ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 6 (1999) 271–277

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

A new enzymatic method of selective phosphorylation of nucleosides [1]

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Received 12 February 1998; revised 13 March 1998; accepted 17 March 1998

Abstract

The phosphorylation of inosine in the 5′-position to produce inosine-5′-monophosphate (5′-IMP) was studied in a number of microorganisms from our culture collection using pyrophosphate (PPi) as the phosphate source. Although many of the microorganisms screened were able to phosphorylate inosine, phosphotransferase activity specific to the 5′-position was found to be distributed among the bacteria belonging to the family Enterobacteriacea. *Morganella morganii* NCIMB10466 was selected for further study of 5′-IMP production. When *M. morganii* intact cells were taken approximately 0.2 mg/ml wet weight, 6.02 mg/ml (11.4 mM) of 5′-IMP were synthesized from 10 mg/ml (37.3 mM) of inosine and 250 mg/ml (560.0 mM) of tetrasodium pyrophosphate decahydrate in 9 h.

Keywords: Screening; Phosphorylation; Nucleoside; 5′-nucleotide; Pyrophosphate; *Morganella morganii*

1. Introduction

Nucleotides are often used as food additives and as pharma intermediates, although their biological activity is related to the position of the phosphate group. Inosine-5′-monophosphate (5′-IMP), for example, has a characteristic taste and is used for a flavour potentiator in various foods, whereas inosine-2′-monophosphate (2′-IMP) and inosine-3′-monophosphate (3′-IMP) are tasteless.

The phosphorylation of nucleosides in the C5′-position can be achieved by both chemical and enzymatic methods. Enzymatic phosphorylation requires only mild reaction conditions and protection of the functional groups is not necessary. The usefulness of this method, however, is usually limited by the narrow specificity of the enzyme.

An enzyme which catalyzes the synthesis of nucleotides by transfer of phosphate groups from organic phosphate to nucleosides was described by Brawermann and Chargaff [1,2]. Most of their works were carried out with the plant enzymes. Marutzky et al. [3] synthesized various nucleoside-5′-monophosphates using a carrot phosphotransferase and phenylphosphate as the phosphate donor. The nucleoside phosphotransferase reaction of bacteria was studied by Mitsugi et al. using *p*-nitrophenylphosphate as the phosphate donor [4–8]. These authors found

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

1381-1177/99/$ - see front matter
PII: S1381-1177(98)00081-2

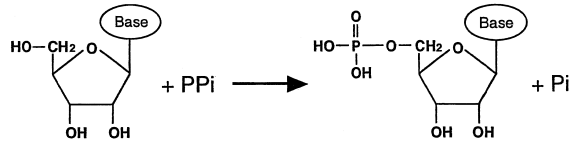

Fig. 1. Selective nucleoside phosphorylation using pyrophospate (PPi) as the phosphate donor.

that the bacteria, which had nucleoside phosphotransferase activity could be divided into C5′-position selective group and C3′(2′)-position selective group, depending on the nucleotide isomers synthesized.

In this current study, nucleoside phosphorylation reaction was studied using the food additive pyrophosphate (PPi) as the phospate donor. Bacteria and yeasts were screened for the ability to phosphorylate inosine in the C5′-position to produce 5′-IMP (Fig. 1).

2. Experimental

2.1. Chemicals

5′-IMP was purchased from Sigma, (St. Louis, MO, USA). 2′ and 3′-IMP were purchased from Yamasa Shoyu, (Chiba, Japan). Tetrasodium pyrophosphate decahydrate ($Na_4P_2O_7 \cdot 10H_2O$) was purchased from Nacalai tesque, (Osaka, Japan). All other chemicals used were commercially available and were of analytical grade.

2.2. Microorganisms and media

The microorganisms screened were from the culture collection held in our laboratory. Bacteria were grown on L-agar which contained 1% peptone, 1% NaCl, 0.5% yeast extract, pH 7.0. Yeasts were grown on YM-agar, which contained 1% glucose, 0.5% yeast extract, 0.3% peptone, 0.3% malt extract, pH 6.0.

