Purification and Properties of Purine Nucleoside Phosphorylase from *Brevibacterium acetylicum* ATCC 954

Hideyuki SHIRAE* and Kenzo YOKOZEKI

Central Research Laboratories of Ajinomoto Co., Ltd.
1–1 Suzuki-cho, Kawasaki-ku, Kawasaki 210, Japan

Received August 17, 1990

Purine nucleoside phosphorylase of *Brevibacterium acetylicum* ATCC 954, which catalyzes the production of ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a potent antiviral agent, from purine nucleoside and 1,2,4-triazole-3-carboxamide in a high yield, was purified 49-fold. This enzyme had a molecular weight of 31,000 and was a monomer. The isoelectric point of the enzyme was 4.7. The optimal temperature and pH of inosine phosphorolyzing reaction catalyzed by the enzyme was around 8.5 and 70°C, respectively. The Michaelis constants for inosine, guanosine, and ribavirin were 1.43 mM, 2.44 mM and 2.08 mM, respectively, at 40°C. This enzyme appeared to be a SH enzyme because it was inactivated by SH reagents, *p*-chloromercuribenzoate and *N*-ethylmaleimide, and $HgCl_2$. In addition, this enzyme was completely inactivated by $AgNO_3$ and was slightly inhibited by $CuSO_4$. It showed nucleoside-phosphorolyzing activity toward inosine, 2′-deoxyinosine, 2′,3′-dideoxyinosine, guanosine, 2′-deoxyguanosine, and xanthosine. However, adenosine and its derivatives could not be phosphorolyzed. This enzyme could not also phosphorolyze various 5′-mononucleotides. According to the amino terminal sequence analysis, the twenty residues from the amino terminal end of this enzyme were identified as follows: MTVNWNETRS-FLECKMQAKPE.

Purine nucleoside phosphorylase (EC 2.4.2.1; PNPase) has been widely detected in eukaryotes[1–7] and prokaryotes,[8–21] and was purified to the crystalline form.[22] PNPase catalyzes the reversible reaction for the phosphorolysis of ribonucleoside, 2′-deoxyribonucleoside and their derivatives as follows:

purine nucleoside + phosphate ⇌
purine base + pentose-1-phosphate

Moreover, this enzyme can also catalyze the transglycosylation reaction of purine nucleosides when another purine base exists in the reaction mixture as follows:

purine(A) nucleoside + purine(B) base ⇌
purine(B) nucleoside + purine(A) base

By the application of this reaction, various nucleoside analogues, such as guanosine 7-N-oxide,[23] bredinin,[24] and allopurinol riboside,[25] could be synthesized.

Recently, Utagawa *et al.*[26] reported on the enzymatic synthesis of ribavirin, an antiviral agent, from inosine and 1,2,4-triazole-3-carboxamide (TCA) by *Enterobacter aerogenes* AJ 11125. But ribavirin could not be produced directly from inosine and TCA because TCA had a much lower affinity (*Km*, 167 mM) for the PNPase of *E. aerogenes* AJ 11125 than hypoxanthine (5.6 mM), which was formed by the phosphorolysis of inosine, and TCA lost the chance to be recognized as a substrate of the PNPase. Therefore, the elimination of ribose-1-phosphate (R-1-P) from the reaction mixture was indispensable to prevent the reverse reaction from hypoxanthine and R-1-P to inosine.

On the other hand, we reported in our previous paper that *Brevibacterium acetylicum* ATCC 954 could produce ribavirin directly from not only guanosine but inosine, and TCA

* Corresponding author.

in a high yield without the elimination of R-1-P.[27] This reaction seemed to be catalyzed by the PNPase in *B. acetylicum* ATCC 954.

In this study, we purified the PNPase from *B. acetylicum* ATCC 954 and characterized the enzyme in detail.

Materials and Methods

Chemicals. All chemicals used were commercially available and of analytical grade.

