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PURIFICATION AND PROPERTIES OF A NON-SPECIFIC NUCLEOTIDASE ACTIVE AGAINST 5′-NUCLEOTIDES AND 3′-NUCLEOTIDES FROM *XANTHOMONAS ORYZAE*[1][2]

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Abstract

A non-specific nucleotidase, active against two different configurations of nucleotides, i. e. 5′-nucleotides and 3′-nucleotides was isolated from the cell wall of *Xanthomonas oryzae.*

The enzyme was extensively purified and its purity was determined by molecular sieving chromatography and disc gel electrophoresis. It has two pH optima, i. e. pH 5 and 7.5. The temperature optimum is at 65°C. Na^+, K^+, Ca^{++}, Mg^{++} or Mn^{++} at concentrations from 1×10^{-4} to 5×10^{-3} M does not effect on the activity, however, Co^{++}, Fe^{++} or Zn^{++} at concentration of 5×10^{-3}M has inhibitory effect on enzyme activity. The enzyme hydrolyzes ribose-5′-nucleotides, ribose-3′-nucleotides and deoxyribose nucleotides.

Non-specificity of enzyme active against 5′-nucleotide and 3′-nucleotide was demonstrated by the identical position of two reactions on the disc gel electrophoresis and molecular sieve chromatography. Further comfirmation was also made by the patterns of the two enzyme activities in different pH conditions, and at different temperatures, by their stabilities to temperatures and the effect of cations.

Introduction

In our study on the incorporation of nucleotides into cells of *Xanthomonas oryzae*, a nucleotide-degrading enzyme located on the surface of bacterial cells was observed. When ^{14}C-labelled nucleotides were mixed with bacterial cells the nucleotides were rapidely degraded into nucleosides. The enzyme was isolated, purified and found to be non-specifically active against 5′-nucleotides and 3′-nucleotides. The 3′-nucleotidases of bacterial system so far reported are substrate specific. 5′-nucleotidase has been demonstrated in an unidentified soil bacterium (Wang, 1954), in *Proteus vulgaris* (Swart *et al.*, 1958), and in *Clostridium sticklandi* (Heman and Wright, 1959). And 3′-nucleotidase was

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described in *Escherichia coli* B (Anraku, 1968) and extensively studied by Neu and Heppel (1964) and Neu (1968). Lohn and Reis (1963) isolated both 5′-nucleotidase and 3′-nucleotidase from *Proteus vulgaris* and demonstrated that 5′-nucleotidase are separated enzymes. Neu (1967, 1968) also isolated 5′-nucleotidase and 3′-nucleotidase from the cell wall of Enterobacteriaceae, these two enzymes were extensively purified and found they are completely different. Non-specific nucleotidase active against both 5′-nucleotides and 3′-nucleotides has not been reported so far in bacterial system. In this investigation the purification of the enzyme and its properties are described. Evidence concerning its surface location and its non-specific activity against 5′-nucleotides and 3′-nucleotides are also presented.

Materials and Methods

Organism

Xanthomonas oryzae 507, a mutant of strain 500 preserved at this laboratory, was used in all experiments. This organism contains less capsule and is more sensitive to lysozyme than wild type.

Chemicals

Nucleotides, lysozyme, bovine serum albumin, EDTA, DEAE-cellulose and 2-mercaptoethanol were purchased from Sigma Chemicals Co., Sephadex G-100 was from Pharmacia Fine Chemicals. Chemicals used for polyacrylamide gel electrophoresis was from Canalco. Inorganic chmicals were of reagent grade.

Media and cultivation of bacteria

The medium used was potato-peptone medium containing potato, 200 g; peptone, 5 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g; $Na_2HPO_4 \cdot 12H_2O$, 2 g; sucrose, 15 g; and water 1000 ml.

For the growth of bacteria, bacteria grown in slant was transfered to potato-peptone medium and incubated at 30° on a rotary shaker for 24 hours. The bacteria was harvested and washed twice with 0.01M tris-buffer at pH 7.5, finally suspended in the same buffer.

