stability of acid phosphotransferase. The purified acid phosphotransferase was pretreated in different buffers with pH ranging from 3.6 to 9.0 at 30 °C for 1 h and the residual activity was tested.

thermostability, the purified enzyme was incubated at 30–60 °C from 5 min to 7 h at the indicated time intervals and residual activity was determined according to the standard enzyme assay method. The residual activity was obtained in accordance with the activity at the 0th h and its natural logarithm value (Ln E_0/E) was plotted against time. Fig. 2b shows the results of the thermostability of acid phosphotransferase. The K_d of the enzyme at 30, 40, 50 and 60 °C were 0.0036, 0.0128, 0.05 and 0.1518 min^{-1}, respectively.

Accordingly, the half-lives ($t_{1/2}$) of the purified enzyme at 30, 40, 50 and 60 °C were calculated to be 3.2, 0.90, 0.23 and 0.076 h, respectively. Indeed, the enzyme seemed to be still sensitive to heat, which can be corroborated by thermostability measurements by CD (Fig. 2c), which demonstrates that this recombinant enzyme was not thermostable and needs further improvements.

3.2.2. Optimal pH and pH stability

To test the optimal pH of the enzyme activity, the reactions were carried out in the following buffers including sodium acetate buffer (pH 3.6–5.8), potassium phosphate buffer (pH 5.8–8.0) and Tris–HCl buffer (pH 8.0–9.0) with a final concentration of 100 mM at 30 °C. The result showed that the optimal pH for the acid phosphotransferase was 5.0 with sodium acetate buffer (Fig. 2d). The pH stability of enzyme was examined by assaying the residual activity under the standard assay conditions. The results indicated that the acid phosphotransferase was more stable at pH 5.8 with about 61% residual activity. In addition, pH higher than 8.5 or lower than 4.0 resulted in a significant decrease in the residual activity of acid phosphotransferase (Fig. 2e), which was similar to the purified enzymes from *P. stuartii*, *M. morganii* and *K. planticola* (Mihara et al., 2001). On the basis of these results, further investigations of the phosphorylation reaction were performed at pH 5.0 and 30 °C.

3.2.3. Effect of metal ions and chemicals on enzyme activity

The effects of various metal ions at a final concentration of 2 mM and other reagents, such as EDTA, DMSO, THF, DTT, Tween-20, Tween-80 and Triton X-100 with concentration of 2%, on the enzyme activity of the acid phosphotransferase were tested (Table 1). The activity assayed in the absence of metal ions and other reagents was considered as control. The results indicated that the activity was partially inhibited by metal ions, such as Hg^{2+}, Ag^{+} and Cu^{2+}. However, no inhibition was observed in the presence of chelating agent EDTA, which illustrated that the acid phosphotransferase obtained in the current study did not required metal ions for its catalysis. It was consistent with acid phosphotransferase production by *M. morganii* (Asano et al., 1999b; Mihara et al., 2000). In addition, the results showed that addition of Tween-20, Tween-80 and Triton X-100 could slightly activate the activity of acid phosphotransferase. However, DTT inhibited the activity of acid phosphotransferase, which showed that thiol groups may play an important role in the function of the active site.

Table 1
Effect of metal ions and other reagents on acid phosphotransferase activity.

Reagent	Relative activity (100%)
Control	100 ± 1.56
$CuCl_2$	77 ± 2.14
$CoCl_2$	80 ± 1.36
$Al_2(SO_4)_3$	84 ± 3.37
$NiCl_2$	68 ± 2.18
$MnCl_2$	66 ± 0.21
$CaCl_2$	94 ± 1.10
LiCl	79 ± 2.08
$MgCl_2$	92 ± 1.42
$BaCl_2$	97 ± 0.16
$ZnSO_4$	88 ± 2.12
$AgNO_3$	6 ± 0.56
$HgCl_2$	38 ± 1.56
$FeCl_3$	82 ± 2.43
$FeSO_4$	85 ± 2.16
EDTA	96 ± 1.56
DMSO	96 ± 0.89
DTT	34 ± 1.21
THF	82 ± 1.05
Tween-20	120 ± 1.46
Tween-80	120 ± 1.26
Triton X-100	144 ± 1.01