Microorganism and culture conditions. Brevibacterium acetylicum ATCC 954 was used as the enzyme source. The cultivation medium consisted of 10 g of meat extract (Wako Chemical Industries, Ltd., Osaka, Japan), 10 g of peptone (Daigo, Osaka, Japan), 5 g of yeast extract (Oriental Yeast, Tokyo Japan), and 5 g of NaCl in a total volume of 1 l and was adjusted to pH 7.0 with 6 N KOH. Cultivation was done as follows: a loopful of cells of *B. acetylicum* ATCC 954 subcultured on a bouillon agar slant was inoculated into 5 ml of the medium in a test tube. After aerobic cultivation at 30°C for 24 hr, 1 ml of the culture broth was transferred to 50 ml of medium in a 500-ml flask, followed by aerobic cultivation at 30°C for 16 hr. The cells were then harvested by centrifugation ($10,000 \times g$) at 4°C for 20 min and rinsed with 0.05 M potassium phosphate buffer (pH 7.0).

Enzyme assay. The standard reaction mixture contains 20 μmol inosine, 50 μmol potassium phosphate buffer (pH 7.5) and an appropriate amount of the enzyme in a total volume of 1 ml. This mixture was incubated at 40°C for 1 min. After the reaction, 100 μl of the reaction mixture was transferred to 900 μl of 0.1 N HCl to stop the reaction. The hypoxanthine formed was measured by high-performance liquid chromatography (HPLC).

One unit of PNPase activity was defined as the amount of enzyme that produced 1 μmol of hypoxanthine per min under these assay conditions. The specific activity was expressed as units/mg protein.

Purification of the PNPase from B. acetylicum ATCC 954. All purification procedures were done at room temperature except for cell disruption at between 5°C and 15°C and dialysis (4°C). Throughout purification steps 1 to 6, 50 mM potassium phosphate buffer (pH 7.5), containing 1 mM dithiothreitol was used and is referred to as "the buffer," unless otherwise specified. All chromatographies were done with LKB HPLC (Pharmacia LKB Biotechnology).

Step 1. Preparation of cell-free extract. Rinsed cells were suspended in 300 ml of the buffer and then disrupted with an ultrasonic oscillator (12 Hz) for 15 min at between 5°C and 15°C. The cell debris was removed by centrifugation at $10,000 \times g$ for 20 min and 290 ml of the supernatant obtained was used for further purification.

Step 2. Heat treatment. The supernatant was kept at 60°C for 2 hr with gentle agitation. After centrifugation ($10,000 \times g$, 4°C, 20 min), 225 ml of supernatant was obtained.

Step 3. DEAE-Toyopearl PAK 650S chromatography. The supernatant was put onto a column of DEAE-Toyopearl PAK 650S (ϕ 2.2 × 20 cm) (Tosoh Co., Tokyo), equilibrated with the buffer before using. After the column was washed with 200 ml of the buffer, the enzyme was eluted with a linear gradient of NaCl from 0 to 0.2 M (0.1 M increase/hr) at a flow rate of 3 ml/min. Each fraction (6.5 ml) was collected and the active fractions (130 ml) were obtained. Ammonium sulfate was added to the solution to 40% saturation with stirring. After centrifugation ($10,000 \times g$, 20 min, 4°C), the supernatant was obtained.

Step 4. Butyl-Toyopearl PAK 650S chromatography. The supernatant was placed on a column (ϕ 2.2 × 20 cm) of Butyl-Toyopearl PAK 650S (Tosoh Co.), equilibrated with the buffer containing 40% saturated ammonium sulfate before using. After the column was washed with 200 ml of the buffer containing 40% saturated ammonium sulfate, the enzyme was eluted with a linear gradient of ammonium sulfate from 40% to 0% (40% decrease/hr) at a flow rate of 2 ml/min. Each fraction (6.5 ml) was collected, and the active fractions (10.4 ml) were obtained. Then the active fractions were dialyzed overnight against the buffer.

Step 5. TSK gel G3000SW filtration, 1st. The dialyzate was concentrated to less than 1 ml using OMEGACELL Units for 10 K of molecular weight limit (Filtron Technology Co., U.S.A.). The concentrated solution was put onto a column of TSK gel G3000SW (ϕ 0.75 × 60 cm) (Tosoh Co.), equilibrated with the buffer containing 0.2 M NaCl. The enzyme was eluted with the buffer containing 0.2 M NaCl at a flow rate of 0.2 ml/min. Each fraction (1.0 ml) was collected, and the active fractions (13.6 ml) were obtained. Then the solution was concentrated to less than 1 ml using OMEGACELL Units.

