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Purification and Characterization of Pyrimidine Nucleotide *N*-Ribosidase from *Pseudomonas oleovorans*

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Abstract Pyrimidine nucleotide *N*-ribosidase (pyrimidine 5'-nucleotide phosphoribo(deoxyribo)hydrolase/pyrimidine 5'-nucleotide nucleosidase, EC 3.2.2.10) catalyzes the breakdown of pyrimidine 5'-nucleotide into pyrimidine base and ribose(deoxyribo)-5-phosphate. However, detailed characteristics of the enzyme have not yet been reported. The enzyme was purified to homogeneity 327.9-fold with an overall yield of 6.1% from *Pseudomonas oleovorans* ATCC 8062. The enzyme catalyzed cytidine monophosphate (CMP) and uridine monophosphate (UMP), but not adenosine monophosphate (AMP) and guanosine monophosphate (GMP). The enzyme optimally metabolized CMP at pH 6.0 and UMP at around 8.5, and the optimum temperature for the overall enzyme reaction was found to be 37°C. The K_m values of the enzyme for CMP (at pH 6.0) and UMP (at pH 8.5) were 1.6 mM and 1.1 mM, respectively. AMP, deoxyCMP, and deoxyUMP were very effective inhibitors of the reaction. Double-reciprocal plots obtained in the absence and in the presence of AMP revealed that this inhibitory effect was of the mixed competitive type with respect to the breakdown of CMP and of the noncompetitive type with respect to the breakdown of UMP. In the presence of AMP, the enzyme followed sigmoid kinetics with respect to each substrate.

Key words: Pyrimidine nucleotide *N*-ribosidase, *Pseudomonas oleovorans*

Generally, the degradation of cytidine monophosphate (CMP) in microorganisms is initiated by the elimination of phosphate, producing cytidine. This degradation occurs through two pathways, one consisting of the sequential catalysis of cytidine deaminase and nucleoside hydrolase (or nucleoside phosphorylase), and the other consisting of the sequential catalysis of nucleoside hydrolase and cytosine deaminase.

Previously, Sakai *et al.* [10–12] and Imada *et al.* [3, 4] demonstrated another CMP-degrading pathway, in which the cleavage of the *N*-ribosyl linkage occurred without the liberation of phosphate, and CMP was directly degraded to ribose-5-phosphate and cytosine. However, cytosine cannot be reused in nucleotides, because microorganisms contain neither CMP: pyrophosphorylase nor cytidine phosphorylase [9]. Moreover, cytosine cannot be an immediate precursor, since it is suggested to be converted to uracil in the first step, and then either reused for the *Salvage* synthesis of cytosine nucleotide or degraded into urea and acetyl-CoA. Certain microorganisms (*i.e.*, *Pseudomonas oleovorans*) possess only this pathway for the direct degradation of CMP. Sakai *et al.* [13] also demonstrated the existence of a novel enzyme catalyzing this pathway, and called it pyrimidine nucleotide *N*-ribosidase (Fig. 1). Pyrimidine nucleotide *N*-ribosidase (pyrimidine 5'-nucleotide

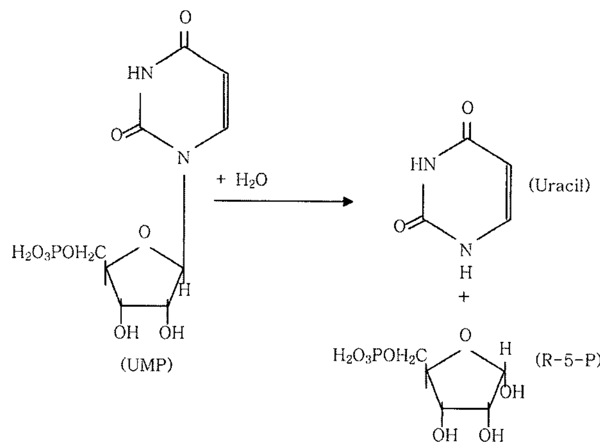

Fig. 1. Hydrolysis of UMP by the action of the pyrimidine nucleotide *N*-ribosidase from *P. oleovorans*.