2.3. Screening

Bacteria and yeasts were grown for 1–2 days at 30°C on the appropriate medium. The reaction mixture was prepared and consisted of 1 ml of 0.1 M potassium phosphate buffer, pH 7.0, or 0.1 M sodium acetate buffer, pH 5.0, each containing 20 mg inosine, 100 mg tetrasodium pyrophosphate decahydrate ($Na_4P_2O_7 \cdot 10H_2O$), and 0.2 mg $MgSO_4 \cdot 7H_2O$, and approximately 50 mg wet weight of bacteria or yeasts cells. The reaction was allowed to proceed at 30°C for 16 h with moderate shaking. The synthesis of 5′-IMP, 3′-IMP and 2′-IMP synthesized were analyzed by thin layer chromatography (TLC) and/or high performance liquid chromatography (HPLC).

2.4. Analysis

Qualitative analysis of 5′-IMP, 3′-IMP and 2′-IMP was carried out by silica-gel TLC with a solvent system of *n*-propanol–NH_4OH–water (20:15:3). IMP isomers were detected by absorption at 254 nm. Quantitative determination of 5′-IMP, 3′-IMP and 2′-IMP was carried out by HPLC with a Cosmos $5C_{18}$-MS column (4.6 × 150 mm, Nacalai tesque) with detection at 245 nm. The mobile phase was 5 mM potassium phosphate buffer (pH 2.8): methanol (95:5, v:v) and the flow rate was 1 ml/min.

2.5. Synthesis of 5′-IMP by intact cells of Morganella morganii

One loopful of *M. morganii* NCIMB10466 subcultured on L-agar, was inoculated into 200 ml of L-broth in 500 ml flasks and cultivated aerobically on a reciprocal shaker at 30°C for 16 h. The cells were harvested by centrifugation at $8000 \times g$ for 20 min and then washed with 0.1 M sodium acetate buffer (pH 5.0). The reaction mixture for 5′-IMP synthesis consisted of 50 ml of 0.1 M sodium acetate buffer (pH 5.0), containing 10 mg/ml of inosine, 50–250 mg/ml of $Na_4P_2O_7 \cdot 10H_2O$, 0.2 mg/ml of $MgSO_4 \cdot 7H_2O$ and the cells harvested from 200 ml of culture broth. The reaction was carried out at 30°C with moderate shaking.

2.6. Isolation of reaction product

Reaction mixture 100 ml containing 5′-IMP (295 mg) was boiled for 10 min, and then centrifuged ($8000 \times g$ for 20 min) to remove the cells. The supernatant was applied onto a column of IRA-400 (Cl^- form, 40 mm × 170 mm), washed with water, and eluted with a concentration gradient of 0.01–0.1 M HCl (total volume 2 l). The fractions containing 5′-IMP (101 mg in total) were collected, neutralized with NaOH, and dried under vacuum.

3. Results

3.1. Screening of microorganisms for the phosphotransferase activity using PPi as the phosphate donor

A total of 217 strains of bacteria and 116 strains of yeast were assayed for their ability to phosphorylate inosine in the C5′ position, at pH 7.0, using PPi as the phosphate donor. Phosphotransferase activity was found to be widely distributed among the bacteria examined. In these bacteria, however, the phosphorylation of inosine was catalyzed not only at the C5′ position, but also at the C3′ and/or C2′ positions. The ratio of the IMP isomers synthesized varied considerably, although *M. morganii* formed 5′-IMP with high regiospecificity. When the effect of pH on phosphotransferase activity was investigated in *M. morganii*, the optimum pH was found to be 5.2 (Fig. 2).

On the basis of this result, phosphotransferase activity was assayed again at pH 5.0. *Escherichia blattae* ATCC33429, *M. morganii* NCIMB10466, *Klebsiella pneumoniae* IFO 3318, and *Enterobacter aerogenes* IFO13534 were all found to have phosphotransferase activity with high regiospecificity to the C5′ position, and all synthesized predominantly 5′-IMP (Table 1). Phosphotransferase activity was also detected in various yeast species, but this activity was weaker than that found in bacteria.

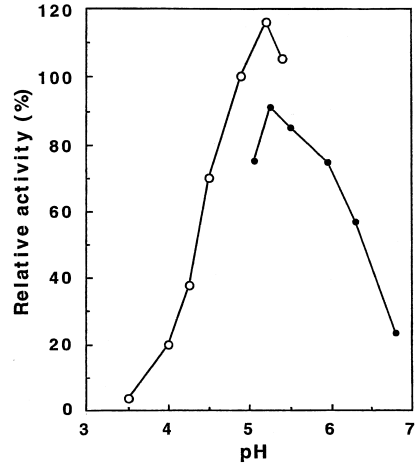

Fig. 2. Effect of pH on phosphotransferase activity of *M. morganii*. The reaction was carried out at 30°C for 16 h in a reaction mixture containing inosine (20 mg), $Na_4P_2O_7 \cdot 10H_2O$ (100 mg), $MgSO_4 \cdot 7H_2O$ (0.2 mg) and 50 mg cells (wet weight), in a total volume of 1 ml. The pH of each reaction was varied using 0.1 M sodium acetate buffer, pH 3.5–5.5 (○) and 0.1 M 2-(*N*-morpholino) ethanesulphonic acid–NaOH buffer, pH 5.0–6.8 (●).