Preparation of cell wall and plasma membrane fractions

The washed bacterial cells were centrifuged down and resuspended in tris-buffer in the presence of 20% sucrose with a ratio of 0.1 gram wet cells per ml buffer solution. 0.16 mg per ml of lysozyme and 0.01 M EDTA were added and incubated at 30° for 40 min. At the end of incubation 99% of bacterial calls became spheroplast. The spheroplasts were precipitated by centrifugation at 10000×g for 10 min. The suspernatant was considered as cell wall fraction.

The spheroplasts were collected and resuspended in 0.01 M tris-buffer at

pH 7.5. Under this condition spheroplasts were broken, and membrance could be separated from cytoplasm by centrifugation. The membrane was washed with buffer and finally resuspended in the same buffer. This fraction was considered as plasma membrane fraction.

Enzyme assay

Nucleotidase activity was assayed by the liberation of inorganic orthophosphate. Reaction mixture contained 5×10^{-3} M disodium ATP, 5×10^{-2} M tris-buffer, pH 7.5, and proper amount of enzyme preparation in a final volume of 1 ml. Reaction was carried out by incubating the mixture at 37° for 10 min and stoped by adding 0.1 ml of 25% TCA. The Pi released was measured according to the procedures described by Fiske and Subbarow (1925). One unit of enzyme activity was defined as the amount of enzyme able to liberate 1 μ mole of Pi per 10 min at 37°. A standard solution for Pi was prepared by dissolving 174 mg of analytical grade K_2HPO_4 in 100 ml H_2O (10 μ mole Pi per ml). One μ mole of Pi has an absorbance of 0.47 at 660 nm.

Disc gel electrophoresis

Electrophoresis was carried out in vertical direction in 6×80 mm gel column. The upper stacking gel was eliminated. The advantage of sharp electrophoretic banding inherent in the stacking gel technique was compensated by giving the sample a lower conductivity than the electrode buffer (Hjerten *et al.*, 1965). In all experiments, a 7.5% acrylamide gel was employed. The preparation of gels and buffers adapted were described by Jovin *et al.*, (1964). Protein sample was pretreated with 2-mercaptoethanol at least 10 min before applied to the gel. A volume of protein solution from 0.01 ml to 0.1 ml with a protein concentration from 10 to 40 μg was used for each column and 2.5 mA per column was employed at room temperature.

Detection of protein on the gel

Protein bands were detected by staining with 0.25% coomassie brilliant blue in 45.4% methanol–9.2% acetic acid solution for 3 to 5 hours at room temperature. Destaining was performed by immersing gels in 7.5% acetic acid–5% methanol solution with gentle stir and several changes of the destaining solution. It takes about 2 to 3 days at room temperature to complete the destaining.

Detection of enzyme activity on the gel

Disc gel electrophoresis was carried out according to the procedures described in above section. Gel after electrophoresis was incubated in a solution containing 5×10^{-3}M substrate (nucleotide) in 0.05 M tris-buffer, pH 7.5 at 40° for 30 min. The gel was rinsed with distilled water to remove the

excess substrate and then transfered to a solution containing 1% ammonium molybdate, 0.25% $FeSO_4$ at room temperature. A blue color will appear at the position where Pi is released in the gel after 2 to 5 min of incubation.

Protein determination

Protein was measured according to the method described by Lowry *et al.*, (1951) with bovine serum albumin as standard.

Results

Location of the enzyme

The cell wall fraction and membrane fraction were preparaded as described previously. Two fractions were assayed for enzyme activity. When ATP was used as subatrate, enzyme activity was detected in both fractions. However, when 3′–nucleotide was used as substrate, enzyme activity was detected only on the cell wall fraction. Later it was found that two enzymes are completely different. The ATP-hydrolyzing enzyme isolated from membrane fraction requires Mg^{++} (Table 1) and very unstable in EDTA solution. On the contrary the enzyme isolated from cell wall fraction does not require any cations and is very stable in EDTA solution. Two enzymes can be easily separated by sephadex G–100 column (unpublished data).

Table 1. *ATP-hydrolyzing activities of the cell wall fraction and plasma membrane from* ***Xanthomonas oryzae***

The concentration of ATP is 5×10^{-3}M, and tris-buffer pH 7.5, 5×10^{-2}M, in a volume of 1.0 ml. Two series of measurements were made, one with Mg^{++}, the other without Mg^{++}.

