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Selective hydrolysis of nucleotides to nucleosides and free bases

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Abstract

The kinetics of the hydrolysis of 2′-deoxyadenosine-5′-monophosphoric acid (dAMP), 2′-deoxycytidine-5′-monophosphoric acid (dCMP), 2′-deoxyguanosine-5′-monophosphoric acid (dGMP) and tymidine-5′-monophosphoric acid (dTMP) was studied in the presence of *Xanthomonas maltophilia* [1]. The reaction products are nucleosides: 2′-deoxyadenosine (dA), 2′-deoxycytidine (dC), 2′-deoxyguanosine (dG) and tymidine (dT), respectively, or the respective free bases. Hydrolysis of dTMP and dGMP proceeded stepwise according to the sequence: nucleotide → nucleoside → free base, whereas no accumulation of the free base was observed during the hydrolysis of dAMP and dCMP.

Keywords: Nucleotides; Selective hydrolysis; *Xanthomonas maltophilia*; *Pseudomonas putida*; *Escherichia coli*; *Lactobacillus acidophilus*

1. Introduction

Nucleosides and free bases commonly used for synthesis of many kinds of drugs, particularly antiviral [2], antitumor [3], antibacterial drugs [4] or immunomodulators [5], are obtained mostly by chemical or enzymatic ways.

For example, Takami et al. [6] described hydrolysis of 5′-nucleotides (AMP, GMP, CMP, UMP) derived from RNA in formate buffer solution. Refluxing for 120 h produced nucleosides with 88% yield. A Japanese Patent [7] discloses a method of hydrolysis of pyridinium nucleotides yielding the respective nucleosides in the presence of 5′-nucleotidase. Another way to obtain nucleosides — the route via intramolecular coupling of bases with 2′-deoxyribosides — was found by Lipshuts et al. [8]. These methods require complex substrates or are labour and time consuming, especially the isolation and purification of enzymes. Another way to obtain nucleosides and free bases is a microbial hydrolysis of nucleotides, without the necessity of isolation of enzymes.

The aim of this work was to find an easy and cost effective way of obtaining nucleosides and free bases by microbial hydrolysis of nucleotides. To obtain the maximum recovery of the product, we compared time-course reactions of the hydrolysis of DNA nucleotides by several different species of bacteria. Hydrolysis of nucleotides in the presence of *Xanthomonas maltophilia* was accomplished and compared with the hydrolysis of tymidine-5′-monophosphoric acid (dTMP) and 2′-deoxyguanosine-5′-monophosphoric acid (dGMP) by *Escherichia coli*, *Pseudomonas putida* and *Lactobacillus acidophilus*. *X. maltophilia* is an environmental Gram-negative bacteria found in soil, water and waste-water; it does not produce resting spores and grows well on typical media in the presence of oxygen. Until now *Xanthomonas* was used to produce xanthan [9].

2. Experimental

2.1. Materials

Microorganisms: *E. coli* ATCC 8739 was obtained from the American Type Culture Collection. *X. maltophilia* was obtained from the Culture Collection of the Pharmaceutical Research Institute in Warsaw and was isolated from water samples in Warsaw. *P. putida*

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was obtained from the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences, Wrocław, Poland. *L. acidophilus* was obtained from the Culture Collection of Biomed, Kraków, Poland.

All solvents used were of HPLC or analytical grade (J.T. Baker Co.). All DNA nucleotides (2′-deoxyadenosine-5′-monophosphoric acid (dAMP), 2′-deoxycytidine-5′-monophosphoric acid (dCMP), dGMP, dTMP), respective nucleosides and bases were obtained from Pharma Waldhof GmbH, Düsseldorf, Germany.

2.2. Microbiological hydrolysis

The culture of a suitable strain was used to inoculate 100 ml of the culture medium in a 500 ml flask containing 0.6% peptone and 0.5% glucose at pH 7.0. The culture was incubated in a rotary shaker at 200 rpm for 24 h, at 30°C, centrifuged at 4000 rpm for 30 min, and the appropriate amount of bacterial mass was resuspended in 50 ml of phosphate buffer solution (pH 7) with glucose (0.5%), and the number of bacteria was estimated by the plate-count method (calculating the colony forming units).

