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Note

Phosphorylation of Guanosine Using Guanosine-inosine Kinase from *Exiguobacterium acetylicum* Coupled with ATP Regeneration

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Guanosine 5′-monophosphate (5′-GMP) and inosine 5′-monophosphate (5′-IMP) are widely used as flavor enhancers. Recently, a novel process for 5′-IMP production by phosphorylation of inosine using guanosine-inosine kinase coupled with ATP regeneration was reported.[1] In this study, we demonstrated the practical possibility of producing 5′-GMP by phosphorylation of guanosine using a guanosine-inosine kinase from *Exiguobacterium acetylicum* coupled with ATP regeneration.

Key words: guanosine 5′-monophosphate; guanosine-inosine kinase; ATP regeneration

Together with inosine 5′-monophosphate (5′-IMP), guanosine 5′-monophosphate (5′-GMP) is used as a flavor enhancer all over the world. 5′-GMP is manufactured by one of two methods: phosphorylation of guanosine using $POCl_3$;[2] or by amination of xanthosine 5′-monophosphate (5′-XMP) using 5′-XMP aminase.[3] The first method is generally used because guanosine fermentation and its phosphorylation using $POCl_3$ can be done efficiently.[2,4] However, this method is complex since it requires three tanks for fermentation, phosphorylation, and purification of guanosine for phosphorylation. Although phosphorylation of nucleoside by acid phosphatase has been reported, further experiments would be required due to its strong dephosphorylating activity.[5,6] Recently, a novel process for 5′-IMP production by phosphorylation of inosine using a guanosine-inosine kinase from *Escherichia coli* coupled with ATP regeneration was reported.[1] Guanosine-inosine kinase (ATP: guanosine 5′-phosphotransferase [EC 2.7.1.73]) catalyzes the phosphorylation of guanosine and inosine to their corresponding monophosphates by the following reaction:

Guanosine, Inosine + ATP
→ 5′-GMP, 5′-IMP + ADP

Application of this method for 5′-GMP production would be useful for constructing a new process for 5′-GMP production that resolves the problems of the traditional one mentioned above and fully exploits the efficiency of guanosine fermentation. Although the process of 5′-GMP production by guanosine phosphorylation using guanosine-inosine kinase coupled with ATP-regeneration has been suggested,[1] there is a need to demonstrate its feasibility in practice. The reason why the process has not been reported might be its enzymatic property being inhibited by by-products of the process, namely GDP and GTP.[7] Since a guanosine-inosine kinase with a novel property, that of not being inhibited by GDP and GTP, has been found in *Exiguobacterium acetylicum*[8] (a strain reclassified from the genus *Brevibacterium* on the basis of 16S rRNA analysis[9]), the possibility of using this enzyme can be investigated.

We expressed the genes that encode guanosine-inosine kinase (*gsk*) from *E. coli*,[10,11] and *Exiguobacterium acetylicum*[12] under the control of the *E. coli trp* promoter in an ATP-regenerating strain, *Corynebacterium ammoniagenes* ATCC 21477. Expression vectors were constructed as follows. For expressing the *E. coli gsk* gene, a DNA fragment carrying the *E. coli trp* promoter and the *E. coli gsk* gene was obtained from the plasmid pUCgsk,[7] which was constructed for expressing the *E. coli gsk* gene under control of the *E. coli trp* promoter in *E. coli*. This fragment, pHSG 298,[13] and the replication origin derived from pHM 1519[14] were ligated. For expressing the *E. acetylicum gsk* gene, it was amplified by PCR using 5′-GGCTGCAGGAATGAATAAAATCGCGGTAAT-3′ as the forward primer (5′-flanking the *Pst*I restriction site) and 5′-GGGCATGCTGGAAAGACATAATACGTTTCG-3′ as the reverse primer (5′-flanking the *Sph*I restriction site). The PCR product was digested with *Pst*I and *Sph*I, and cloned into pUC18 digested with *Pst*I and *Sph*I. The synthetic *E. coli trp* operon promoter was inserted between the *Bam*HI site and the *Pst*I site of the plas-