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phosphoribo(deoxyribo)hydrolase/pyrimidine 5'-nucleotide nucleosidase, EC 3.2.2.10) catalyzes pyrimidine 5'-nucleotide to pyrimidine base and ribose(deoxyribo)-5-phosphate. However, the properties of the enzyme have not yet been reported in detail.

In this study, the purification and characteristics of this enzyme in *P. oleovorans* ATCC 8062 as well as this novel pathway for the degradation of CMP were studied.

Materials and Methods

Chemicals

Peptone, corn steep liquor (CSL), and meat extract were purchased from Difco Co., U.S.A. Cytidine monophosphate (CMP), uridine monophosphate (UMP), nucleotides, nucleosides, and bases were all obtained from the Sigma Co., U.S.A. All other chemicals used were of analytical reagent grade.

Microorganism and Culture

Pseudomonas oleovorans ATCC 8062 was used throughout this study. *P. oleovorans* was grown either solely with 11-alkoxyundecanoic acid or with 6-alkoxyhexanoic acid [6].

The microorganism was inoculated into a 500-ml flask, which contained 100 ml of medium containing 2% sodium fumarate, 1% peptone, 5% CSL, 0.03% $MgSO_4 \cdot 7H_2O$, 0.01% KH_2PO_4, 0.01% K_2HPO_4, 1% NH_4Cl, and 0.2% yeast extract, at pH 7.0 [15]. The subculture was carried out at 28°C for 24 h under reciprocal shaking. Each 500 ml of this subculture was in turn inoculated into a 20-l jar fermentor containing 10 l of the medium and cultivation proceeded for 48 h at 28°C with aeration (10 l/min). The cells were harvested by a continuous-flow centrifuge and then washed three times with 0.9% saline.

Preparation of Cell-Free Extract

The cell paste (400 g) obtained from 100 l of the medium was suspended in 0.05 M phosphate buffer (pH 6.5) to make a suspension of about 1 g/10 ml. The suspension was divided into 500-ml portions, and each portion was subjected to ultrasonic oscillation (20 kHz) for 30 min. The cell debris was removed by centrifugation at 19,000 ×*g* for 30 min. The supernatant solution, as cell-free extract, was dialyzed for 24 h against 10 l of 0.01 M phosphate buffer containing 5×10^{-4} M EDTA (pH 6.5).

Enzyme Assay

Enzyme activity was assayed by measuring the amount of ribose-5-phosphate (R-5-P) formed from UMP. In order to determine R-5-P quantities, the following procedure was used. The reaction mixture contained 10 µmoles of UMP, 100 µmoles of Tris-HCl buffer, pH 7.0, and the enzyme at pH 7.0. The total volume was 1.0 ml. The reaction was carried out at 37°C for 30 min with gentle shaking and was stopped by heating for 3 min in a boiling water bath. After cooling, the precipitate formed was removed, and the supernatant was submitted to analysis. The amount of R-5-P was determined according to Somogy's method [14]. Ribose, ribose-1-phosphate, and ribose-5-phosphate were detected by the method of Agarwal *et al.* [1].

In the enzyme assays, reaction mixtures without substrate were run as a control. One unit of enzyme activity was defined as the amount of enzyme to degrade 1 µmole of UMP per hour under the assay conditions described. Specific activity is expressed as unit per milligram of enzyme protein.

Protein Determination

Protein concentration was determined according to the method of Lowry *et al.* [8].

Polyacrylamide Gel Electrophoresis

Acrylamide gel electrophoresis was performed by the method of Davis [2] with some modifications: stacking and running gels were polymerized in a glass tube (7×80 mm), and about 20 µg of protein in 15% sucrose solution was laid on the gel.

Purification of the Enzyme

All procedures were performed at below 5°C unless otherwise noted.