Enzyme source	Total protein (mg)	Activity			
		units		specific activity	
		$+Mg^{++}$	$-Mg^{++}$	$+Mg^{++}$	$-Mg^{++}$
cell wall fraction	0.25	1.2	1.18	4.80	4.72
plasma membrane	2.5	1.2	0.3	0.48	0.12

In order to prevent the possible contamination of nucleotidase from membrane fraction, the bacterial cells were treated with 0.1 M EDTA for 2 hrs. Under this mild treatment bacterial cells do not change to spheroplasts, but only small part of cell wall component released. When treated cells were examined under microscope there are no change in both morphology and number of bacteria. After the bacterial cells were precipitated with centrifugation, the enzyme activity was detected in the supernatant. In addition the enzyme is not extracellular and whole bacterial cells can be directly used as enzyme. It seems that enzyme is located on the surface of cell wall.

Purification procedures

Nucleotidase in "cell wall fraction" was prepared as described previously. The crude enzyme preparation was concentrated to about 1/4 by freezing lyophylizer and dialysed against 0.01 M tris-buffer at PH 7.5. After dialysis, the enzyme preparation was further concentrated by ultrafiltration (PM 30, Amicon) and then loaded on a DEAE-cellulose column (2.6×26 cm) equilibrium with 0.01 M tris-buffer at pH 7.5. The enzyme was eluted linear with a 0 to 0.25 M NaCl (200 ml) in the same tris-buffer. The fractions containing nucleotidase were pooled and concentrated by ultrafiltration in the presence of 0.1% 2-mercaptoethanol. The enzyme was furhter purified by Sephadex G-100 gel filtration column (gel bed, 2.6×85 cm) equilibrium with 0.01 M tris-buffer pH 7.5 at 6° with a flow rate of 10.5 ml per hour. A summary of the purification steps were shown in Table 2.

Table 2. *Purification of nucleotidase from* ***Xanthomonas oryzae***

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude cell wall preparation	52	240	4.6	100
DEAE-cellulose	3.7	218	59.0	90.8
Sephadex G-100	2.1	193	87.1	80.5

* Started from 20 g of wet cells.

Determination of purity of enzyme preparation

The purity of enzyme was tested by polyacrylamide gel electrophoresis. As shown in Fig 1, when purified enzyme was applied on polyacrylamide gel, only one clear sharp protein band was detected. Direct assay of enzyme activity was also conducted on the polyacrylamide gel. As shown in Fig 2. only one Pi liberation band was detected, and this band was exactly coincident with protein band.

The purified enzyme preparation was also loaded on a Sephadex G-100 column (gel bed, 2.6×85 cm) equilibrium with 0.01 M tris-buffer at pH 7.5 with a flow rate of 10.5 ml per hour. As showed in Fig. 3, only one protein peak was obtained and the protein peak was again coincident with the pattern of enzyme activity.

Properties of the nucleotidase

1. *Substrate specificity*

Various nucleotides were tested for the substrate specificity of the nucleotidase. As shown in Table 3, the enzyme hydrolyzes ribose 5′-nucleotide, ribose 3′-nucleotide or deoxyribose 5′-nucleotide. Ribose 5′-triphosphonucleotides are the best substrates.

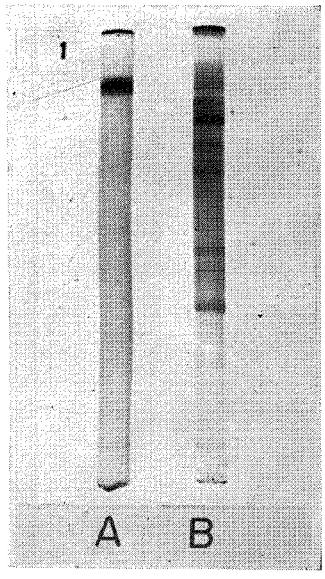

Fig. 1. Disc gel electrophoresis patterns of purified nucleotidase and crude cell wall extract. (A) purified nucleotidase, (B) crude cell wall extract.