Step 6. TSK gel G3000SW filtration, 2nd. The concentrated solution was put onto a column of TSK gel G3000SW by the same method described in step 5. Finally, 13.2 ml of the enzyme solution including 5.8 mg protein was obtained.

Analysis. Bases and nucleosides were measured by HPLC at room temperature as follows: column, CAPCELL PAK C_{18} (ϕ 4 × 250 mm: Shiseido, Tokyo, Japan); solvent, 0.1 M sodium perchlorate with 0.1% (v/v) phosphoric acid; flow rate, 1.0 ml/min; detecter, UV monitor 210 nm for ribavirin and TCA and 254 nm for various natural bases and nucleosides. Protein was measured by the Coomassie brilliant blue G-250 dye-binding method of Bradford[28] using the dye reagent supplied by Bio-Rad.

Electrophoresis in polyacrylamide gel. Sodium dodecyl sulfate (SDS) electrophoresis was done in 4–20% polyacrylamide gradient gel using the Tris/glycine buffer.[29] Protein in the gel was stained by the silver stain method using a kit (Wako Chemical Industries Ltd.). The molecular weight of the enzyme and its subunit were calculated from the mobilities of standard proteins (LMW kit E; Pharmacia LKB Biotechnology).

Kinetic properties. The Michaelis constants for various substrates were measured under the following conditions: the reaction mixture containing 0.11 μg of the purified enzyme, 50 μmol potassium phosphate buffer (pH 7.5), and various amounts of nucleosides in a total volume of 1 ml, was incubated at 40°C. The reaction was stopped by adding 4 ml of 0.1 N HCl to the reaction mixture. The amount of nucleoside base formed in the reaction mixture was measured by HPLC.

Metal ions and inhibitors. The standard reaction mixture containing 20 μmol nucleoside, 50 μmol potassium phosphate buffer (pH 7.5), 0.11 μg the purified enzyme, and various metal ions or inhibitors described below, was incubated at 60°C for 1 min. The reaction was stopped by adding 4 ml of 0.1 N HCl to the reaction mixture. The amount of nucleoside base formed in the reaction mixture was measured by HPLC.

The following metal ions and inhibitors were tested: metal ions (mM); NaCl (20), KCl (20), $MgCl_2 \cdot 6H_2O$ (1), $FeSO_4 \cdot 7H_2O$ (1), $MnCl_2$ (1), $CaCl_2$ (1), $AlCl_3$ (1), $NiCl_2 \cdot 6H_2O$ (1), $CoCl_2$ (1), $BaCl_2$ (1), $CuSO_4 \cdot 5H_2O$ (1), $Na_2MoO_4 \cdot 2H_2O$ (1), LiCl (1), $ZnSO_4$ (1), $NaHAsO_4 \cdot 7H_2O$ (1), $AgNO_3$ (1), $SnCl_2$ (1), $CdCl_2$ (1), $FeCl_3$ (1), $PbCl_2$ (1), and $HgCl_2$ (1), and inhibitors (mM); *N*-ethylmaleimide (1), iodoacetic acid (1), iodoacetamide (1), *p*-chloromercuribenzoate (0.1), sodium azide (1), hydroxylamine (1), phenylhydrazine (1), D,L-penicillamine (0.1), semicarbazide (1), 8-hydroxyquinoline (1), α,α′-dipyridyl (1), ethylenediamine tetraacetate (1), *O*-phenanthroline (1), and KCN (1).

Amino acid sequencing. Amino acid sequencing was done by automated Edman degradation with an Applied Biosystems 470A gas-liquid phase protein sequencer.[30] The PTH derivatives of amino acids were separated and identified with an on-line PTH analyzer model 120A (Applied Biosystems) on a PTH-C_{18} column.

Results

Purification of the PNPase

Using the purification procedures described in Materials and Methods, the enzyme was purified approximately 49-fold with a yield of 6% (Table I). The enzyme catalyzed the phosphorolysis of inosine to hypoxanthine and R-1-P at 195.1 $\mu mol\,min^{-1}$ (mg protein)$^{-1}$ under the standard assay conditions.