Step I. Ammonium Sulfate Fractionation. Solid ammonium sulfate was added to 3,700 ml of the crude extract to 30% saturation. After being allowed to stand for 5 h, the precipitate formed was removed by centrifugation at 19,000 ×*g* for 20 min. The concentration of ammonium sulfate in the supernatant was increased to 50% saturation by the addition of solid ammonium sulfate. After being kept overnight, the precipitate formed was collected by 30 min of centrifugation at 19,000 ×*g*, and was dissolved in a small amount of 0.01 M phosphate buffer at pH 6.5. The solution was dialyzed for 24 h against 10 l of 0.01 M phosphate buffer at pH 6.5.

Step II. Streptomycin Treatment. Into 250 ml of the above dialysate, 10 ml of a solution, containing 1 g of dihydrostreptomycin sulfate, was added. After 60 min, the precipitate formed was removed by centrifugation at 19,000 ×*g* for 30 min.

Step III. Heat Treatment. The supernatant (250 ml) obtained in step II was dialyzed overnight against 0.01 M phosphate buffer (pH 7.0), and the dialysate was kept at 53°C for 7 min and then cooled. The precipitate formed was discarded by centrifugation at 19,000 ×*g* for 30 min.

Step IV. First DEAE-Cellulose Column Chromatography. The supernatant obtained in the preceding step was dialyzed overnight against 10 l of 0.04 M phosphate buffer (pH 6.5)

and applied to a DEAE-cellulose column (5×50 cm) that had been equilibrated with 0.04 M phosphate buffer (pH 6.5). The enzyme preparation was placed on the column, and the column was washed with 0.04 M phosphate buffer (pH 6.5), removing much of the inactive protein. The enzyme was eluted with 0.08 M phosphate buffer (pH 6.5) at a flow rate of 1 ml per minute. Five ml fractions were collected. The fractions that exhibited enzyme activity were combined, and solid ammonium sulfate was added to a saturation of 55%. After overnight, the precipitate formed was collected by centrifugation at 19,000 ×*g* for 30 min, dissolved in 0.1 M Tris-HCl buffer (pH 7.0), and dialyzed for 24 h against 5 l of Tris-HCl buffer (pH 7.0).

Step V. Second DEAE-Cellulose Column Chromatography. The enzyme solution prepared in the preceding step was applied to a DEAE-cellulose column (3.3×22 cm) that had been equilibrated with 0.1 M Tris-HCl buffer (pH 7.0). The enzyme solution was placed on the column, and the column was washed with 0.1 M Tris-HCl buffer (pH 7.0) and then eluted with 0.1 M Tris-HCl buffer containing 0.1 M NaCl (pH 7.0) at a flow rate of 1 ml per minute. The fractions that exhibited enzyme activity were combined and concentrated by the addition of solid ammonium sulfate to a saturation of 55%.

The resultant precipitate was collected by centrifugation at 19,000 ×*g* for 30 min and dissolved in 0.05 M phosphate buffer (pH 6.5).

Step VI. Sephadex G-200 Gel Filtration. The enzyme solution obtained in the previous step was subjected to Sephadex G-200 gel filtration. The gel was packed in a column (2.5×95 cm) and equilibrated with 0.05 M phosphate buffer (pH 6.5). The enzyme solution was then placed at the bottom of the column, the buffer was allowed to flow by the ascending method at a flow rate of 10 ml per hour, and 5-ml fractions were collected. The fractions that contained enzyme activity were combined and concentrated by the addition of solid ammonium sulfate to 55% saturation. The resultant precipitate was collected by centrifugation at 19,000 ×*g* for 30 min and dissolved in a minimal amount of 0.1 M phosphate buffer (pH 6.5). The enzyme solution was then dialyzed for 24 h against two changes of 2 l of 0.1 M phosphate buffer (pH 6.5).

Step VII. Hydroxylapatite. The dialyzed enzyme solution (5 ml) was applied to a hydroxylapatite column (3×5 cm) that had been equilibrated with 0.1 M phosphate buffer (pH 6.5). The column was washed with 0.1 M phosphate buffer (pH 6.5), and elution was carried out with 0.3 M phosphate buffer (pH 6.5) at a flow rate of 10 ml per hour.

