ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2009, Vol. 35, No. 6, pp. 739–745. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © S.A. Taran, K.N. Verevkina, S.A. Feofanov, A.I. Miroshnikov, 2009, published in Bioorganicheskaya Khimiya, 2009, Vol. 35, No. 6, pp. 822–829.

Enzymatic Transglycosylation of Natural and Modified Nucleosides by Immobilized Thermostable Nucleoside Phosphorylases from *Geobacillus stearothermophilus*

S. A. Taran[a, b,1], K. N. Verevkina[a], S. A. Feofanov[a], and A. I. Miroshnikov[a]

[a] *Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Pushchino Division, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia*

[b] *Pushchino State University, Pushchino, Moscow oblast, 142290 Russia*

Received March 23, 2009; in final form, April 2, 2009

Abstract—Natural and modified purine nucleosides have been synthesized using the recombinant thermostable enzymes purine nucleoside phosphorylase II (E. C. 2.4.2.1) and pyrimidine nucleoside phosphorylase (E. C. 2.4.2.2) from *Geobacillus stearothermophilus* B-2194. The enzymes were produced in recombinant *E. coli* strains and covalently immobilized on aminopropylsilochrom AP-CPG-170 after heating the cell lysates and the removal of coagulated thermolabile proteins. The resulting preparations of thermostable nucleoside phosphorylases retained a high activity after 20 reuses in nucleoside transglycosylation reactions at 70–75°C with a yield of the target products as high as 96%. Owing to the high catalytic activity, thermal stability, the ease of application, and the possibility of repeated use, the immobilized preparations of thermostable nucleoside phosphorylases are suitable for the production of pharmacologically important natural and modified nucleosides.

Key words: purine nucleoside phosphorylase, pyrimidine nucleoside phosphorylase, transglycosylation, immobilized enzymes, modified nucleosides

DOI: 10.1134/S1068162009060107

INTRODUCTION

Natural nucleosides and their modified analogues find wide application in the production of chemotherapeutic drugs for the treatment of viral and cancer diseases.[2] Also, they are used in the synthesis of, and the search for, new compounds that are of interest in other fields of pharmacology, as well as precursors in oligonucleotide synthesis in diagnostic and therapeutic purposes.

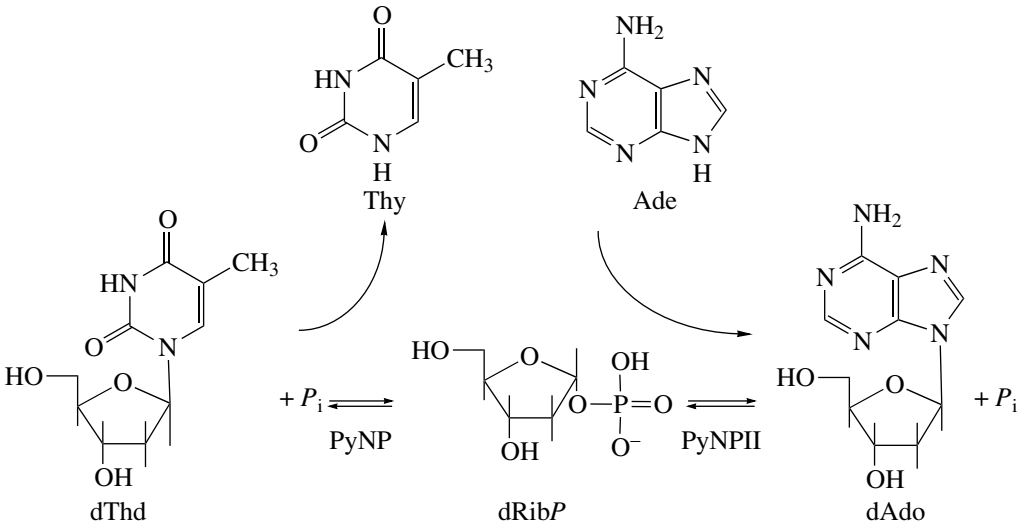
Nucleosides containing modifications in the base or at the ribofuranose residue are obtained, as a rule, by multistage chemical synthesis. This synthesis is very laborious and gives a low yield of target products, owing to the formation of a mixture of regio- and stereoisomers in the reaction [1, 2]. An alternative way of obtaining natural nucleosides and their modified analogues is the biosynthetic process catalyzed by *N*-deoxyribosyltransferases or nucleoside phosphorylases from microorganisms. Owing to a wide substrate specificity, nucleoside phosphorylases are more suitable biocatalysts for industrial application [3–5].