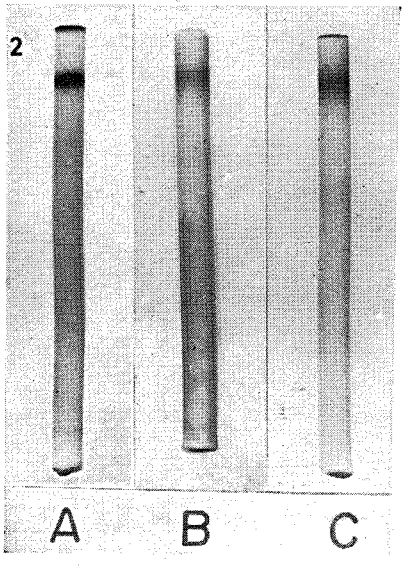

Fig. 2. Electrophoretic analysis of the purified nucleotidase active against 3′-AMP and 5′-AMP. (A) protein band for purified nucleotidase (B) Pi liberation band of the purified nucleotidase active against 3′-AMP, (C) Pi liberation band of the purified nucleotidase active against 5′-AMP.

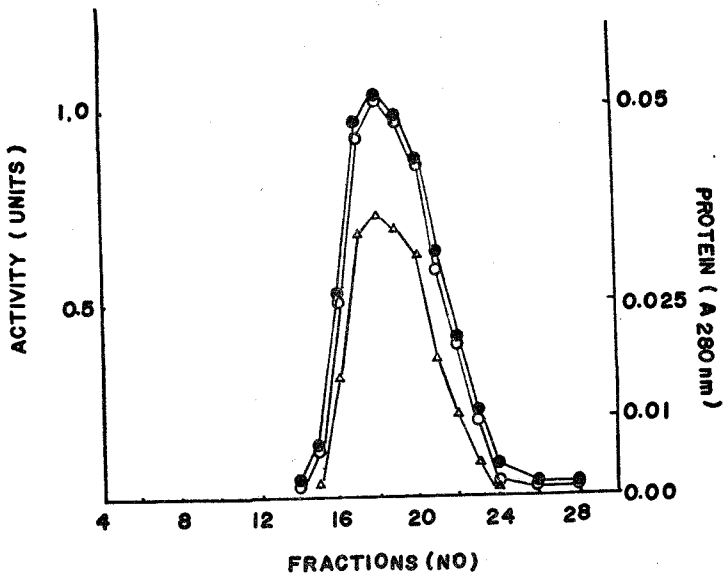

Fig. 3. Sephadex G-100 chromatography of the purified nucleotidase and its activity against 3′-AMP and 5′-AMP. ○—○—○=3′-AMP, ●—●—●=5′-AMP, △—△—△ =protein.

Table 3. *Substrate specificity of purified nucleotidase*

Incubation was carried out at 37° for 10 min. with 20 μg of the purified nucleotidase in a volume of 1.0 ml containing 5×10^{-3}M of subtrate and 5×10^{-2}M tris-buffer, at pH 7.5.

Subtrate	Relative activity (%)
ATP	100
CTP	125
GTP	120
ADP	109
dGMP	48
5′-AMP	73
3′-AMP	69
3′-UMP	65
3′-CMP	72

2. *Stability*

The nucleotidase is stable for several months at −5°. There is no lose of activity after 1 week at 4° to 6°. At room temperature it loses about 10% of activity after 24 hrs. The enzyme is stable during concentraction by freezing lyophylizer. It lose 80% of activity when concentration by ultrafiltration at room temperature, but stable at 4°. The enzyme activity is not affected by

EDTA. It loses about 50% of activity in 6 M urea after 10 min incubation at room temperature. Reducing agents such as 2-mercaptoethanol, glutathione, cysteine or ascobic acid increases its activity to about 20%. As shown in Fig. 4. the enzyme loses its 80% activity when it was incubated at 70°C for 10 min. before assay.