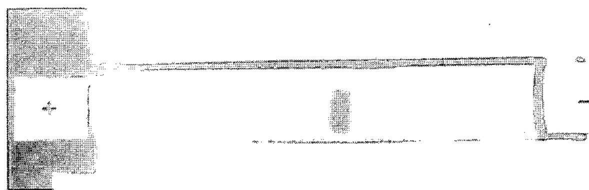

Fig. 2. Acrylamide gel electrophoresis of the purified pyrimidine nucleotide *N*-ribosidase.

Purified enzyme (about 20 μg) was subjected to electrophoresis at a current of 2.5 mA for 2 h in Tris-glycine buffer (pH 8.9). The direction of migration is from the cathode to the anode.

Table 1. Effect of metal ions on the activity of the pyrimidine nucleotide *N*-ribosidase from *P. oleovorans*.

Metal	Relative activity (%)	
	10 mM	1 mM
None	100	100
None*	79	–
$MgCl_2$	64	85
$CaCl_2$	78	105
$MnCl_2$	8	56
$FeCl_2$	4	82
$FeCl_3$	50	89
$NiCl_2$	21	84
$CuCl_2$	5	44
$ZnCl_2$	4	4
$CoCl_2$	5	80
$BaCl_2$	89	104
$HgCl_2$	21	35
$SnCl_2$	12	85

Reaction mixture contained 5 units of EDTA-treated enzyme and various metal ions as indicated.

EDTA-treated enzyme was prepared as follows: the purified enzyme was mixed with EDTA to make a final concentration of 10 mM and allowed to stand for 10 min at 5°C. Afterwards, the enzyme was dialyzed overnight against several changes of distilled water.

*Nontreated enzyme was used in the enzyme reaction.

RESULTS

Homogeneity of Preparation

The pyrimidine nucleotide *N*-ribosidase of *Pseudomonas oleovorans* ATCC 8062 was purified 327.9-fold from 33,000 units of total activity with an overall yield of 6.1%. The elution patterns of the final step in the hydroxylapatite column chromatography demonstrated a single symmetrical protein peak and the activity was entirely associated with the peak (data not shown). The enzyme preparation obtained in the step VII produced a single band on polyacrylamide gel electrophoresis (Fig. 2). These results indicate that the enzyme was indeed purified to homogeneity.

Substrate Specificity

Cell-free extract of the microorganism exhibited enzyme activities involved with catalysis of the conversion of UMP, CMP, IMP, GMP, and AMP to R-5-P and corresponding bases, but the purified enzyme did not catalyze IMP, GMP,

and AMP during the purification. The UMP-cleaving/CMP-cleaving activity ratio was constant at each step of the purification. Twenty-one compounds, including 5'-UMP, 5'-CMP, 5'-AMP, 5'-GMP, 5'-IMP, 5'-deoxyAMP, 5'-deoxyGMP, 5'-deoxyUMP, 5'-deoxyCMP, 3'-CMP, 2',3'-CMP, uridine, cytidine, thymidine, guanosine, adenosine, inosine, deoxyuridine, deoxycytidine, deoxyguanosine, and deoxyadenosine, were investigated for their ability to serve as substrates for the purified enzyme.

However, only UMP and CMP were found to be the substrate of this purified enzyme.

Stoichiometry

The materials and methods applied were identical to those in the previous paper [13]. When 10 μmoles of UMP were subjected to degradation by the purified enzyme under the standard assay conditions, 3.65 μmoles of UMP were consumed, while 3.24 μmoles of uracil and 559.98 μg/ml of ribose-5-phosphate as D-ribose were formed (data not shown). These results suggested that this enzyme reaction seems to proceed stoichiometrically.

Stability

The purified enzyme was completely inactivated after storage (at pH 7.0) for one month at 5°C and was more rapidly inactivated by freezing. The enzyme was, however, relatively heat-stable; its activity persisted without loss when kept at 53°C for 10 min (pH 7.0), although more than 80% of the activity was lost when kept for 5 min at temperatures higher than 55°C. In these cases, the enzyme activities for CMP and UMP were inactivated at identical rates (Fig. 3).