Nucleoside phosphorylases are a group of key enzymes involved in the synthesis and exchange of purine and pyrimidine nucleosides in living organisms, from bacteria to mammals [6]. In the presence of phosphate ions, these enzymes catalyze the reversible phosphorolysis of ribo-, arabino-, and deoxyribonucleosides to pentoso-1-phosphate and the corresponding bases. In the transglycosylation reaction catalyzed by two phosphorylases having different substrate specificity, the phosphate-dependent transfer of pentose between the bases and/or nucleosides present in the mixture takes place, which results in the formation of nucleosides different from the starting compounds. In particular, the enzymes uridine phosphorylase (UP, E. C. 2.4.2.3) and purine nucleoside phosphorylase (PNP, E. C. 2.4.2.1) can be used together for obtaining the natural nucleosides and their analogues in transglycosylation reac-

[1] Corresponding author; phone: 8 (4967) 73-17-80; e-mail: correspondent@rambler.ru.

[2] Abbreviations: aAdo, 9-β-D-arabinofuranosyladenine; aUrd, 1-β-*D*-arabinofuranosyluracil; 2ClAde, 2-chloroadenine; 2ClAdo, 2-chloroadenosine; 2CldAdo, 2-chloro-2′-deoxyadenosine; dThd, thymidine; dRib*P*, 2-deoxyriboso-1-phosphate; 2FAdo, 2-fluoroadenosine; 2FaAdo, 9-β-D-arabinofuranosyl-2-fluoroadenine; IEP, immobilized enzyme preparation; IPTG, isopropylthio-β-D-galactopyranoside; PuNPII, purine nucleoside phosphorylase from *G. stearothermophilus*; PyNP, pyrimidine nucleoside phosphorylase from *G. stearothermophilus*; Thy, thymine.

tions using, e.g., uridine, 2'-deoxyuridine, dThd, or aUrd as the donors of the sugar residue and a wide spectrum of natural and modified purine bases as acceptors [7] (see the scheme).



Transglycosylation with the formation of dAdo.

The major advantage of bioconversion over chemical synthesis is the absolute regio- and stereospecificity of processes catalyzed by nucleoside phosphorylases. In addition, these reactions do not require any cofactors and can proceed in ecologically and technologically safe water buffers. However, the limitations on the stability of biocatalysts may play an important role if special reaction conditions are required. Thus, to provide high concentrations of weakly soluble purine nucleosides and their modified analogues, transglycosylation processes must be conducted at elevated temperatures and pH; however, these conditions usually lead to the destabilization of the quaternary and tertiary structures of the protein and in the final analysis the loss of the enzymatic activity. Enzymes of thermophilic bacteria stabilized by immobilization seem to be attractive as biocatalysts in the nucleotide synthesis, since they offer a number of advantages over the enzymes of mesophilic microorganisms [8] and whole bacterial cells [4, 9–11] that are usually used for these purposes.

The goal of this study was to obtain IEPs of recombinant thermostable nucleoside phosphorylases PuNPII (E. C. 2.4.2.1) and PyNP (E. C. 2.4.2.2) from the thermophilic bacterium *Geobacillus stearothermophilus* B-2194 and develop a highly effective biocatalytic process based on the use of these preparations in transglycosylation reactions for the synthesis of a number of natural and modified nucleosides.

RESULTS AND DISCUSSION

It was shown in papers devoted to the technological production of nucleosides that a temperature of 50–70°C is preferable for conducting the transglycosylation, since it corresponds to the optimum of enzymatic activity of nucleoside phosphorylases, including those from mesophiles [4, 12]. Also, these conditions permit one to introduce into the reaction nucleosides and bases at higher concentrations, which ultimately increase the yield of the target product. As sources of nucleoside phosphorylases, the authors of numerous papers generally used bacterial strains that were either selected from different bacterial collections by microbiological screening [12, 13] or were obtained using the classical selection and mutagenesis methods [9]. In some investigations, purified enzymes isolated primarily from mesophiles were used [5]. However, these examples of transglycosylation cannot be considered as universal for the preparation of a wide spectrum of nucleosides owing to the thermal instability of these preparations and the nonoptimal ratio of enzymatic activities in them.