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Abbreviations: 5′-IMP, inosine 5′-monophosphate; 5′-XMP, xanthosine 5′-monophosphate

mid. The DNA fragment carrying the *E. coli trp* promoter and the *E. acetylicum gsk* gene obtained from the resulting plasmid, pHSG 298,[13] and the replication origin derived from pHM 1519[14] were ligated. *C. ammonagenes* cells were transformed by electroporation as described previously.[15]

We measured the guanosine-inosine kinase activity of recombinant cells grown in the medium described previously[16] were as follows[8] (Table 1). Recombinant cells were grown on a CM2G[17] agar plate (pH 7.2) containing 0.1 g/l adenine and 50 μg/ml kanamycin for 24 h at 32°C. The cells from one-sixth of the plate were transfered to 50 ml of medium (pH 7.2) containing 50 g/l glucose, 1 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l $CaCl_2 \cdot 2H_2O$, 5 g/l urea, 1 g/l KH_2PO_4, 3 g/l K_2HPO_4, 0.01 g/l $FeSO_4 \cdot 7H_2O$, 0.001 g/l $ZnSO_4 \cdot 7H_2O$, 0.004 g/l $MnSO_4 \cdot 4$–$6H_2O$, 0.005 g/l thiamine-HCl, 0.01 g/l Ca-D-pantothenic acid, 30 μg/l biotin, 0.02 g/l L-Cys-HCl, 5 g/l $(NH_4)_2SO_4$, 10 g/l polypepton, 10 g/l yeast extract, 0.3 g/l adenine, 50 μg/ml kanamycin. After 8 h of cultivation at 32°C, the cells were harvested and washed twice with 0.9% NaCl. The cells were then suspended in 2 ml of the buffer (50 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM dithiothreitol) and disrupted by sonication. Cell debris was removed by centrifugation at 18000 × *g* for 30 min, after which the supernatant was recovered and filtered on a PD-10 gel column (Pharmacia, Uppsala, Sweden). The measurement of guanosine-inosine kinase activity was done in a reaction mixture containing 100 mM Tris-HCl (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 0.2 mM [^{14}C]inosine (Moravec Biochemicals, Brea, CA, USA), and cell-free extract. The reaction mixture was incubated for 10 min at 30°C after which 2 μl of it was spotted onto an Art 5715 silica gel plate (Merck, Whitehouse, NJ, USA), developed in an eluent of 1-butanol-ethanol-water (2:1:1), and air-dried. Radiolabeled compounds were detected and measured using a BAS2000 Bio-Image analyzer (Fujifilm, Tokyo, Japan). The protein concentration was measured with a protein assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard.

Phosphorylation of inosine and guanosine using recombinant cells was done as follows. Recombinant cells were grown on a CM2G agar plate containing 0.1 g/l adenine and 50 μg/ml kanamycin for 24 h at 32°C. The cells from one-sixth of the plate were transfered to 50 ml of the medium described above and cultured for 24 h at 32°C. The phoshorylation reaction was done with 20 ml of a mixture containing 50 g/l inosine or 25 g/l guanosine, 20 g/l KH_2PO_4, 30 g/l glucose, 5 g/l $MgSO_4 \cdot 7H_2O$, 10 ml/l xylene, 4 g/l Nymeen S-215 (polyoxyethylene stearylamine, Nippon Oil and Fats, Tokyo, Japan), 10 g/l phytic acid, and 200 g wet cells/l recombinant cells at 32°C and pH 7.2 in a 200-ml flask with vigorous agitation at 700 rpm by a magnetic stirrer.[1] During the reaction, the pH of the reaction mixture was occasionally adjusted to 7.2 with 4 M NaOH. Since inorganic phosphate was incorporated into 5′-IMP or 5′-GMP, an equimolar amount of KH_2PO_4 relative to consumed inorganic phosphate was added at intervals. As shown in Table 2, both the strain that expressed the *E. coli gsk* gene and the strain that expressed the *E. acetylicum gsk* gene were able to efficiently phosphorylate inosine to 5′-IMP. The difference in phosphorylation efficiency between the two strains would have been caused by the difference in expression level between them, as shown in Table 1. By contrast, only the strain that expressed the *E. acetylicum gsk* gene was able to efficiently phosphorylate guanosine to 5′-GMP (Table 2). The strain that expressed the *E. coli gsk* gene could only produce traces of 5′-GMP. Even using the strain that expressed the *E. acetylicum gsk* gene, guanosine phosphorylation efficiency was lower than in the case of inosine phosphorylation. This lower efficiency might be explained by further phosphorylation of 5′-GMP to guanosine 5′-diphosphate (5′-GDP) and guanosine 5′-triphosphate (5′-GTP) catalyzed by GMP kinase and nucleoside diphosphate kinase, because of the presence of whole cells and ATP in the reaction mixture. Indeed, both 5′-GDP and 5′-GTP were detected in the reaction mixture along with the increase in 5′-GMP levels (Fig. 1). The reason why the total amount of guanine nucleotides detected was lower than the total amount of the substrate might be the substrate degrading activity of this strain or further conversion of GDP and GTP, for example to ppGpp, because guanosine was not detected in the third sample withdrawal (data not shown). In Fig. 1, maximal accumulation of 5′-GDP might have occurred between the withdrawal of the third and fourth samples. Inhibition of *E. acetylicum* guanosine-inosine kinase by 5′-GDP and by 5′-GTP has not been observed,[8] although, strong inhibition of *E. coli*