The hydrolysis reaction was started by the addition of 50 mg of the respective nucleotide (solid) to the bacteria suspension in a 300-ml flask. The mixture was incubated at 30°C in a rotary shaker at 200 rpm for a time period required to hydrolyse the nucleotide to nucleoside or free base. The respective times depend on the particular nucleotide.

2.3. Methods of analysis

Samples of 0.5 ml were taken in different time intervals. The aliquot was centrifuged (7 rpm, 15 min), then diluted 40 times in a phosphate buffer (pH 4) and filtered through the membrane filter (0.45 μm, $\varnothing = 4$ mm, Cole-Palmer). The sample was injected into the 20 μl loop of the HPLC.

The samples were analysed by HPLC (Shimadzu spectrophotometric detector UV–Vis) using a LC-18S column (Supelco; length 25 cm, 4.6 mm × 5 μm); mobile phase: aqueous buffer (NaH_2PO_4, pH 4); flow rate 1 ml/min; injection volume 20 μl; detection at $\lambda = 254$ nm. The quantitive determination of reaction products was done by the external standard calibration method. Results are shown in Figs. 1–10. The concentration of

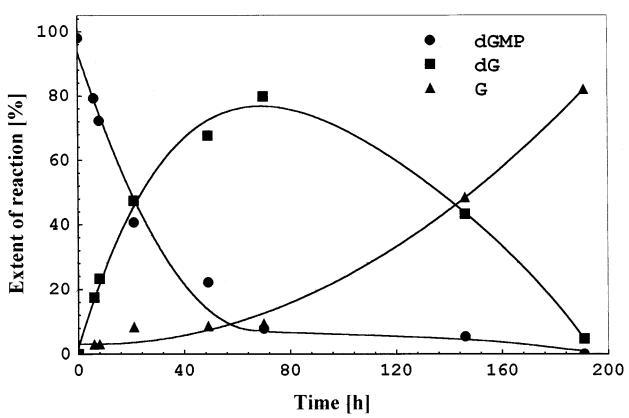

Fig. 1. Hydrolysis of dGMP by *X. maltophilia*.

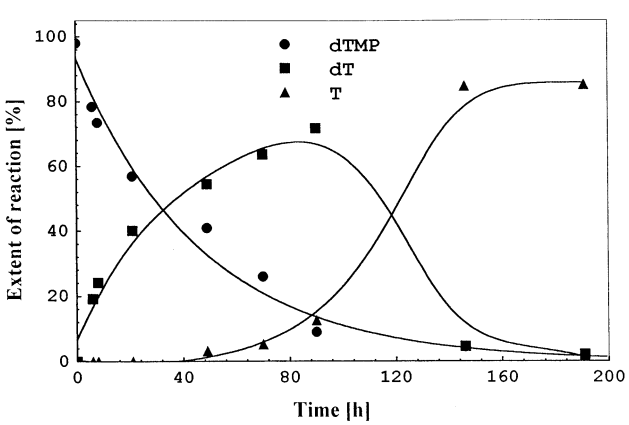

Fig. 2. Hydrolysis of dTMP by *X. maltophilia*.

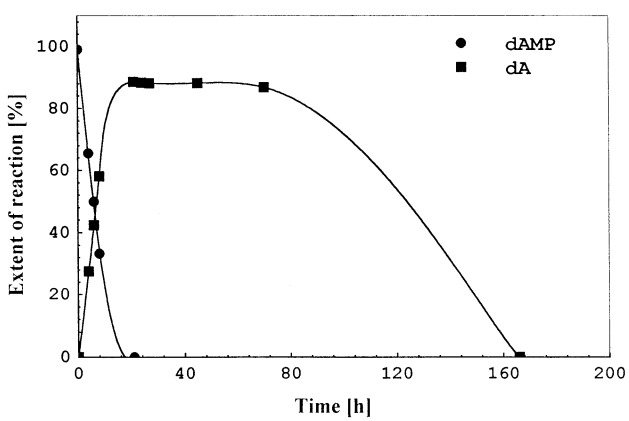

Fig. 3. Hydrolysis of dAMP by *X. maltophilia*.