The *E coli* strains BL21(DE3)/pBstPNPII and BL21(DE3)/ pBstPYNP, which produce, respectively, PuNPII and PyNP of *G. stearothermophilus* BKM-2194, were created in our laboratory (data not published) for obtaining thermostable nucleoside phosphorylases and the study of their properties with the aim of finding approaches to the synthesis of natural and modified nucleosides. During the cultivation of the producers on the rich nutrient medium TB [14], both enzymes accumulated in the cytoplasm in the soluble state and the yield of nucleoside phosphorylases upon the induction of the culture by 0.2 mM IPTG was 40% of the total cellular protein, as indicated by SDS-PAGE. Owing to the high thermal stability of recombinant *G. stearother-*

Table 1. Characteristics of preparations of *G. stearothermophilus* nucleoside phosphorylases isolated from recombinant *E. coli* strains

Strain	Enzyme preparation	Protein concentration, mg/ml carrier	Specific activity, unit/mg protein
BL21(DE3)/pBstPNPII	PuNPII	8.3 ± 1.2	55 ± 4
BL21(DE3)/pBstPYNP	PyNP	7.8 ± 1.4	112 ± 7

Notes: * The activity was determined by the phosphorolysis of the corresponding substrates (inosine for PuNPII and thymidine for PyNP); one unit of activity was defined as that amount of the enzyme that converts 1 μmol of nucleoside in 1 min at 37°C.

mophilus nucleoside phosphorylases, we were able to simplify the multistage procedure of purification of the enzymes and separate them from the host proteins by heating the cell lysates for 30 min to 70°C with the subsequent sedimentation of thermolabile aggregates. The characteristics of the nucleoside phosphorylase preparations obtained are given in Table 1.

Thus, the genetically engineered bacterial strains provide a high level of production of recombinant nucleoside phosphorylases of the thermophilic bacterium *G. stearothermophilus*, which opens up wide possibilities for their use, together with *E coli* nucleoside phosphorylases [8] and whole-cell biocatalysts [9, 11], as suitable sources of phosphorylases for the synthesis of natural or modified nucleosides in transglycosylation reactions.

The preparations of recombinant nucleoside phosphorylases of *G. stearothermophilus* and *E. coli* were immobilized on aminopropylated macroporous glass AP-CPG-170. Preliminarily, the sorbent was treated with glutaraldehyde as described in [15]. In the presence of sodium boron hydride, a covalent bond between the aldehyde groups of glutaraldehyde and the amino groups of the sorbent and enzymes is formed [16]. Attempts to immobilize *Bacillus stearothermophilus* nucleoside phosphorylases by reversible sorption on an anion-exchange resin were reported [17]. Despite small losses in the nucleoside phosphorylase activity during the immobilization, this approach, nevertheless, did not enable one to create a biocatalyst stable enough for the synthesis of, e.g., deoxyguanosine, which requires extreme conditions of temperature and pH. At the same time, there is evidence indicating that the covalent immobilization of nucleoside phosphorylases stabilizes the quaternary structure of enzyme complexes, which extends the range of conditions of applying IEPs toward higher temperatures and a wider range of pH values [18, 19].

In this study, we optimized the conditions of the covalent immobilization of nucleoside phosphorylases on a solid support by adding stabilizing (inosine, dThd, $MgCl_2$) and adjuvant agents (Tween-20, BSA) to the reaction. This enabled one, on one hand, to retain the enzyme activity (about 70% of the initial activity) and, on the other hand, to provide a complete absence of protein desorption from the support (Table 2) upon further use. In addition, owing to the availability of individual enzymes isolated from producer strains, it became possible to obtain both mono- and bienzymatic IEPs and rapidly vary the ratio of activities of immobilized enzymes during the coimmobilization of proteins on the support (see the Experimental section).

In the course of the study, we obtained data on the stability of IEPs of *G. stearothermophilus* nucleoside phosphorylases in a wide range of pH and temperature values. The pH optimum for IEP in the transglycosylation reaction coincided essentially with the optimum of unimmobilized nucleoside phosphorylases (pH 7.5–8.0 [20]); however, the range of values at which the enzyme retained complete stability was wider (pH 6.5–11.5). The temperature optimum in the synthesis of dAdo varied from 80°C (at the PuNPII to PyNP activity ratio 1 : 2) to 86°C (at the PuNPII to PyNP activity ratio 2 : 1),

Table 2. Characteristics of IEPs of *G. stearothermophilus* nucleoside phosphorylases

Immobilized enzyme preparation	Protein concentration, mg/ml	Specific activity, unit/mg protein
PuNPII	18.8 ± 0.8	36 ± 6*
PyNP	19.0 ± 0.5	74 ± 8*
(PuNPII + PyNP) 1 : 1	18.9 ± 0.7	15 ± 2**
(PuNPII + PyNP) 2 : 1	18.4 ± 0.7	12 ± 2**
(PuNPII + PyNP) 1 : 2	19.1 ± 0.9	19 ± 3**

Notes: * The activity was determined by the phosphorolysis of the corresponding substrates (see the note to Table 1).