3.3. Determination of kinetic parameters

The kinetic data for the recombinant enzyme obtained by varying the concentration of inosine (3–45 mM) at 30 °C were plotted according to the Lineweaver–Burk equation and the Michaelis constant K_m and V_{max} values were estimated as well. The values of apparent K_m and V_{max} are 40 mM and 3.5 U/mg for inosine, respectively, while the values of K_m and V_{max} are 0.32 mM and 24.4 U/mg for 5′-IMP, respectively (Table 2). It is reported that the acid phosphotransferase activity of *M. morganii* was much improved, the K_m value for inosine was decreased and the V_{max} value for inosine was increased by introducing mutations G92D/I171T and S72F (Mihara et al., 2001; Suzuki, Ishikawa, Mihara, Shimba, & Asano, 2007). In the present study, we found that after three mutations S72F/G74D/I153T were introduced into the enzyme, the acid phosphotransferase activity was enhanced. One key distinction of the mutant was a reduced K_m value from 202 to 40 mM, compared to the wild type, for inosine in the transphosphorylation reaction (Suzuki et al., 2007). Another distinction was a decrease of V_{max} value in the dephosphorylation reaction, and almost the same K_m value was exhibited for inosine as that of the *M. morganii* G74D/I153T mutant (43 mM) (Mihara et al., 2000). In addition, the K_m value for inosine in the transphosphorylation reaction was much higher than that for 5′-IMP (0.32 mM) in the dephosphorylation reaction. The V_{max} value for dephosphorylation reaction appeared to be higher than that for transphosphorylation reaction. Due to these changes in catalytic properties, the productivity of the synthesised 5′-IMP may have been improved.

3.4. Production and isolation of 5′-IMP

3.4.1. Production of 5′-IMP by the purified enzyme

Using the purified enzyme, the time course of 5′-IMP synthesis from different concentrations of inosine and pyrophosphate was examined (Fig. 3). The data in Fig. 3 showed that as the reaction time was extended, the dephosphorylation of 5′-IMP occurred and the synthesised 5′-IMP was hydrolysed to inosine. In addition, as the concentration of inosine increased, the rate of hydrolysis of 5′-IMP decreased and the amount of 5′-IMP synthesised increased. When 37.3 mM of inosine and 560 mM of disodium hydrogen pyrophosphate was added to the reaction mixture, 16.83 mM of 5′-IMP were produced from 37 mM of inosine in 40 min, and the molar yield of 5′-IMP from inosine was 45.5%, while *M. morganii* exhibited lower yield from higher concentration of inosine (80 mM) (Asano et al., 1999b). Additionally, the enzyme from *Enterobacter aerogenes* IAM 1183 synthesised 5.85 mg/ml of 5′-IMP, which was lower than the yield in this study. Mihara reported that by introducing 11 mutations, the AP/PTase activity was much improved and 156 g/L of 5′-IMP was obtained (Mihara et al., 2004). As the purified nucleoside phosphorylating enzyme was used in the reaction, dephosphorylation of synthesised 5′-IMP appeared to be catalysed by the same enzyme. In order to enhance the productivity of 5′-IMP in the transphosphorylation reaction, it is necessary to suppress the phosphatase activity. In addition, to achieve efficient phosphorylation, pyrophosphate from phosphate should be recycled.

3.4.2. Isolation and identification of 5′-IMP

After isolation and purification according to the method described in materials and methods (Section 2.8.2), 5′-IMP with 98.6% of purity and 39.8% of total yield was obtained. The FTIR spectrum of the 5′-IMP separated is presented in Fig. 4a. The sharp peaks present at 1679 cm^{-1} can be assigned to P = O stretching

Table 2
Kinetic constants for the transphosphorylation reactions.

	Activity	Substrate	K_m (mM)	V_{max} (U/mg)
Wild type	Acid phosphotransferase	Inosine	202	1.83
S72F/G74D/I153T	Acid phosphotransferase	Inosine	40	3.5
Wild type	Acid phosphatase	5′-I MP	0.18	49.4
S72F/G74D/I153T	Acid phosphatase	5′-I MP	0.32	24.4

Note: The enzyme activities were assayed as described in Section 2. Initial velocities were determined, and steady-state kinetic constants were calculated from Lineweaver–Burk plots.