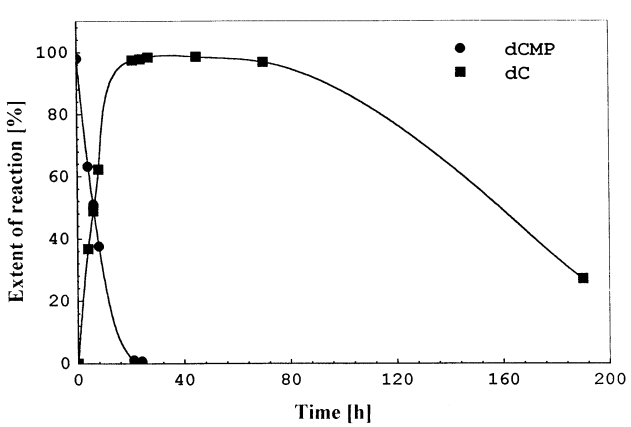

Fig. 4. Hydrolysis of dCMP by *X. maltophilia*.

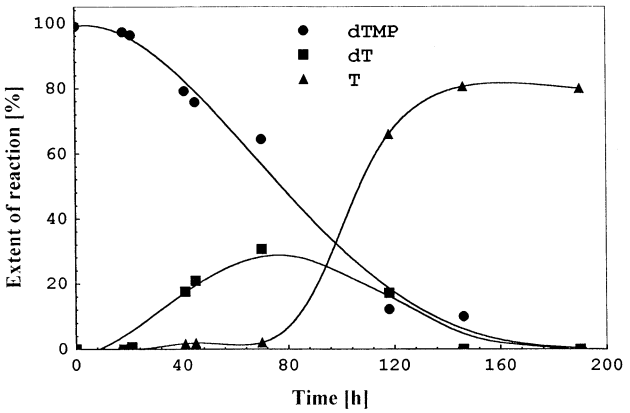

Fig. 5. Hydrolysis of dTMP by *P. putida*.

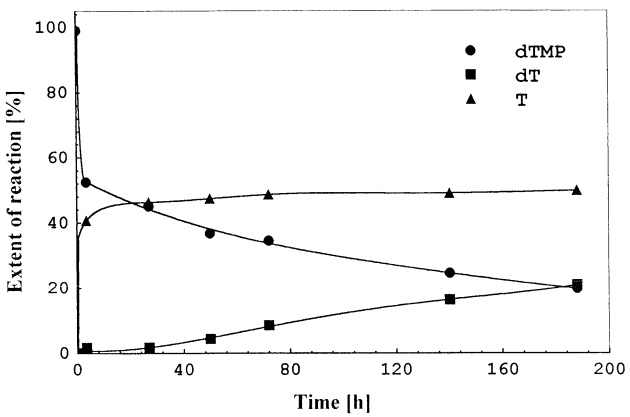

Fig. 6. Hydrolysis of dTMP by *E. coli*.

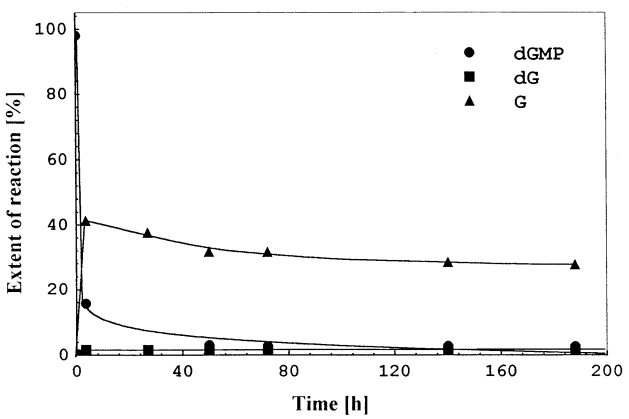

Fig. 7. Hydrolysis of dGMP by *E. coli*.

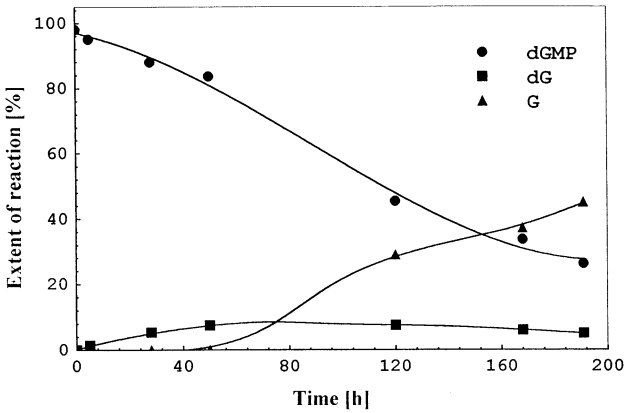

Fig. 8. Hydrolysis of dGMP by *L. acidophilus*.