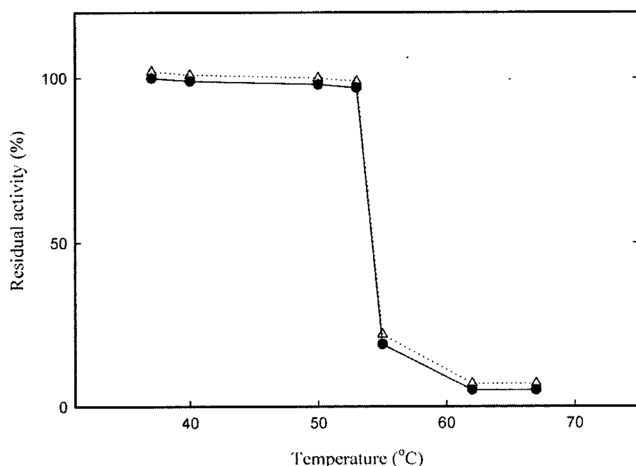

Fig. 3. Heat stability of the purified pyrimidine nucleotide *N*-ribosidase.
The purified enzyme (30 units) in 0.1 M Tris-HCl buffer (pH 7.0), was kept for 5 min at the indicated temperature, and residual activity was then measured. △, enzyme activity with UMP as a substrate; ●, enzyme activity with CMP as a substrate.

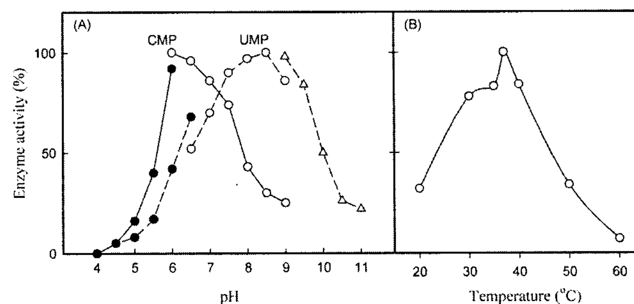

Fig. 4. Effect of pH (A) and temperature (B) on the pyrimidine nucleotide *N*-ribosidase activity using CMP and UMP as substrates.
Enzyme activity was assayed under standard conditions, except that (A) pH was varied using a reaction mixture containing 100 μmoles of buffers or (B) reaction temperature was varied (buffer used: pH 7.0, Tris-HCl). (A) Buffer used: ●, citrate buffer (pHs 4.0 to 6.5); ○, Tris-HCl buffer (pHs 6.0 to 9.0); △, glycine buffer (pHs 9.0 to 11.0); —, enzyme activity with CMP as a substrate; --, enzyme activity with UMP as a substrate.

Effect of pH and Temperature on the Enzyme Activity

The enzyme reactions with CMP and UMP were carried out in different buffers with overlapping ranges of pH [Fig. 4(A)]. Plots of enzyme activities against pH showed that the enzyme has an optimum pH of around 6.0 for CMP and around 8.5 for UMP.

The effects of temperature on the enzyme activity at pH 7.0 are shown in Fig. 4(B). The optimum temperature for the enzyme reaction with UMP and CMP was found to be 37°C. The degree of uracil formation was proportional to the amounts of enzyme (1 to 2.5 units/ml) in the reaction mixture tested, and was also found to be proportional to reaction time (5 to 60 min).

Effect of Metal Ions on the Enzyme Activity

In order to ascertain the requirement for metal ions, reactions were carried out using the EDTA-treated enzyme both with and without metal ions. As indicated in Table 1, no metal ion was found to accelerate the reaction, but the enzyme activity was strongly inhibited by Mn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Sn^{2+} at 10 mM concentration, and EDTA treatment activated the enzyme.

Effect of Various Compounds on the Enzyme Activity

The effects of phosphate, sulfate, sulfite, nitrate, carbonate, acetate, citrate, fumarate, pyruvate, succinate, arsenate, and borate were also studied. Of these, borate, sulfite, sulfate, and phosphate at 10 mM concentration were found to inhibit the enzyme reaction by 32%, 50%, 22%, and 15%, respectively.