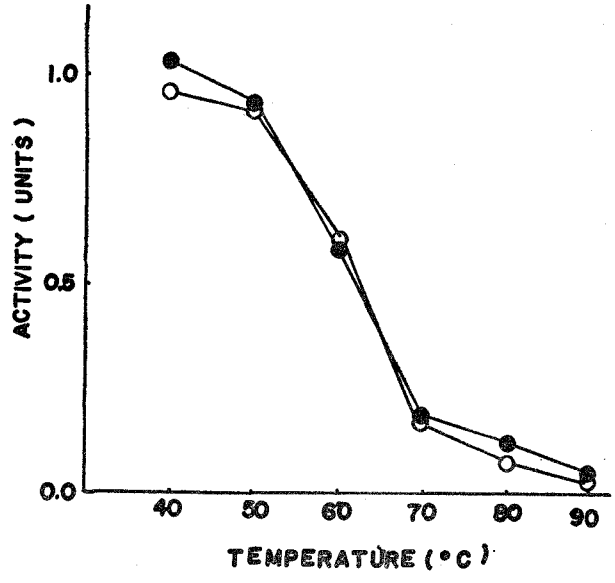

Fig. 4. Effect of the temperature on the stability of purified nucleotidase active against 3'-AMP and 5'AMP. ○—○—○=3'AMP, ●—●—●= 5'-AMP.

3. *Effect of cation on the enzyme activity*

The effect of various concentrations of cations on enzyme activity was tested. As shown in Fig. 5. Na^+, K^+, Ca^{++}, Mn^{++}, Mg^{++} at concentrations of 1×10^{-4}—5×10^{-3} M does not effect on enzyme activity. However Fe^{++} at 5×10^{-4} M inhibites 29% enzyme activity, increasing concentration of Fe^{++} does not show any proportionally inhibitory effect. Zn^{++} at concentration of 5×10^{-3} M inhibites 70% enzyme activity and Co^{++} at same concentration inhibites 30%.

4. *pH optimum*

The effect of pH on nucleotidase activity was investigated. The pH range used was between 4 and 9. As shown in Fig. 6, two pH optima at 5 and 7.5 were obtained.

5. *Temperature optimum*

The reaction mixtures were placed in test tubes and incubated in various temperatures from 10° to 80°C. As shown in Fig. 7. optimum temperature is at 65°C.

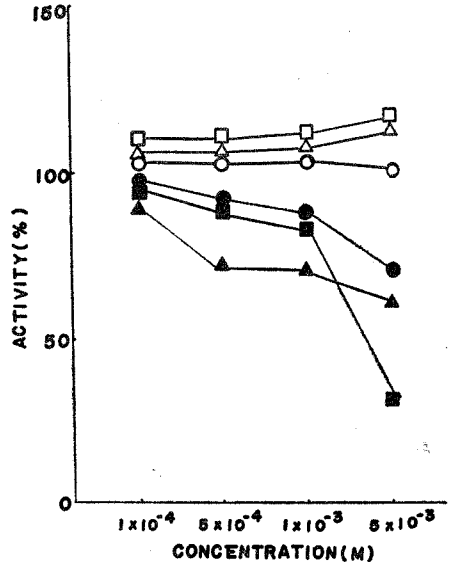

Fig. 5. Effect of various cations on the activity of purified nucleotidase against ATP. □—□—□=Ca^{++}, △—△—△=Mg^{++}, ○—○—○=Mn^{++}, Na^{+}, K^{+}, ●—●—●=Co^{++}, ■—■—■=Zn^{++}, ▲—▲—▲=Fe^{++}.