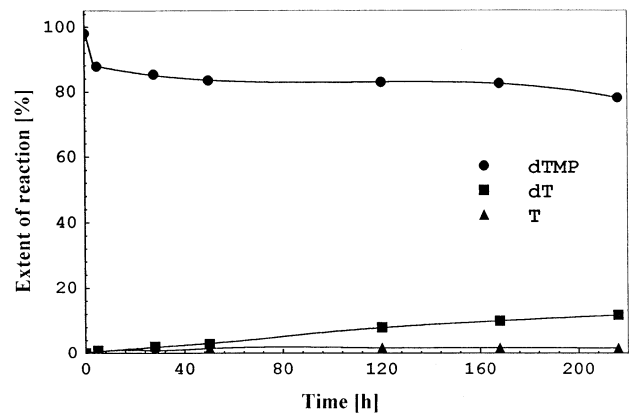

Fig. 9. Hydrolysis of dTMP by *L. acidophilus*.

the reaction products in the reaction mixture was expressed as a percentage of the initial concentration of the substrate (nucleotide). 'Extent of reaction' is the percentage of the particular component in the reaction mixture.

3. Results and discussion

X. maltophilia hydrolysed dGMP in two separate steps. Initially formed deoxyguanosine was subsequently hydrolysed to free guanine (Fig. 1).

X. maltophilia hydrolysed dTMP in two steps too. Initially formed thymidine was subsequently hydrolysed to free thymine (Fig. 2).

Surprisingly, when we tried to hydrolyse dAMP and dCMP with *X. maltophilia*, we were not able to obtain free bases. The hydrolysis of dAMP to deoxyadenosine was performed very quickly, within 12 h (Fig. 3). The same occurred for dCMP. Deoxycytidine was obtained by *Xanthomonas* within 20 h (Fig. 4). To explain this phenomenon we suggest that *X. maltophilia* degrades dAMP and dCMP through a deamination step. A similar way of adenosine degradation was found in the case of *Aspergillus terricola* [10], which deaminates adenosine to inosine, then hydrolyses inosine to ribose and hypoxanthine.

We would like to see whether the hydrolysis pattern described for *X. maltophilia* is also common for the

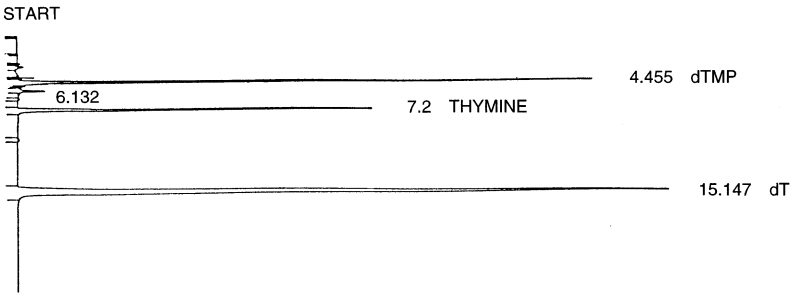

Fig. 10. Chromatogram of hydrolysis of dTMP by *X. maltophilia* after 3.5 days.

other bacteria from the Pseudomonadaceae family, thus we followed the hydrolysis of dTMP by *P. putida*. In this case the hydrolysis proceeded in a similar way, but the yield of thymidine was much lower (30%) and degradation of dTMP was slower (Fig. 5).

Another question was whether the hydrolysis pattern described for *Xanthomonas* is also common for the bacteria of other families: we followed the hydrolysis of dTMP and dGMP by human symbionts, *E. coli* and *L. acidophilus*. *E. coli* degraded dTMP very quickly and no high accumulation of thymidine was observed, but only accumulation of thymine to 50% (Fig. 6). In a similar manner *E. coli* degraded dGMP, even quicker; no accumulation of deoxyguanosine was observed, only guanine (30%) was found (Fig. 7). *L. acidophilus* degraded dGMP much slower than *E. coli*, and similarly no accumulation of deoxyguanosine was observed, but slow accumulation of guanine (to about 50%) (Fig. 8). No accumulation of thymine and thymidine was observed during incubation of dTMP with *L. acidophilus* (Fig. 9), there was no degradation.