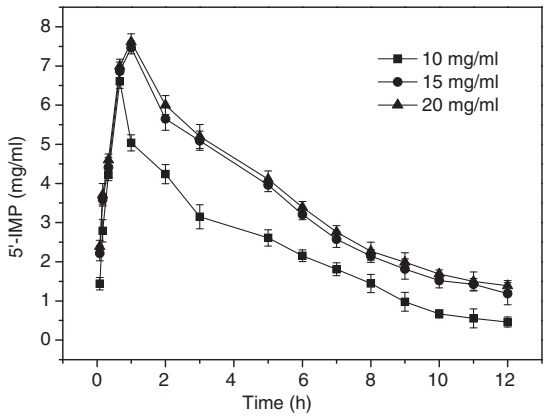

Fig. 3. Time course of 5′-IMP synthesis from inosine and pyrophosphate by the purified enzyme. Reaction was carried out at different concentrations of inosine; enzyme activities were determined according to the standard enzyme assay method. ■, 10 mg/ml inosine; ●,15 mg/ml inosine; ▲, 20 mg/ml inosine.

vibration. The strong and broad absorption band between 3200 and 3400 cm^{-1} is due to N–H stretching vibration and the peak appearing at 2875 cm^{-1} is due to O–H stretching vibration of the phosphate group. The ^{13}C and ^{1}H NMR spectra were used to characterise 5′-IMP dissolved in D_2O solution. When ^{13}C NMR was performed, the isolated product gave double signals at the C4′ position which resulted from ^{13}C–^{31}P coupling, which demonstrated that isolated IMP was phosphorylated at the C5′ position (Fig. 4b). Fig 4c shows seven different kinds of H atoms, which correspond to the structure of 5′-IMP, such as two peaks at chemical shifts δ = 8.1877, 8.5563 ppm, represent two H atoms of purine ring at C2 and C8 position. Accordingly, it was concluded that the isolated product was 5′-IMP.

3.5. Homology modelling and molecular docking

The secondary and tertiary structures analysis showed that AP/PTase obtained in this study consisted of 13 α-helices, which is different from some reported AP/PTases (Strater, Klabunde, Tucker, Witzel, & Krebs, 1995). Crystal structure of *E. blattae* AP/PTase (PDB Accession Code 1EOI, resolution 2.4 Å) was used as the template. After the transition-state analogue molybdate of the template was replaced by phosphate, a hypothetical binding mode of inosine to the phosphoenzyme intermediate could be modelled (Fig. 5a). As shown in Fig. 5a, the active site contains Lys 115, Arg 122, Ser 148, Gly 149, His 150, Arg 183, His 189 and Asp 193, which is the same as previous reports (Hemrika & Wever 1997; Neuwald 1997; Stukey & Carman, 1997). These residues can be found in the conserved motif KX_6RP-(X_{12-54})-PSGH-(X_{31-54})-SRX_5HX_3D (Stukey & Carman, 1997; Thaller, Schippa, & Rossolini, 1998). The reaction mechanism can be proposed as follows: the conserved residue His 189 in motif 3 (SRX_5HX_3D) was predicted to attack the phosphate group, NE2 of His 189 to form a covalent bond with phosphate, subsequently a phosphoenzyme catalytic intermediate was formed. Simultaneously, the conformation of the residues His 150, Arg 183, Arg 122 around His 189 was changed. Direct hydrogen bonds are formed between HZ1 of Lys 115 and O1 of phosphate, HH22 of Arg 122 and O1 of phosphate, HE of Arg 183 and O2, O3 of phosphate, HN of His 150 and O2 of phosphate, HD1 of His 150 and O4 of phosphate. The hydrogen bonds could stabilise the phosphoenzyme catalytic intermediate. When phosphate acceptor inosine attacks the binding pocket of AP/PTase, two hydrogen bonds were formed between 2′,3′-hydroxyl group of the ribose ring and the carboxyl group of Glu 104. It leads to the distance between O1 of inosine and O4 of phosphate group being nearer by 3.1 Å. In the last step of the catalytic reaction, a water attacks the phosphoenzyme catalytic intermediate, in order to cleave the covalent bond between His 189 and the phosphate; consequently, phosphate group is released from the enzyme. Later the released phosphate attacks the inosine which is close to it and replaces the water by inosine; subsequently, 5′-IMP was produced.