Molecular weight

As shown in Fig. 1, a single band was observed by SDS-polyacrylamide gel electrophoresis whether the heat treatment at 100°C for 5 min in the presence of 2-mercaptoethanol (1 mM) was done or not. This shows that the PNPase is a monomer. The molecular weight was estimated to be 31,000.

Isoelectric point

The isoelectric point of the PNPase was estimated to be pH 4.7 by isoelectric focusing (Phast system, Pharmacia LKB Biotechnology).

Effects of temperature, pH, and phosphate concentration

The optimal temperature and pH for inosine phosphorolysis catalyzed by the PNPase were

Table I. PURIFICATION OF PNPASE FROM *B. acetylicum* ATCC 954

The reaction was done under the standard conditions using inosine as a substrate.

Purification	Volume (ml)	T.A[a] (units)	Protein (mg)	S.A[b] (units/mg)	Fold	Yield (%)
Cell-free extract	290	18189	4524	4.0	1	100
Heat treatment	225	14004	579	24.2	6	77
DEAE-Toyopearl	130	8622	142	60.8	15	47
Butyl-Toyopearl	10.4	2281	34	66.4	17	13
Gel filtration 1st	13.6	1943	12.5	155.3	39	11
Gel filtration 2nd	13.2	1134	5.8	195.1	49	6

[a] T.A, total activity; [b] S.A, specific activity.

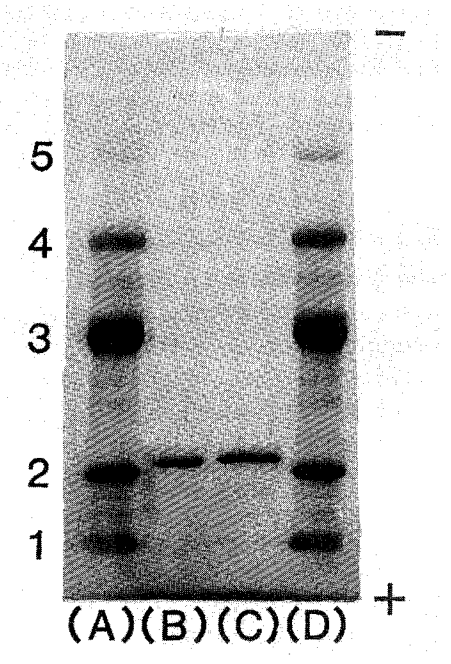

Fig. 1. SDS-slab Gel Electrophoresis of PNPase from *B. acetylicum* ATCC 954.

The conditions for SDS-slab gel electrophoresis are given in Materials and Methods. (A), (D) Marker proteins: 1, soybean trypsin inhibitor (20,000); 2, carbonic anhydrase (30,000); 3, ovalbumin (43,000); 4, bovine serum albumin (67,000); 5, phosphorylase b (94,000). (B) The native enzyme, 4.4 μg. (C) The enzyme, 4.4 μg, was first incubated at 100°C for 5 min in the presence of 1 mM 2-mercaptoethanol.

around 70°C and 8.5, respectively (Fig. 2). The optimal phosphate concentration for this reaction was around 200 mM, although the change in the activity level was only slight in a range of more than 100 mM of the phosphate concentration (Fig. 3).

Substrate specificity and kinetic properties

The results on substrate specificity are summarized in Table II. The enzyme showed the highest activity toward inosine and the activity decreased in the order of 2′-deoxyinosine, xanthosine, 2′,3′-dideoxyinosine, ribavirin, guanosine, and 2′-deoxyguanosine. It showed no activity toward adenosine, 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR), or various 5′-mononucleotides. The *Km* of the PNPase for inosine, guanosine, and

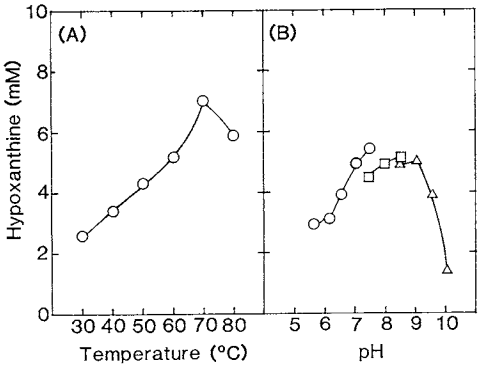

Fig. 2. Effects of Temperature and pH.