Table 1. Expression of Guanosine-inosine Kinase Genes in *C. ammoniagenes*

Origin of the gene introduced	Specific activity (nmol/min/mg protein)
Vector	0.02
E. coli	139
E. acetylicum	414

Table 2. Phosphorylation of Inosine and Guanosine Using Guanosine-inosine Kinase

Origin of the gene introduced	IMP (mM)	GMP (mM)
Vector	n.d.	n.d.
E. coli	44	0.8
E. acetylicum	150	14

186 mmol/l of inosine and 88.3 mmol/l guanosine were used as substrates
n.d., not detected.

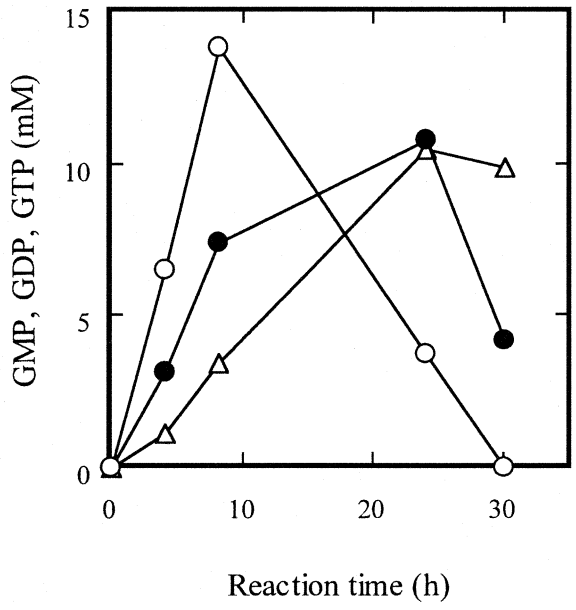

Fig. 1. 5′-GDP and 5′-GTP Formation Occurring with 5′-GMP Production.

Phosphorylation of guanosine was done as described in the text. Sample withdrawals were done at the times indicated. ○: GMP, ●: GDP, △: GTP.

guanosine-inosine kinase by 5′-GDP and by 5′-GTP has been reported.[7,18] Using the strain that expressed the *E. coli gsk* gene, 5′-GDP and 5′-GTP could not be detected in the reaction mixture; however, a small amount of 5′-GDP and 5′-GTP would be produced and would inhibit *E. coli* guanosine-inosine kinase.

In this study we demonstrated practically the feasibility of the process of 5′-GMP production by phosphorylation of guanosine using guanosine-inosine kinase coupled with ATP regeneration. We also showed the usefulness of guanosine-inosine kinase from *E. acetylicum* for this purpose. One of the most critical problems in applying this method as an industrial process may be reducing further phosphorylation of 5′-GMP to 5′-GDP and 5′-GTP.

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