A binding mode of inosine to the mutated phosphoenzyme intermediate could be modelled (Fig. 5b). The result in Fig. 5b showed that after mutation, the interaction between some residues near His 189, such as Lys 115, Arg 122, His 150, Arg 183 and phosphate, have little changes. However, the structure of S72F/G74D/I153T altered the interaction between inosine and phosphoenzyme intermediate. Besides the previously formed hydrogen bonds between the carboxyl group of Glu 104 and the hydroxyl group of the ribose, NH of Leu 70, NH of Ser 71, NH of Asp 108, and OH of Asn 143 form hydrogen bonds with inosine. These newly-formed hydrogen bonds may enhance the affinity of enzyme to inosine, accordingly reducing the K_m value for inosine. In addition, the distance between inosine and phosphate was from 3.1 to 2.5 Å, which could help the released phosphate to attack inosine more easily and enhance the catalytic activity. As the structure of mutated protein S72F/G74D/I153T was constructed, the result revealed that the overall structure of mutant is identical to that of the wild type, which indicated that the difference in the K_m between mutant and wild type can be ascribed to the changes in the binding pocket (Mihara et al., 2001).

In the inosine binding model, it is found that Thr 153 is situated near the inosine binding site (Fig. 5a and b). Mutation at position 153 may cause a subtle structural change in the inosine binding site; the reduced K_m could be ascribed to a difference in the interaction between the inosine and the inosine binding site. On the other hand, after mutation at position Asp 74, the flexibility of the region ranging from Asn 69 to Val 75 is increased in the G74D/I153T structure, compared to the wild type. The increased flexibility of this region may enhance the tightness of binding and the affinity of the enzyme to inosine Moreover, the G74D/I153T mutations can reduce the K_m without either a significant structural change or a new direct interaction, which suggests that amino acid substitution around the inosine binding site can reduce the K_m. The S72F mutation can lead to the formation of aromatic–aromatic interaction between the base of a nucleoside and the side-chain of an aromatic residue of the enzyme. The loop containing Phe 72 has a notable conformational change on the formation of phosphoenzyme intermediate; therefore, a much better