** The activity was determined from the ability to catalyze transglycosylation in the synthesis of dAdo under conditions specified in the Experimental section; one unit of activity in the transglycosylation reaction was defined as that amount of the enzyme incorporated in IEP that provides the synthesis of 1 μmol of dAdo in 1 h at 75°C.

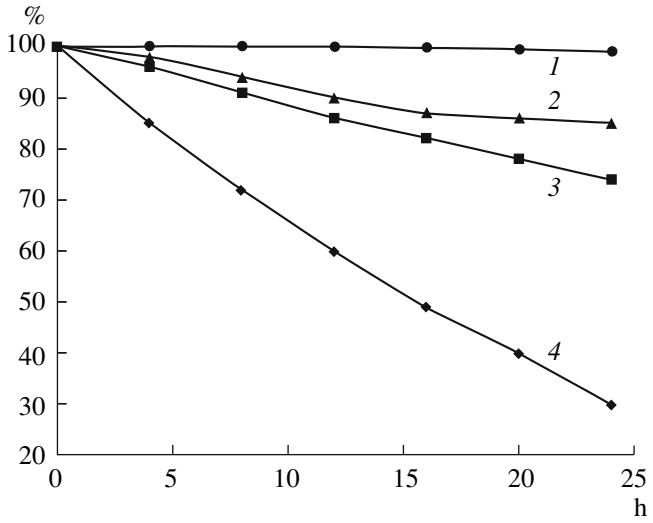


Fig. 1. Stability at 70°C of immobilized (*1*, *3*) and unimmobilized liquid enzyme preparations of *G. stearothermophilus* nucleoside phosphorylases (*2*, *4*): PuNPII (*1*, *2*) and PyNP (*3*, *4*) (see the Experimental section). The maximum degree of the conversion of the substrate by the enzyme at the initial moment of its temperature incubation was taken as 100%.

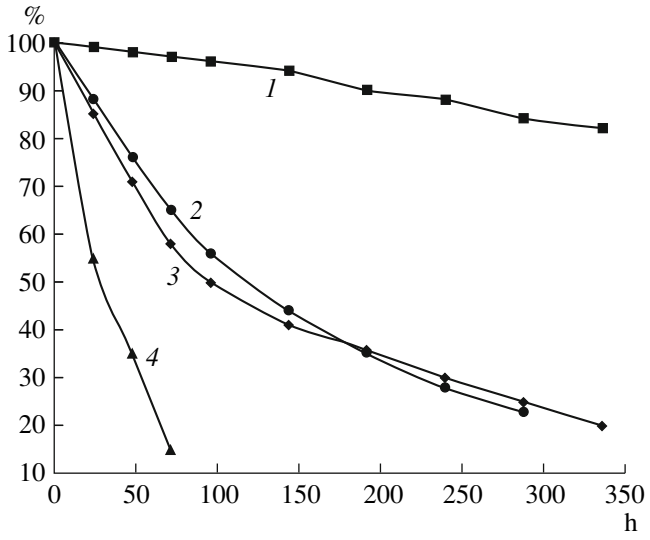


Fig. 2. Thermal stability of *G. stearothermophilus* (*1*, *2*) and *E coli* (*3*, *4*) IEPs in the synthesis of dAdo at 60 (*1, 3*) and 70°C (*2, 4*) (see the Experimental section). The theoretically calculated yield of the target dAdo upon complete conversion of adenine introduced to the reaction was taken as 100%.

which is 5–10°C higher than the temperature optimum determined for unimmobilized *G. stearothermophilus* nucleoside phosphorylases.

The thermal stability of IEPs also significantly increased compared with that of liquid preparations of nucleoside phosphorylases (Fig. 1). The thermal stability of IEPs of *G. stearothermophilus* nucleoside phosphorylases was examined in a series of transglycosylation reactions with the formation of dAdo at 60 and 70°C in comparison with IEPs of *E. coli* nucleoside phosphorylases. For each temperature value, one and the same sample of the biocatalyst was used, and the total time of incubation was about 340 h (14 days). The results obtained demonstrate a relative decrease in the catalytic activity of IEP of *G. stearothermophilus* nucleoside phosphorylases within 24 h by about 1% at 60°C and by about 9% at 70°C. At the same time, the IEP of *E. coli* nucleoside phosphorylases under similar conditions lose 8 and 45% of activity, respectively (Fig. 2). These data confirm a higher stability of enzyme preparations of thermophilic microorganisms in nucleoside transglycosylation reactions. This is most pronounced in the case of pyrimidine-specific phosphorylases whose thermal stability is a limiting factor in the bioconversion reaction.