Effect of Substrate Concentration on Enzyme Activity

The effect of CMP and UMP concentration on the enzyme activity was examined. The double-reciprocal plots [7] of enzyme activity and substrate concentration are given in

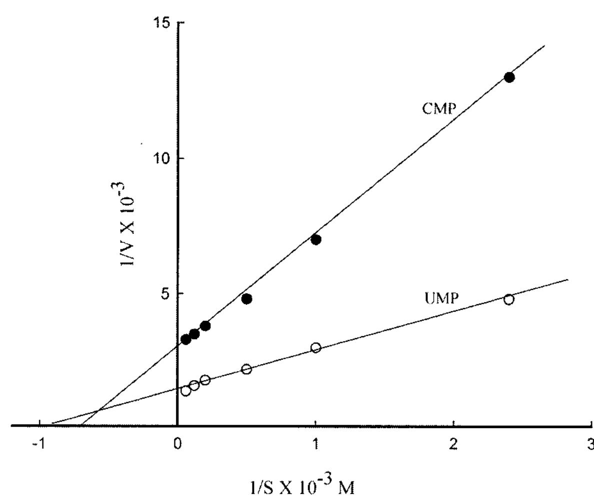

Fig. 5. Double-reciprocal plots of the initial velocities of the purified pyrimidine nucleotide *N*-ribosidase with CMP and UMP as the concentration-variable substrates.
●, CMP was used as a substrate; ○, UMP was used as a substrate.

Fig. 5. The K_m values for CMP (at pH 6.0) and UMP (at pH 8.5) were 1.6 mM and 1.1 mM, respectively.

Effect of Nucleotides and Their Related Compounds
The enzyme reaction was inhibited by a variety of nucleotide-related compounds. Out of the 16 kinds of substances tested in this experiment, the substances that proved to inhibit the enzyme reaction are listed in Table 2. AMP, deoxyCMP, and deoxyUMP, in particular, were very effective

Table 2. Effect of nucleotides and their related compounds on the activity of the pyrimidine nucleotide *N*-ribosidase from *P. oleovorans*.

Addition	Relative activity (%)
None	100
AMP	54
GMP	86
IMP	79
deoxyAMP	82
deoxyGMP	100
deoxyCMP	49
deoxyUMP	0
Deoxyadenosine	107
Deoxyguanosine	166
Deoxyinosine	80
Cytidine	100
Uridine	100
Adenine	100
Guanine	51
Hypoxanthine	47
Cytosine	58

Reaction mixture contained 5 units of purified enzyme, 5 μmoles of UMP, 100 μmoles of Tris-acetate buffer (pH 8.5), and 5 μmoles of the materials indicated.

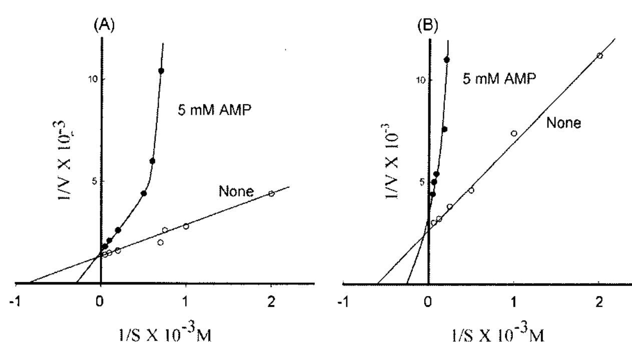

Fig. 6. Inhibitory effect of AMP on the degradation of UMP (A) and CMP (B).
Enzyme activity was assayed in the absence or in the presence of 5 mM AMP. The reaction mixture contained UMP (A) or CMP (B) as the concentration-variable substrate, and pHs were 8.5 and 6.0, respectively. ○, none; ●, 5 mM AMP.

inhibitors. Double-reciprocal plots obtained in both the absence and presence of AMP showed that the inhibition was of the mixed competitive type with respect to CMP and of the noncompetitive type with respect to CMP and UMP, respectively, and that the enzyme followed sigmoid kinetics with respect to each substrate in the presence of AMP (Fig. 6).