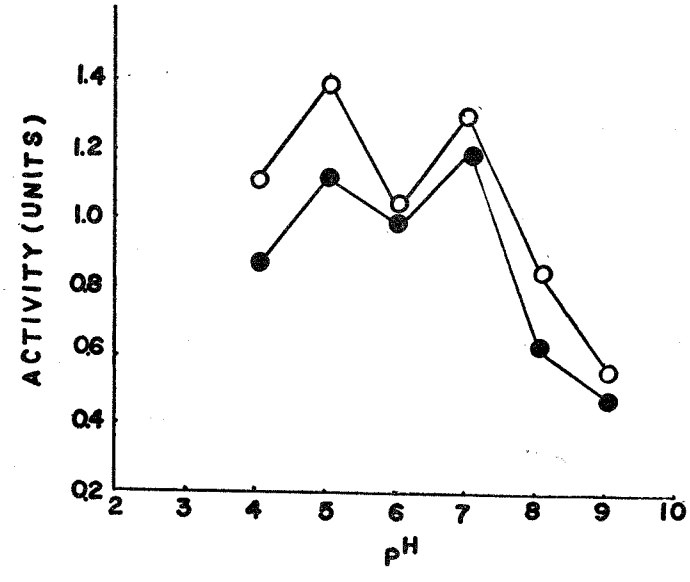

Fig. 6. Effect of pH on the purified nucleotidase active against 3′-AMP, and 5′-AMP. The reaction mixtures were adjusted with 0.05 M citrate buffer for ranges between pH 4 to 6.0; with tris-Cl buffer for pH 7 to 9. ●—●—●=3′-AMP, ○—○—○=5′-AMP.

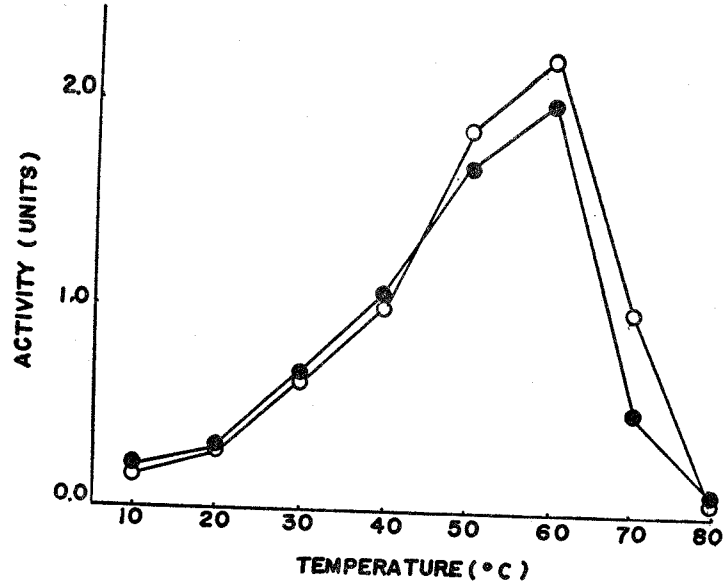

Fig. 7. Effect of the temperature on the nucleotidase active against 3′-AMP and 5′-AMP. ○—○—○=3′-AMP, ●—●—●=5′-AMP.

Evidences for the non-specific activity of nucleotidase against 5′-nucleotide and 3′-nucleotide

The most important properties of the enzyme is its non-specific activity against two nucleotides with different configuration. Several lines of evidence are shown in the following experiments.

Purified enzyme was applied on polyacryamide gel. After electrophoresis the gels were respectively incubated in the solutions containing 5×10^{-3}M 3′-AMP or 5′-AMP. After 30-minute incubation, the liberation of Pi on the gel was detected by the method described previously. A blue band for Pi liberation could be observed. As shown in Fig. 2, this enzyme reacts with both 5′-AMP and 3′-AMP.

The enzyme activities active against 3′-AMP or 5′-AMP were also measured on the fractions of Sephadex G-100 column chromatography. As shown in

Table 4. *Effect of metals on the activity of the purified nucleotidase active against 5′-AMP and 3′-AMP*

Substrate	Relative activity (%)				
	no addition	$+Ca^{++}$	$+Mg^{++}$	$+Co^{++}$	$+Fe^{++}$
5′-AMP	100	122	110	96	45
3′-AMP	100	120	110	90	51

The concentration of cation added is 5×10^{-3}M.

Fig. 3, the positions of the enzyme activity against 5′–AMP and enzyme activity against 3′–AMP were coincident.

Since the enzyme possesses its characteristic pH optima, temperature optimum, stability to heating and the effect of cations. These characteristic properties were also examined on the enzyme active against 3′–AMP or 5′–AMP. As shown in Fig. 4, 6, 7, and Table 4. No matter 5′–AMP or 3′–AMP was used as substrate the reaction patterns were identical.