(A) The reaction mixture, containing 20 μmol inosine, 50 μmol potassium phosphate buffer (pH 7.5), and 0.22 μg PNPase in a total volume of 1 ml, was incubated at the indicated temperature for 1 min. (B) The reaction mixture, containing 20 μmol inosine, 0.22 μg PNPase, and the indicated buffer in a total volume of 1 ml, was incubated at 60°C for 1 min. Fifty mM potassium phosphate buffer, (○); 50 mM Tris–HCl buffer plus 50 mM K_2HPO_4, (□); 50 mM glycine–NaOH buffer plus 50 mM K_2HPO_4, (△).

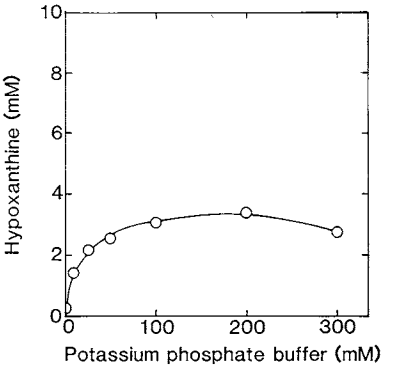

Fig. 3. Effects of Phosphate Concentration.

The reaction mixture, containing 20 μmol inosine, 0.22 μg PNPase, and the indicated concentrations of potassium phosphate buffer (pH 7.5) in a total volume of 1 ml, was incubated at 60°C for 1 min. Hypoxanthine, (○).

ribavirin were 1.43 mM, 2.44 mM, and 2.08 mM, respectively, at 40°C.

Equilibrium of the phosphorolyzing reaction catalyzed by the PNPase

As shown in Fig. 4, phosphorolysis by the PNPase was reached the equilibrium when 21% of the inosine or 15% of the ribavirin was phosphorolyzed in the presence of 20 mM

Table II. SUBSTRATE SPECIFICITY OF PNPASE FROM *B. acetylicum* ATCC 954

The reaction mixture, containing 20 μmol nucleoside or nucleotide, 50 μmol potassium phosphate buffer (pH 7.5) and 0.22 μg PNPase in a total volume of 1 ml, was incubated at 60°C for 1 min.

Nucleosides	Relative activity (%)
Inosine	100
Guanosine	21
Xanthosine	26
Adenosine	0
2′-Deoxyinosine	87
2′-Deoxyguanosine	11
2′-Deoxyadenosine	0
2′,3′-Dideoxyinosine	22
2′,3′-Dideoxyadenosine	0
Inosine-5′-monophosphate	0
Guanosine-5′-monophosphate	0
Xanthosine-5′-monophosphate	0
Adenosine-5′-monophosphate	0
AICAR[a]	0
Ribavirin	22
Acyclovir	0

[a] AICAR, 5-amino-4-imidazolecarboxamide ribonucleoside.

inosine or ribavirin, and 50 mM potassium phosphate buffer at 40°C.

Effects of metal ions and inhibitors

The effects of various metal ions and inhibitors on the inosine phosphorolysis by the PNPase were examined. Among them, the sulfhydryl (SH) reagents inactivated the PNPase activity. *p*-Chloromercuribenzoate (*p*CMB), $HgCl_2$, and $AgNO_3$ strongly inhibited the PNPase, and *N*-ethylmaleimide (NEM) also inhibited and $CuSO_4$ slightly inhibited the enzyme. However, iodoacetic acid and iodoacetoamide, which are also SH reagents, showed no inhibition. The other metal ions and inhibitors tested had no effect on the PNPase activity.

Amino terminal sequence analysis

The amino terminal sequence of the PNPase protein was analyzed. Twenty residues from the amino terminal end were identified as follows: MTVNWNETRSFLECKMQAKPE (one-letter symbols of amino acids).

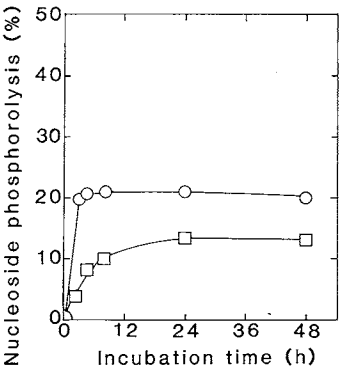

Fig. 4. Equilibrium of the Reaction Catalyzed by the PNPase of *B. acetylicum* ATCC 954.