The ability of *Pseudomonas trifolii* IAM-1555, *Achromobacter superficialis* IAM1433 and *Flavobacterium odoratum* ATCC4615 to phosphorylate nucleosides at the C5′ position has already been documented, and *E. coli* B-25 and *Aeromonas punctata* IAM1646 have been shown to have phosphotransferase activity at the C2′ and C3′ position using *p*-nitrophenylphosphate as the phosphate donor [4]. When the phosphotransferase activity of these strains was assayed using PPi as the phosphate no phosphotransferase activity was detected. It is suggested, therefore, that these enzymes that phosphorylate nucleosides using PPi as the phosphate donor are different from those that use *p*-nitrophenylphosphate as the source of phosphate.

The regiospecificity of phosphotransferase activity to the C5′-position was distributed widely among the bacteria belonging to the family Enterobacteriaceae [9]. Among these, *E. blattae* ATCC33429 produced the high level of 5′-IMP with the highest regiospecificity. *M. morganii* NCIMB10466 produced the highest

Table 1
Screening of microorganisms for phosphotransferase activity using PPi as the phosphate donor

Strains	IMP (μg/ml)			Ratio of IMP
	5′-IMP	3′-IMP	2′-IMP	5′:3′:2′:
Bacteria				
Achromobacter parvulus IFO13181	213	517	N.D.	100:242.3:0
Brevibacterium linens IFO12141	3608	1849	176	100:51.2:4.9
Cedecea davisea JCM1685	3047	118	47	100:3.9:1.5
Chromobacterium fluviatile IAM13652	1478	206	73	100:14.0:5.0
Chromabacterium violaceum IFO12614	1933	310	99	100:16.0:5.1
Corynebacterium rathayi IFO12161	2584	1379	160	100:53.4:6.2
Edwardsiella ictaluri JCM1680	110	4	N.D.	100:3.2:0
E. aerogenes IFO13534	2196	43	14	100:2.0:0.6
Erwinia herbicola IAM1584	518	37	N.D.	100:7.1:0
E. blattae ATCC3342	3900	8	N.D.	100:0.2:0
Hafnia alvei IFO3731	304	7	2	100:2.4:0.7
K. pneumoniae IFO3318	3613	37	8	100:1.0:0.2
Klebsiella planticola IFO14939	4128	84	24	100:2.0:0.6
M. morganii NCIMB10466	4526	163	61	100:3.4:1.4
Pseudomonas maltopholia IFO12020	98	108	12	100:110.4:12.2
Pseudomonas diminuta IAM1513	58	122	N.D.	100:209.4:0
Pseudomonas synxantha IFO3906	123	118	16	100:95.3:13.1
Serratia grimesii IFO13537	456	233	60	100:51.0:13.1
Serratia ficaria IFO13537	153	5	3	100:3.02:2.1
Yeasts				
Candida lipolytica IFO0746	105	77	60	100:73.0:57.1
Candida oleophila ATCC20177	103	5	33	100:5.1:31.5
Cryptococcus laurentii IFO0609	125	15	N.D.	100:12.2:0
Hansenula anomala IAM4561	228	39	N.D.	100:17.2:0
Hansenula miso IAM4116	283	43	N.D.	100:15.2:0
Hansenula beckii IFO0803	140	33	N.D.	100:23.9:0
Pichia polymorpha IFO1670	223	36	N.D.	100:16.3:0
Pichia miso IAM4682	105	4	N.D.	100:3.7:0
Pichia haplophila IFO0947	80	48	N.D.	100:60.1:0
Saccharomycopsis lipolytica IFO1209	142	67	N.D.	100:47.0:0
Wickerhamia fluorescens IFO1116	130	19	N.D.	100:14.2:0

The reaction was carried out at 30°C for 16 h with moderate shaking in a reaction mixture consisting of 0.1 M sodium acetate buffer (pH 5.0), containing inosine (20 mg), $Na_4P_2O_7 \cdot 10H_2O$ (100 mg), $MgSO_4 \cdot 7H_2O$ (0.2 mg), and approximately 50 mg (wet weight) of cells, in a total volume of 1 ml.
N.D., not detected.

level of 5′-IMP with high regiospecificity, and was selected for further study of 5′-nucleotide synthesis using PPi as the phosphate source.