By providing a high stability of IEPs, we obtained a reproducible yield of dAdo with the repeated use (more than 20 cycles) of one and the same sample of nucleoside phosphorylase IEPs. The separation of IEP from the reaction mixture was carried out by filtration through a glass filter; then, the IEP was transferred into the next bioconversion reactions. It follows from the data obtained that the transglycosylase activity of the enzyme preparation upon 20 reuses decreased approximately by 0.6–0.7% per cycle at 70°C, which is, in our opinion, a very good indicator and provides good technological prospects for these IEPs. In addition, owing to the high specific activity of IEP, we managed to achieve a more than 80% degree of conversion over a period of 4–10 h with the content of the biocatalyst in the reaction mixture being as low as 1–5% (by volume). This enabled us to effectively use the IEPs for conducting transglycosylation reactions with a wide spectrum of substrates, including weakly soluble substrates, such as guanine, as well as for the synthesis of pharmaceutically significant preparations of 2CldAdo and 2FaAdo (Table 3).

Thus, we obtained preparations of *G. stearothermophilus* nucleoside phosphorylases covalently immobilized on aminopropylated macroporous glass AP-CPG-170. The novel preparations of two immobilized thermostable nucleoside phosphorylases exhibit high enzymatic activity and stability, which makes them suitable for repeated use as biocatalysts in transglycosylation reactions. In our opinion, the application of immobilization-stabilized nucleoside phosphorylases of thermophilic microorganisms, obtained from recombinant producer strains, is the most optimal approach to the biotechnological synthesis of nucleosides and their analogues necessary for the needs of national medicine and other applications.

Table 3. Parameters of transglycosylation in the synthesis of natural and modified nucleosides using the IEPs of *G. stearothermophilus* nucleoside phosphorylases*

Target product	IEP (activity of PuNPII : PyNP), ml	Pentose donor, mM	Base, mM	KH_2PO_4, mM	*t*, °C	pH	Reaction time, h	Yield**, %
dAdo	(1 : 2), 0.1	dThd, 15	Ade, 5	5	75	7.5	2	89
dGuo	(2 : 1), 0.5	dThd, 15	Gua, 5	5	70	10.0***	10	96
2CldAdo	(1 : 2), 0.2	dThd, 15	2ClAde, 5	5	75	7.5	4	86
aAdo	(1 : 2), 0.1	aUrd, 15	Ade, 5	5	75	7.5	4	88
2FaAdo	(1 : 1), 0.2	aUrd, 15	2FAde, 5	5	75	7.5	8	85

Notes: * The reaction volume is 10 ml.
** According to the data of HPLC.
*** The reaction was conducted in a 50-mM glycine-NaOH buffer (pH 10.0 at 70°C); for more detail, see the Experimental section.

EXPERIMENTAL

The following preparations were used: deoxyadenosine, deoxyguanosine, thymidine, inosine, sodium boron hydride, and 2-mercaptoethanol (Sigma, United States). 2ClAdo, 2ClAde, 2F-adenosine, and aUrd were synthesized in the Laboratory of Biotechnology (Institute of Bioorganic Chemistry, Russian Academy of Sciences) by an ingenious method [21, 22]. Other chemical reagents were purchased from Promega (United States), Serva (Germany), Fluka (Switzerland), and Bio-Rad (United States) and were of special purity or chemical grade. Aminopropylated glass AP-CPG-170 was used as a carrier for enzyme immobilization (Fluka, Switzerland).

The *E. coli* BL21(DE3)/pBstPNPII and BL21(DE3)/pBstPYNP producer strains have been deposited in the collection of the Group of Technology of Nucleic Acids and Their Components (Institute of Bioorganic Chemistry, Russian Academy of Sciences, Pushchino Division). Plasmids pBstPNPII and pBstPYNP were obtained previously in our laboratory (data not published). They are the derivatives of plasmids of the pET series (Novagen, United States) and contain, respectively, the genes of PuNPII and PyNP of the thermophilic bacterium *G. stearothermophilus* BKM-2194 under the control of the promoter of bacteriophage T7.