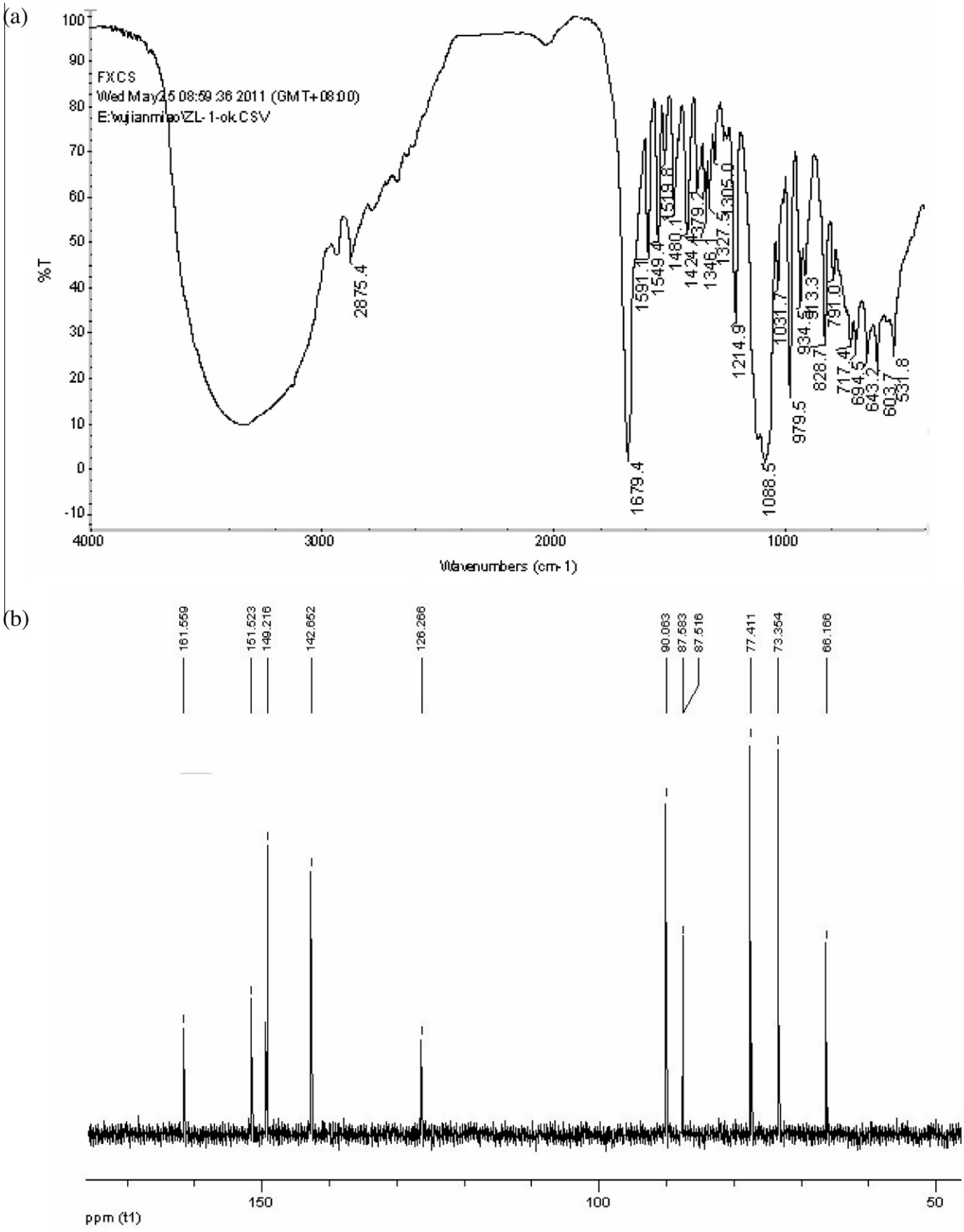

Fig. 4. Identification of 5′-IMP. (a) Infrared spectroscopy of 5′-IMP. (b) Identification of the isolated product performed on ^{13}C NMR. (c) Identification of the isolated product performed on ^{1}H NMR.

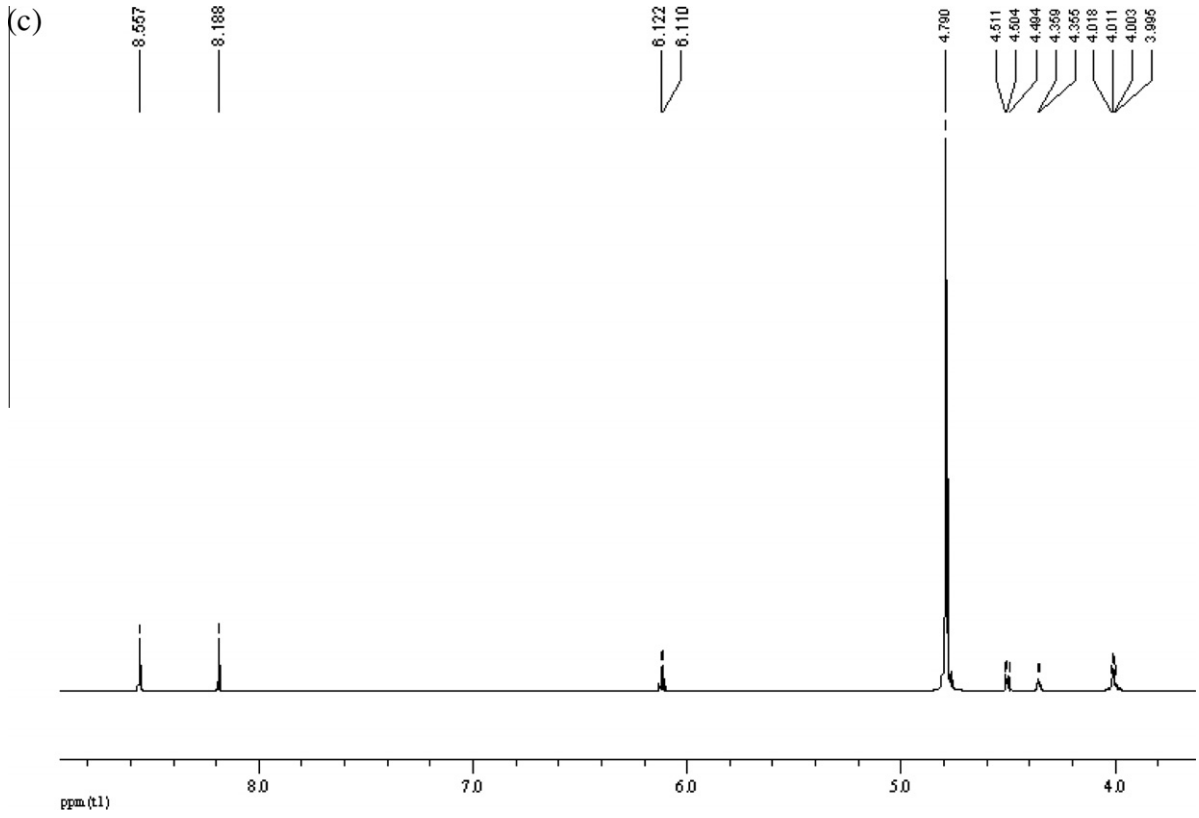

Fig. 4 (*continued*)