3.2. Reaction conditions for 5′-IMP production by M. morganii

Phosphotransferase activity was found in intact cells of *M. morganii*, but not in the culture supernatant. The activity did not increase when PPi or phosphate was added to the medium, indicating that the enzyme was produced constitutively by the strain.

The optimum reaction conditions for 5′-IMP production were investigated using intact cells of *M. morganii*. The optimum pH was found to be 5.2 and the optimum temperature was around 35°C. The reaction pH and temperature did not affect the ratio of IMP isomers synthesized. When the reaction was carried out at 30°C, little hypoxantine was detected, whereas more hypoxantine was formed from inosine as the reaction temperature increased.

3.3. Substrate specificity

The ability of intact cells of *M. morganii* to phosphorylate inosine was examined using several phosphate compounds. As shown in Table 2, 5′-IMP was synthesized from various phosphate donors, although phosphoric acid was inert. Energy rich compounds like carbamylphosphate and acetylphosphate were very effective donors, whereas tripolyphosphate was less effective as a donor than PPi.

Various nucleosides were subjected to phosphorylation using intact cells of *M. morganii* (Table 3). In addition to inosine, the other nucleosides tested were also phosphorylated; hence, the enzyme had a broad specificity with regard to the phosphate acceptor. Each phosphorylated product was confirmed to be mainly nucleoside-5′-monophosphate by HPLC.

3.4. Synthesis of 5′-IMP by intact cells of M. morganii

The time course of 5′-IMP synthesis from inosine (10 mg/ml) and $Na_4P_2O_7 \cdot 10H_2O$ (50–250 mg/ml) was examined using intact cells of *M. morganii* (Fig. 3). In the reaction, phosphorylated products were mainly 5′-IMP and the amount of IMP isomers was very small. *M. morganii* also had phosphatase activity. The hydrolysis was directed primarily towards PPi, but with an extended reaction time synthesized 5′-IMP was hydrolyzed to inosine and phosphate. As the concentration of PPi increased, the

Table 2
Phosphoryl donor specificity of the phosphotransferase reaction with intact cells of *M. morganii*

Phospate compounds	Relative activity (%)
Pyrophosphate	100
Tripolyphosphate	22
Polyphosphoric acid	91
p-Nitrophenylphosphate	99
Phenylphosphate	113
Carbamylphosphate	246
Acetylphosphate	211
Glucose-6-phosphate	41
Glucose-1-phosphate	4
ATP	31
ADP	31
AMP	72
Phosphoric acid	0

The reaction was carried out at 30°C for 16 h with moderate shaking in a reaction mixture consisting of 0.1 M sodium acetate buffer (pH 5.0), consisting 40 μmol of inosine, 1 μmol $MgSO_4$, 50 mg cells (wet weight), and 112 μmol of each phosphate compound, in a total volume of 1 ml.
Polyphosphate was a mixture of $H_{n+2}P_nO_{3n+1}$.
A total of 38 mg/ml of polyphosphate (Nacalai tesque) was added to the reaction mixture.

Table 3
Phosphoryl acceptor specificity of the phosphotransferase reaction with intact cells of *M. morganii*

Nucleoside	Relative activity (%)
Inosine	100
Adenosine	41
Guanosine	51
Xanthosine	61
Cytidine	39
Uridine	47

The reaction was carried out at 30°C for 16 h with moderate shaking in a reaction mixture consisting of 0.1 M sodium acetate buffer (pH 5.0), containing 100 μmol $Na_4P_2O_7$, 1 μmol $MgSO_4$, 50 mg cells (wet weight), and 5 μmol of each nucleoside, in a total volume of 1 ml.