An example chromatogram of hydrolysis of dTMP by *X. maltophilia* is shown in Fig. 10.

As is known, bacteria are a source of a wide range of enzymes and are able to extracellularly release hydrolases [11], in the present case phosphohydrolases and glycosidases. The fast increase of the yield of deoxynucleosides at the beginning of the incubation period accompanied by the slow increase of the yield of the free bases suggests that phosphohydrolases may be the constitutive enzymes, whereas glycosidases may be substrate-induced enzymes in *X. maltophila*. However, this needs to be confirmed experimentally.

Hydrolysis of nucleotides to nucleosides in the presence of *X. maltophilia* was inhibited when nucleotide solutions were buffered at pH 4 or 10.

4. Conclusions

X. maltophilia is a useful bacterium to obtain dA, dC, dT, dG, thymine and guanine from nucleotides. Hydrolysis of dTMP and dGMP proceeds stepwise according to the following sequence: nucleotide → nucleoside → free base. *P. putida* hydrolyses dTMP similarly to *X. maltophilia*. Human symbionts, *L. acidophilus* and *E. coli*, hydrolyse dTMP and dGMP in a different way and give small amounts of nucleosides and free bases.

References

[1] U. Chmielowiec, H. Kruszewska, J. Cybulski, A. Głowacka, Method of hydrolysis of nucleotides derived from DNA or RNA and production of nucleosides and free amines, Polish Patent Application P 315995 (1996).
[2] W.S. Mungall, L.J. Lemmen, K.L. Lemmen, J.K. Dethmers, L.L. Norling, Nucleoside 5′-monophosphate analogues. Synthesis of 5′-sulfamino-5′-deoxynucleosides, J. Med. Chem. 21 (1978) 704–706.
[3] A. Matsuda, A. Don, N. Mikanawa, S.J. Tregear, S. Okazaki, Y. Sugimato, T. Sasaki, Synthesis of 1-(2-deoxy-2-isocyano β-D-arabinofuranosyl)-cytosine and related nucleosides as potential antitumor agents, J. Med. Chem. 36 (1993) 4190–4194.
[4] H. Osada, K. Isono, Mechanisms of action and selective toxicity of ascamycin, a nucleoside antibiotic, Antimicrob. Agents Chemother. 27 (1985) 230–233.
[5] G.E. Hardart, G.W. Sullivan, H.T. Carper, G.L. Mandell, Adenosine and 2-phenylaminoadenosine (CV-1808) inhibit human neutrophil bactericidal function, Infect. Immun. 59 (1991) 885–889.
[6] A. Takami, M. Imazawa, M. Irie, Ch. Ukida, A preparation of nucleosides by the hydrolysis of 5′-nucleotides, Yakugaku Zasshi 85 (1965) 658–661 [Chem. Abstr. 67 (1967) 44047h].
[7] K. Shichiro, T. Shuichi, S. Akio, Pharmaceutical pyridine nucleoside derivatives — prepared by treating pyridine nucleotide derivatives with nucleotidase, Japanese Patent Application, Japan Kokai 7826396 (29840A/16; J5 3026-396) (1976) [Chem. Abstr. 89 (1978) P44137s].
[8] B.H. Lipshuts, H. Hayakawa, K. Kato, R.F. Lowe, K.L. Stevens, A novel route to nucleosides via intramolecular coupling of bases with 2′-deoxyribosides: quick and stereospecific… but with a 'twist', Synthesis 12 (1994) 1476–1484.
[9] H. Umashankar, G. Annadurai, M. Chellapandian, Influence of nutrients on cell growth and xanthan production by *Xanthomonas campestris*, Bioprocess Eng. 14 (1996) 307–309.
[10] A.M. Elshafei, M.R. Abbu-Shady, F.M. El-Beih, L.A. Mohamed, Mode and extent of degradation of adenosine and guanosine by extracts of *Aspergillus terricola*, Microbiol. Res. 150 (1995) 291–295.
[11] B.M. Grohs, B. Kunz, Untersuchungen zum Mikrobiellen Purinabbau, Z. Ernährungswiss. 33 (1994) 120–127.