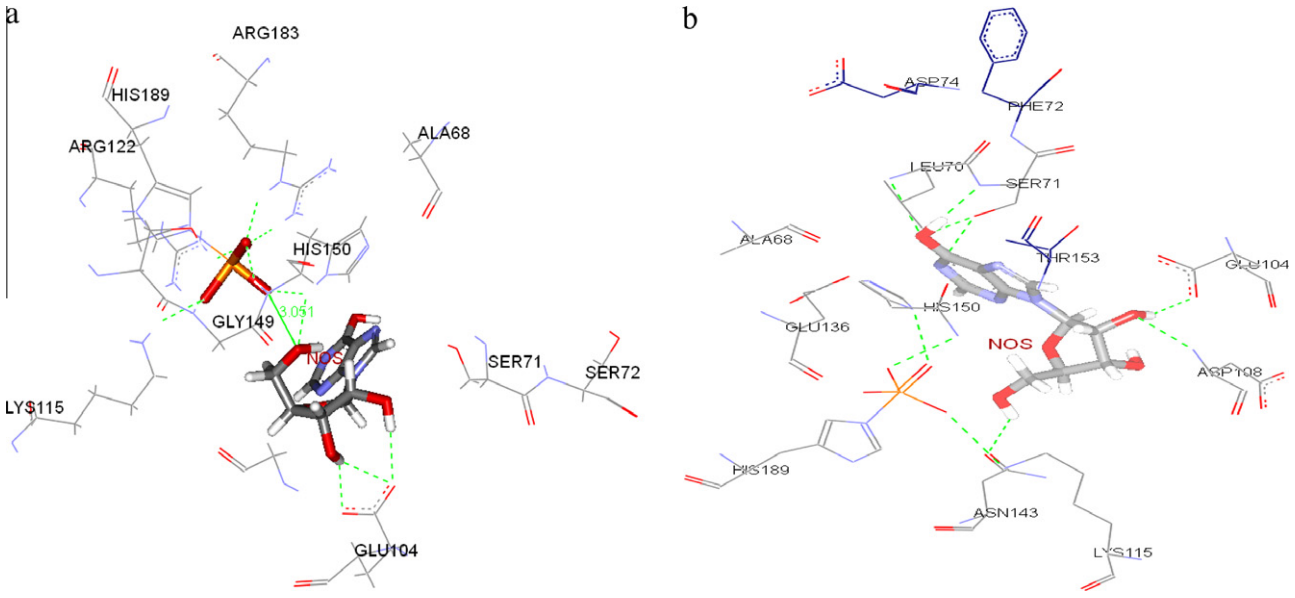

Fig. 5. A stereo view of the putative intermediate. (a) Inosine (NOS) docking into the active site of native acid phosphatase/phosphotransferase and its catalytic triad with inosine. (b) Inosine docking into the active site of the mutative acid phosphatase/phosphotransferase.

aromatic–aromatic interaction is formed and results in lower K_m and higher activities (Suzuki et al., 2007).

4. Conclusions

In this current study, an acid phosphatase with strictly regioselective nucleoside phosphotransferase from *E. blattae* was expressed and evaluated. Characterisation of this recombinant enzyme revealed that this enzyme has an excellent function on the transphosphorylation of inosine. The investigation of the characteristics of the purified acid phosphotransferase showed that the optimal temperature and pH of this enzyme were 30 °C and 5.0, respectively. The enzyme activity could not be activated by the metal ions tested in this study, and was partially inhibited by Hg^{2+}, Ag^{+} Cu^{2+}, but slightly activated by Tween-20, Tween-80

and Triton X-100. The values of K_m and V_{max} for inosine were 40 mM and 3.5 U/mg, respectively. The time course showed that 16.83 mM of 5′-IMP was synthesised from 37 mM of inosine and the molar yield reached 45.5% after 40 min reaction. The 5′-IMP obtained in this study was further isolated and characterised. Further investigations on improvement of phosphotransferase activity for industrial production of 5′-IMP are in progress in our laboratory.

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