E. coli purine nucleoside phosphorylase and thymidine phosphorylase isolated in the Laboratory of Biotechnology (Institute of Bioorganic Chemistry, Russian Academy of Sciences) from *E. coli* strains BL21(DE3)/pERPUPH01 and BL21(DE3)/pERTPH01 [8], respectively, were used as control enzymes.

Manipulations of plasmid DNA, the preparation of competent cells of *E. coli* strain BL21(DE3), and transformation were carried out by standard methods [23].

Thermostable nucleoside phosphorylases of *G. stearothermophilus* BKM-2194. A night inoculate of the recombinant *E. coli* strain BL21(DE3)/pBstPNPII or BL21(DE3)/pBstPYNP (5 ml) was added to 100 ml of medium TB [14] containing bacto tryptone (Difco, United States) (1.2 g), bacto yeast extract (Difco, United States) (2.4 g), K_2HPO_4 (0.95 g), KH_2PO_4 (0.22 g), glycerol (0.8 g), and kanamycin, pH 7.0, (5 mg), and the culture was grown on a shaker (200–220 rpm) at 30°C for 4 h until the optical density of A_{550} = 1.0 was attained. Then, IPTG was added to a concentration of 0.2 mM, and the culture was incubated under the same conditions for an additional 6 h. Optical absorption was measured at 550 nm on a UV-1800 spectrophotometer (Shimadzu, Japan) using the software package UV Probe. The level of expression was estimated by electrophoresis in gradient (5–25%) SDS-PAGE by monitoring the appearance of the major bands of 27 and 46 kDa for PuNPII and PyNP, respectively.

Isolation of nucleoside phosphorylases. A cell culture was centrifuged at 4000–5000 g for 15 min. Sedimented cells were suspended in two (weight/volume) volumes of a homogenization buffer (25 mM K-phosphate, pH 7.5, 0.9% NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol) and disrupted by ultrasound on a VC130 disintegrator (Cole-Parmer, United States). The homogenate was clarified by centrifugation for 20 min at 10000 g, and the supernatant was transferred to a glass vessel and incubated for 30 min in a water bath at 70°C with gentle stirring. The suspension was cooled to 4°C and centrifuged to sediment denatured thermolabile cellular proteins of *E. coli* at 10000 g for 20 min. In the supernatant, the protein concentration was determined by the Bradford method [24] and the specific activity of nucleoside phosphorylases was estimated by the phosphorolysis of the corresponding substrates. One unit of activity of PuNPII was defined as that amount of the enzyme that converts 1 μmol of inosine to hypoxanthine in 50 mM potassium phosphate, pH 7.5, for 1 min at 37°C. One unit of activity of PyNP was defined as that amount of the enzyme that converts 1 μmol of thy-

midine to thymine in 50 mM potassium phosphate, pH 7.5, for 1 min at 37°C. The resulting liquid enzyme preparations were used for immobilization.

For the immobilization of nucleoside phosphorylase preparations, aminopropylated glass AP-CPG-170 and *E. coli* and *G. stearothermophilus* nucleoside phosphorylase preparations were essentially prepared as described earlier [25]. Liquid preparations of PuNPII and PyNP of *G. stearothermophilus* BKM-2194 obtained from the strains BL21(DE3)/pBstPNPII and BL21(DE3)/pBstPYNP were diluted to a concentration of the protein of 5 mg/ml by a buffer containing 50 mM K-phosphate, pH 7.5, 50 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.2% Tween-20, and 0.1 mg/ml of BSA. Also, inosine and thymidine were added to the buffer to a concentration of 10 mM for PuNPII and PyNP, respectively. Aminopropylsilochrom AP-CPG-170 prepared for immobilization and liquid preparations of nucleoside phosphorylases were mixed in a ratio of 20 mg of the protein of the enzyme preparation per 1 mg of the carrier. Mixtures for immobilization were prepared as monoenzymatic for each of the nucleoside phosphorylases or multienzymatic with different ratios of specific activities of PuNPII and PyNP by varying the ratio of liquid enzyme preparations. The immobilization was carried out for 16 h at room temperature under gentle stirring. Then, sodium boron hydride was added to the reaction mixture to a final concentration of 1 mg/ml and incubation was continued for 1 h. The preparation was washed five times with two volumes of a 50-mM K-phosphate buffer, pH 7.5. IEPs were stored in a 50-mM K-phosphate buffer supplemented with sodium azide (0.2%) at 2–4°C.