DISCUSSION

Sakai *et al.* [11, 13] and Yu [5, 15] earlier observed that some microorganisms are able to produce ribose-5-phosphate (R-5-P) from UMP or CMP, and they attempted to elucidate the pathway by which R-5-P can be produced from pyrimidine nucleotides.

Three tentative pathways are possible concerning the formation of R-5-P from UMP, as shown in the following scheme.

In pathway (1), R-5-P is produced from phosphoribosyl-pyrophosphate (PRPP), which is in turn produced from UMP by nucleotide pyrophosphorylase. Inorganic pyrophosphate (PPi) may function as a co-substrate in this reaction. In pathway (2), ribose-l,5-diphosphate (R-1,5-PPi) is produced by nucleotide phosphorylase in the presence of inorganic phosphate (Pi), and R-5-P is then produced after the liberation of phosphate. Pathway (3) is catalyzed by the nucleotide *N*-ribosidase. Because the reaction was affected by neither Pi nor by PPi, it is clear that this reaction is catalyzed by the novel enzyme pyrimidine nucleotide *N*-ribosidase. In this study, the enzyme was purified, in order to confirm the presence of the enzyme that catalyzes R-5-P formation from pyrimidine nucleotides.

The enzyme catalyzing the hydrolysis of the *N*-ribosidic linkages of pyrimidine nucleotides was purified about 327.9-fold from the cell-free extract of *P. oleovorans*.

The purified enzyme was homogeneous, as determined by polyacrylamide gel electrophoresis.

The cell-free extract of *P. oleovorans* revealed enzyme activities that catalyze the hydrolytic split of the *N*-ribosidic linkages of AMP, GMP, UMP, and CMP. Enzyme activities toward purine nucleotides were lost, while the activities of UMP and CMP persisted after purification. The ratio of the activity for UMP to that for CMP (at pH 7.0) is about 2.0 at each purification step (data not shown).

According to these results, the hydrolysis of the *N*-ribosidic linkages of both UMP and CMP appeared to be catalyzed by the same enzyme. The purified enzyme showed the activity for only UMP and CMP.

The enzyme reaction is inhibited by a variety of nucleotide-related compounds. The sigmoidal shape of the substrate saturation curves, showing inhibition by AMP, is of special interest. This observation suggests that AMP is an allosteric inhibitor, and that the enzyme is an allosteric enzyme.

Previously, Imada *et al.* [4] discovered an enzyme, which catalyzed the hydrolysis of the *N*-ribosidic linkages of UMP, CMP deoxyUMP, deoxyTMP, and deoxyCMP, in *Streptomyces virginiae*. Detailed purification of the enzyme was not performed, and it is uncertain whether this reaction was catalyzed by one or more enzymes. Enzymes of the same type have been found to occur in some other species, notably those of *Pseudomonas, Alcaligenes* [10], and *Streptomyces* [4]. As shown in this study, *P. oleovorans* does not possess a pyrimidine nucleoside-degrading enzyme, therefore, cytosine must be produced from CMP in that organism.

In summary a new enzyme, pyrimidine nucleotide *N*-ribosidase, was purified 327.9-fold from the cell-free extract of *P. oleovorans* ATCC 8062. The enzyme catalyzes the stoichiometric conversion of UMP and CMP to ribose-5-phosphate and their respective bases, but catalyzes neither the 3'-isomers nor their deoxyribonucleotide. This enzyme activity is inhibited by many nucleotides and their related compounds, and especially by AMP, deoxyUMP, cytosine, guanine, and hypoxanthine.

Therefore, in these organisms, cytosine deaminase seems to play an important role of shunting cytosine into metabolic pathways. AMP appears to function as an allosteric inhibitor of the pyrimidine nucleotide *N*-ribosidase activity of *Pseudomonas oleovorans* ATCC 8062.

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