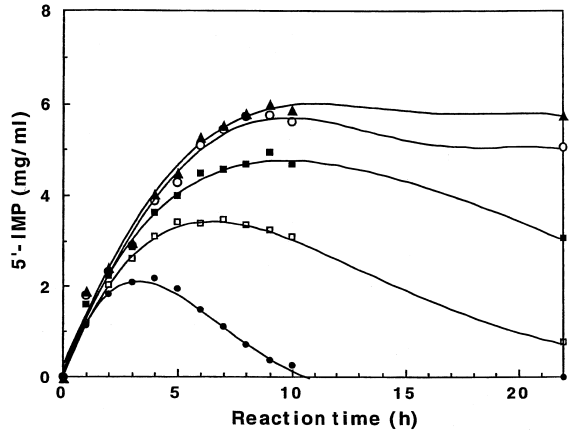

Fig. 3. Time course of 5′-IMP synthesis from inosine and PPi by intact cells of *M. morganii*. The reaction was carried out at pH 5.0 and 30°C for 22 h in a reaction mixture consisting of 0.1 M sodium acetate buffer (pH 5.0) containing inosine (10 mg/ml), $Na_4P_2O_7 \cdot 10H_2O$ (50–250 mg/ml), $MgSO_4 \cdot 7H_2O$ (0.2 mg/ml) and *M. morganii* cells harvested from 200 ml of culture broth, in a total volume of 50 ml. $Na_4P_2O_7 \cdot 10H_2O$ was added to the reaction mixture at 50 (●), 100 (□), 150 (■), 200 (○) and 250 (▲) mg/ml.

rate of hydrolysis of 5′-IMP decreased and the amount of 5′-IMP synthesized increased. The yield of 5′-IMP was dependent on the concentration of PPi. When 250 mg/ml (560.0 mM) of $Na_4P_2O_7 \cdot 10H_2O$ was added to the reaction mixture, 6.02 mg/ml (11.4 mM) of 5′-IMP were produced from 10 mg/ml (37.3 mM) of inosine in 9 h, and the molar yield of 5′-IMP from inosine was approximately 32%.

When the reaction product, which accumulated in the reaction mixture was isolated, a single peak with a retention time of 3.9 min was obtained on HPLC. This was identical to that of commercially available 5′-IMP. When ^{13}C-nuclear magnetic resonance (NMR) was performed, commercially available 5′-IMP and the purified product gave identical spectra, and each sample gave similar doublet signals at the C4′ position which resulted from $^{13}C-^{31}P$ coupling. This indicated that purified IMP was phosphorylated at the C5′ position. From these results, it was concluded that the purified product was 5′-IMP.^{13}C-NMR (D20) δ ppm: 162.11 (C6), 151.66 (C4), 149.72 (C2), 142.72 (C8), 126.41 (C5), 90.22 (C1′), 87.60, 87.52 (C4′), 77.48 (C2′), 73.41 (C3′), 66.31 (C5′).

4. Discussion

The regiospecificity of the nucleoside phosphorylating activity to the C5′-position belonged to bacteria of the family Enterobacteriacea. 5′-IMP could also be synthesized from inosine and PPi using intact cells of *M. morganii* NCIMB10466.

PPi is a safe compound and is used as a food additive. Tetrasodium pyrophosphate, for example, is used to regulate the consistency of cold puddings and ice cream, and disodium hydrogen pyrophosphate is used as an acid carrier in baking powders. PPi is also cheap and can be synthesized easily from phosphate. Tetrasodium pyrophosphate is produced by calcination of disodium hydrogen phosphate at 300–900°C [10]. PPi could, therefore, be used as a phosphate donor in the phosphorylation of nucleosides, and efficient phosphorylation could be achieved by recycling PPi from phosphate formed as a by-product in this reaction.

Some purine nucleotides, such as 5′-xanthylic acid [11] and 5′-IMP [12], can be produced by fermentation. However, nucleotides are usually difficult to accumulate by direct fermentation because of their poor permeability [12]. On the other hand, some purine nucleosides, such as inosine [13] and guanosine [14] can be produced efficiently by fermentation. With this knowledge, a novel process for producing 5′-nucleotides could be achieved by a combination of nucleoside fermentation and enzymatic phosphorylation using PPi as the phosphate source.

In the phosphorylation of inosine by *M. morganii*, small amounts of 2′- and 3′-IMP were formed as by-products. It is currently unknown whether one enzyme phosphorylates nucleosides with low regiospecificity or whether other enzymes with a different regiospecificity are also present. Several phosphoryl compounds could be used as phosphate donors, suggesting that phosphorylation was catalyzed by a phosphatase or phosphotransferase.

Further investigations are in progress to characterize this enzyme and to optimize the conditions for the phosphorylation reaction.

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