For the determination of stability at 70°C, monoenzymatic IEPs and liquid enzyme preparations of *G. stearothermophilus* nucleoside phosphorylases were incubated for 24 h at 70°C. At regular intervals (every 4 h), an equal volume of an enzyme preparation was taken and added to 1 ml of the reaction of phosphorolysis of inosine and thymidine for PuNPII and PyNP, respectively. After 15 min, the residual content of nucleoside was determined by HPLC. The maximum degree of the conversion of the substrate by the enzyme at the initial moment of its temperature incubation was taken as 100%.

2'-*Deoxyadenosine (dAdo).* A reaction mixture (10 ml) containing a 5-mM K-phosphate buffer (pH 7.5), 5 mM adenine, 15 mM thymidine, 10% DMSO (v/v), and 0.1 ml of IEP of nucleoside phosphorylases (a PuNPII/PyNP activity ratio of 1 : 2) was incubated for 2 h at 75°C with gentle stirring. The yield of dAdo was 89%. For repeated use, the IEP was separated from the reaction mixture by filtration through a glass filter and washed on the filter with a 50-mM K-phosphate buffer.

2'-*Deoxyguanosine (dGuo).* A reaction mixture (10 ml) containing a 50-mM glycine-NaOH buffer (pH 10.0 at 70°C), 5 mM K_2HPO_4, 15 mM thymidine, 20% DMSO (v/v), IEPs of nucleoside phosphorylases (0.5 ml) (a PuNPII/PyNP activity ratio of 2 : 1), and guanine (7.5 mg, 0.05 mmol) was incubated for 10 h at 70°C with gentle stirring. The yield of dGuo was 96%.

2-Chloro-2'-deoxyadenosine (2ClAdo, cladribine). A reaction mixture (10 ml) containing a 5-mM K-phosphate buffer (pH 7.5), 5 mM 2ClAde, 15 mM thymidine, 10% DMSO (v/v), and IEPs of nucleoside phosphorylases (0.2 ml) (the PuNPII/PyNP activity ratio 1 : 2) was incubated for 4 h at 75°C with gentle stirring. The yield of 2ClAdo was 86%.

9-β-D-Arabinofuranosyladenine (aAdo). A reaction mixture (10 ml) containing a 5-mM K-phosphate buffer (pH 7.5), 5 mM adenine, 15 mM aUrd, 10% DMSO (v/v), and IEPs of nucleoside phosphorylases (0.1 ml) (a PuNPII/PyNP activity ratio of 1 : 2) was incubated for 4 h at 75°C with gentle stirring. The yield of aAdo was 88%.

9-β-D-Arabinofuranosyl-2-fluoroadenine (2FaAdo, fludarabine). A reaction mixture (10 ml) containing a 5-mM K-phosphate buffer (pH 7.5), 5 mM 2FAde (or 2FAdo), 15 mM aUrd, 20% DMSO (v/v), and IEPs of nucleoside phosphorylases (0.2 ml) (a PuNPII/PyNP activity ratio of 1 : 1) was incubated for 8 h at 70°C with gentle stirring. The yield of 2FaAdo was 85%.

Analysis of reaction products was performed by TLC on Kiselgel 60-F_{254} plates (Merck, Germany) in the solvent system chloroform–ethanol (4 : 1) and by HPLC using a 254-nm detector (Beckman, United States) in the isocratic operation mode on Separon SGX C18 5 μl columns (3 × 150 mm). As an eluent, 5% MeCN in 10 mM KH_2PO_4 was used; the elution rate was 0.5 ml/min. After separation by TLC and HPLC, the fractions of individual products were analyzed on a UV-188 spectrophotometer (Shimadzu, Japan) by measuring the spectra in the wavelength range from 200 to 340 nm and comparing them with standard or reference data.

ACKNOWLEDGMENTS

The authors are grateful to the research workers of the Laboratory of Biotechnology (Institute of Bioorganic Chemistry, Russian Academy of Sciences), R.S. Esipov for providing the *E. coli* nucleoside phosphorylase preparations, V.B. Berzin for the synthesis of 2Cl-adenosine and 2F-adenosine, and K.V. Antonov for the synthesis of 1-β-*D*-arabinofuranosyluracil, which were necessary for performing the experiments.

This work was supported by the Russian Foundation for Basic Research, project nos. 08-04-12143, 08-04-01842) and the Federal Education Agency (project of the Thematical Plan no. 1.1.09).