The reaction mixture, containing 20 μmol inosine or ribavirin, 50 μmol potassium phosphate buffer (pH 7.5), and 0.22 μg PNPase in a total volume of 1 ml, was incubated at 40°C. Hypoxanthine, (○); TCA, (□).

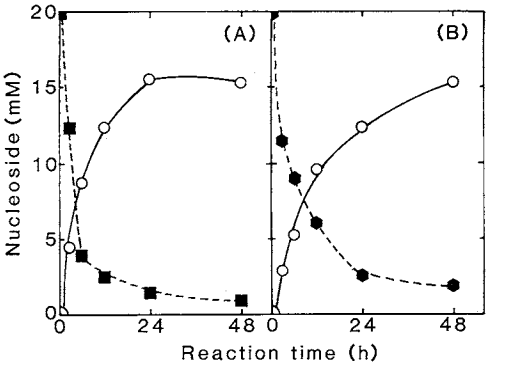

Fig. 5. Ribavirin Production by the Purified PNPase.

The reaction mixture, containing 20 μmol (A) guanosine or (B) inosine, 50 μmol TCA, 50 μmol potassium phosphate buffer (pH 7.0), and 22 μg PNPase in a total volume of 1 ml, was incubated at 60°C. Guanosine, (■); inosine, (●); ribavirin, (○).

Ribavirin production by the purified PNPase

In our previous paper,[27] we reported on ribavirin production using resting cells of *B. acetylicum* ATCC 954. In this study, we attempted to produce ribavirin by the purified PNPase of *B. acetylicum* ATCC 954. Figure 5 shows that ribavirin could be formed not only

from guanosine but also from inosine, in the presence of TCA, by the purified PNPase alone. Moreover, the amount of ribavirin formed from guanosine and TCA was almost the same as that formed from inosine and TCA after 48 hr of incubation. However, the rate of ribavirin formation from guanosine was faster than that from inosine.

Discussion

PNPase was extracted from *B. acetylicum* ATCC 954, which was a potent producer of ribavirin, and purified 49-fold. PNPase can be classified into two types according to the abilities for adenosine phosphorolysis.[16] The enzyme purified in this study may belong to the type of inosine-guanosine phosphorylase because this enzyme could not phosphorolyze adenosine.[12] This could also phosphorolyze xanthosine, but could not phosphorolyze AICAR or Acyclovir (an acyclic nucleoside analogue).

It seemed that this enzyme might have a sulfhydryl residue in the active center because this was inhibited by some SH reagents, such as *p*CMB, NEM, and $HgCl_2$. Moreover, the narrow range of optimal pH (around pH 8.5) also suggested that the enzyme might be a SH-enzyme because Hori *et al.* had shown that PNPase having no SH residue at the active center had a broad range of optimal pH.[20,21]

As the result of SDS-polyacrylamide electrophoresis, the PNPase purified from *B. acetylicum* ATCC 954 was found to be a monomer ($M=31{,}000$). There have already been many reports on PNPase, and most of those showed that PNPase had some subunits. However, the only exception is Lewis's report, in which the PNPase purified from rat liver might be a monomer (39,000 according to SDS-polyacrylamide electrophoresis). This enzyme is also inactivated by *p*CMB.

Utagawa *et al.* reported that ribavirin could not be produced directly from inosine and TCA by *E. aerogenes* AJ 11125 because of the lower affinity of TCA for the PNPase of *E. aerogenes* AJ 11125 than that of hypoxanthine. On the other hand, we investigated the *Km*s values of inosine, guanosine, and ribavirin for the PNPase of *B. acetylicum* ATCC 954, and found that that of ribavirin (*Km*, 2.08 mM) was the same level as those of inosine (1.43 mM) and guanosine (2.44 mM). Therefore, it seems that *B. acetylicum* ATCC 954 can produce ribavirin not only from guanosine but also from inosine in the presence of TCA.

Figure 5 shows that ribavirin is produced from guanosine faster than from inosine in the presence of TCA. This difference in rate of ribavirin formation might be due to the difficulty in solubilizing guanine in water compared with hypoxanthine.