Discussion

Although several nucleotidase in bacterial system have been studied, however the location of the enzymes was not clarified, (Neu and Wang, 1954, Swertz *et al.* 1958, Heman and Wright 1959, Momose *et al.*, 1964, Anraku, 1964). Neu and Heppel (1964) first described that 5′–nucleotidase and 3′–nucleotidase of *Escherichia coli* is located at the cell surface. Later, cell surface location of *E. coli* 5′–nucleotidase was conclusively proved by Electron Microscopy (Nisonson *et al.*, 1969). From the release of the nucleotidase by the mild treatment of EDTA or lysozyme from *X. oryzae* cells, the nucleotidase of *X. oryzae* seems located on the surface of cell wall.

In most reports nucleootidase has been active in the absence of cations. Addition of cation stimulated the enzyme activities. The degree of cation stimulation varies from less than double to about 10 fold. A 5′–nucleotidase of *Clostridium sticklandi* has been reported to be stimulated by Fe^{++} (Herman and Wright, 1959). And the activities of a 5′–nucleotidase and a 3′–nucleotidase of *Escherichia coli* are stimulated by Co^{++} (Neu 1967, 1968). The activity of nucleotidase isolated from *X. oryzae* is not stimulated by any cations but inhibited by Zn^{++}, Co^{++}, and Fe^{++}. From this aspect *X. oryzae* nucleotidase is different from other nucleotidases reported.

The other unusual property of *X. oryzae* nucleotidase is its substrate specificity. The enzyme is active against both 3′–nucleotide and 5′–nucleotide. The purity of the enzyme was confirmed by disc gel electrophoresis and molecular sieve chromatography. The results obtained showed it is one protein enzyme. The comparison was made on the effect of pH or temperature on the 5′–nucelotidase and the 3′–nuleotidase, it is seen that both activities run parallel depending on the change of the pH or the temperature (Fig. 6, 7). The inactivation by heat of both enzyme activities over the same period of time are also parallel (Fig. 4). Furthermore both enzyme activities are inhibited by Zn^{++}, Co^{++} and Fe^{++}, in the same way (Fig. 5). These results strongly suggest that both enzyme activities are associated with the same protein.

The physiological role of this enzyme has not been studied. Neu (1967) suggested that the role of surface located nucleotidase *in vivo* is the hydrolysis

of nucleotides which exist in the periplasmic space however, the nucleotidases of *Escherichia coli* require cobalt for its full activities. It was suspected that whether the concentration of cobalt was sufficient in reaction site to allow these activities to take place. *X. oryzae* nucleotidase possesses a broad substrate specificity, pH optimum and independent of cations. It appears that the enzyme is more favor for the utilization of nucleotides.

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Xanthomonas oryzae 細胞壁上的一種同時能分解 5′-和3′-核苷酸的核苷酸分解酵素

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自細菌所分離到的核苷酸分解酵素，其特異性一般很高，5′-核苷酸分解酵素祇能分解5′-核苷酸而 3′-核苷酸分解酵素祇能分解 3′-核苷酸，自水稻白葉枯病病原細菌細胞壁上可以分離到一種可以分解 3′-和 5′-核苷酸的核苷酸分解酵素。此酵素可經 DEAE-Cellulose 及 gel filtration 純化。所純化之蛋白質在 disc gel 上呈單一的 band。

純化的核苷酸分解酵素，其最適 pH 值爲 5.0 和 7.5，最適之反應溫度爲 65°C。單價的鈉，鉀和二價的鈣，鎂，錳等離子，在 1×10^{-4}M 到 5×10^{-3}M 的濃度時對酵素的活性沒有影響，二價的鐵，鈷及鋅在 5×10^{-3}M 濃度時對酵素活性有抑制作用。

根據酵素在 disc gel 中對5′-和 3′-AMP 的活性，在 gel filtration chromatography 中酵素蛋白的位置跟其分解 5′-和 3′-AMP 的位置，pH 值，溫度和不同離子對酵素分解5′-和 3′-AMP 的影響，以及溫度對酵素的隱定性等證明此酵素同時具有分解 5′-和 3′-核苷酸的能力。