REFERENCES

1. Kawakami, H., Matsushita, H., Naoi, Y., Itoh, K., and Yoshikoshi, H., *Chem. Lett.*, 1989, vol. 1, pp. 235–238.

2. Witkowski, J.T., Robins, R.K., Sidwell, R.W., and Simon, L.N., *J. of Med. Chem*, 1972, vol. 15, pp. 1150–1154.
3. Hanrahan, J.R. and Hutchinson, D.W., *J. Biotechnol.*, 1992, vol. 23, pp. 193–210.
4. Utagawa, T., *J. Molec. Catalysis B: Enzymatic*, 1999, vol. 6, pp. 215–222.
5. Krenitsky, T.A., Koszalka, G.W., and Tuttle, J.V., *Biochemistry*, 1981, vol. 20, pp. 3615–3621.
6. Pugmire, M.J. and Ealick, E., *Biochem. J.*, 2002, vol. 361, pp. 1–25.
7. Hatchinson, D.W., *Trends Biotechnol.,* 1990, vol. 8, pp. 348–353.
8. Esipov, R.S., Gurevich, A.I., Chuvikovsky, V., Chupova, L.A., Muravyova, T.I., and Miroshnikov, A.I., *Protein Express. Purif.*, 2002, vol. 24, pp. 56–60.
9. Barai, V.N., Zinchenko, A.I., Eroshevskaya, L.A., Kalinichenko, E.N., Kulak, T.I., and Mikhailopulo, I.A, *Helv. Chim. Acta*, 2002, vol. 85, pp. 1901–1908.
10. Spoldi, E., Ghisotti, D., Cali, S., Grisa, M., Orsini, G., Tonon, G., and Zuffi, G., *Nucleosides Nucleotides*, 2001, vol. 20, pp. 977–979.
11. Taran, S.A., Verevkina, K.N., Esikova, T.Z., Feofanov, S.A., and Miroshnikov, A.I., *Appl. Biochem. Microbiol.*, 2008, vol. 44, pp. 162–166.
12. Utagawa, T., Morisawa, H., Yamanaka, S., Yamazaki, A., Yoshinaga, F., and Hirose, Y., *Agric. Biol. Chem.*, 1985, vol. 49, pp. 3239–3246.
13. Yokozeki, K. and Tsuji, T., *J. Molec. Catalysis B: Enzymatic*, 2000, vol. 10, pp. 207–213.
14. Tartoff, K.D. and Hobbs, C.A., *Bethesda Res. Lab. Focus*, 1987, vol. 9, p. 7.
15. Juekiewicz, M., Alegret, S., and Fabregas, E., *Anal. Chim. Acta*, 1998, vol. 370, pp. 47–58.
16. Kito, M., Tawa, R., Takeshima, S., and Hirose, S., *J. Chromatogr.*, 1990, vol. 528, pp. 91–99.
17. Hori, N., Watanabe, M., Sunagawa, K., Uehara, K., and Mikami, Y., *J. Biotechnol.*, 1991, vol. 114, pp. 121–131.
18. Zuffi, G., Ghisotti, D., Ilaria, O., Capra, E., Frascotti, G., Tonon, G., and Orsini, G., *Biocatal. Biotransform.*, 2004, vol. 22, pp. 25–33.
19. Ubiali, D., Rocchietti, S., Scaramozzino, F., Terreni, M., Albertini, A., Fernandez-Lafuente, R., Guisan, J.M., and Pregnolato, M., *Adv. Synth. Catal.,* 2004, vol. 346, pp. 1361–1366.
20. Hori, N., Watanabe, M., Yamazaki, Y., and Mikami, Y., *Agric. Biol. Chem.*, 1989, vol. 53, pp. 3219–3224.
21. Berzin, V.B., Dorofeeva, E.V., Leonov, V.N., Lutonina, O.I., and Miroshnikov, A.I., RF Patent no. 2324698, from February 5, 2007.
22. Konstantinova, I.D., Antonov, K.B., Berzin, V.B., Dorofeeva, E.V., Fateev, I.V., Muzyka, I.S., and Miroshnikov, A.I., RF Patent Application no. 2008126 081 from June 27, 2008.
23. Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, New York: Cold Spring Harbor, Cold Spring Harbor Lab. Press, 1989.
24. Bradford, A.T., *Anal. Biochem.*, 1976, vol. 72, pp. 248–254.
25. Konstantinova, I.D., Leont'eva, N.A., Galegov, G.A., Ryzhova, O.I., Chuvikovskii, D.V., Antonov, K.V., Esipov, R.S., Taran, S.A., Verevkina, K.N., Feofanov, S.A., and Miroshnikov, A.I., *Bioorg. Khim.*, 2004, vol. 30, pp. 613–620 [*Russ. J. Bioorg. Chem.* (Engl. Transl.), vol. 30, pp. 553–560].