Acknowledgments. We are indebted to Dr. I. Maeda and Dr. K. Kubota of their laboratories for encouragement during the course of this work.

References

1) H. M. Kalckar, *J. Biol. Chem.*, **167**, 477 (1947).
2) E. D. Korn and J. M. Buchanan, *J. Biol. Chem.*, **217**, 183 (1955).
3) B. K. Kim, S. Cha and R. E. Parks, Jr., *J. Biol. Chem.*, **243**, 1763 (1968).
4) T. P. Zimmerman, N. B. Gersten, A. F. Ross and R. P. Miech, *Can. J. Biochem.*, **49**, 1050 (1971).
5) A. S. Lewis and M. D. Glantz, *J. Biol. Chem.*, **251**, 407 (1976).
6) A. S. Lewis and M. D. Glantz, *Biochemistry*, **15**, 4451 (1976).
7) R. L. Miller, C. L. K. Sabourin and T. A. Krenitsky, *Biochem. Pharmacol.*, **36**, 553 (1987).
8) A. L. Koch, *J. Biol. Chem.*, **223**, 535 (1956).
9) J. L. Ott and C. H. Werkman, *Arch. Biochem. Biophys.*, **69**, 264 (1957).
10) Y. Tazuke and H. Yamada, *Agric. Biol. Chem.*, **27**, 625 (1963).
11) R. Gardner and A. Kornberg, *J. Biol. Chem.*, **242**, 2383 (1967).
12) H. L. Engelbrecht and H. L. Sadoff, *J. Biol. Chem.*, **244**, 6228 (1969).
13) R. W. Gilpin and H. L. Sadoff, *J. Biol. Chem.*, **246**, 1475 (1971).
14) K. F. Jensen and P. Nygaard, *Eur. J. Biochem.*, **51**, 253 (1975).
15) S. Senesi, G. Falcone, U. Mura, F. Sgarrella and P. L. Ipata, *FEBS lett.*, **64**, 353 (1976).
16) K. F. Jensen, *Biochim. Biophys. Acta*, **525**, 346 (1978).
17) K. Hammer-Jespersen, R. S. Buxton and T. D. Hansen, *Mol. Gen. Genet.*, **179**, 341 (1980).

18) T. A. Krenitsky, G. W. Koszalka and J. V. Tuttle, *Biochemistry*, **20**, 3615 (1981).
19) G. W. Koszalka, J. Vanhooke, S. A. Short and W. W. Hall, *J. Bacteriol.*, **170**, 3493 (1988).
20) N. Hori, M. Watanabe, Y. Yamazaki and Y. Mikami, *Agric. Biol. Chem.*, **53**, 2205 (1989).
21) N. Hori, M. Watanabe, Y. Yamazaki and Y. Mikami, *Agric. Biol. Chem.*, **53**, 3219 (1989).
22) R. P. Agarwal and R. E. Parks, Jr., *J. Biol. Chem.*, **244**, 644 (1969).
23) M. Nishii, Y. Fujihara, J. Inagaki, K. Ogata, F. Nohara, M. Ubukata and K. Isono, *Agric. Biol. Chem.*, **50**, 2697 (1986).
24) K. Mizuno, S. Yaginuma, M. Hayashi, M. Takeda and N. Muto, *J. Ferment. Technol.*, **53**, 609 (1975).
25) D. J. Nelson, S. W. LaFon, J. V. Tuttle, W. H. Miller, R. L. Miller, T. A. Krenitsky and G. B. Elion, *J. Biol. Chem.*, **254**, 11544 (1979).
26) T. Utagawa, H. Morisawa, S. Yamanaka, A. Yamazaki, F. Yoshinaga and Y. Hirose, *Agric. Biol. Chem.*, **49**, 3239 (1985).
27) H. Shirae, K. Yokozeki, M. Uchiyama and K. Kubota, *Agric. Biol. Chem.*, **52**, 1777 (1988).
28) M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
29) J. King and U. K. Laemmli, *J. Mol. Biol.*, **62**, 465 (1971).
30) J. E. Stricker, M. W. Hunkapiller and K. J. Wilson, *Anal. Biochem.*, **140**, 553 (1984).