Kor. J. Appl. Microbiol. Biotechnol.
Vol. 20, No. 1, 40-45 (1992)

Characterization of Acidic Nucleotidase from *Aspergillus niger*

Kim, Gi-Nahm and In-Shik Park
Department of Food Science and Nutrition, Dong-A University, Pusan 604-714, Korea

*Aspergillus niger*의 Acidic Nucleotidase의 특성

김기남 · 박인식*
동아대학교 식품영양학과

Abstract — Acidic nucleotidase from *Aspergillus niger* has been partially purified by Sepharose CL-6B gel filtration and DEAE-Sephacel ion exchange chromatography. The optimum pH and temperature for the enzyme reaction with 5'-AMP or 3'-AMP as a substrate were 4.5 and 55℃, respectively. However, the optimum temperature became 70℃ when *p*-nitrophenyl phosphate was used as a substrate. The enzyme was stable at acidic pH. The enzyme activity was not affected by addition of various nucleotides, nucleosides and inorganic phosphates. Ferric, aluminium, vanadate and molybdate ions inhibited the enzyme activity dramatically. In kinetic studies, K_m values for 3'-AMP, 5'-AMP and *p*-nitrophenyl phosphate were 1.39 mM, 1.5 mM and 5.77 mM, respectively. The substrate efficiency (V_{max}/K_m) shows 3'-AMP is the prefered substrate for the enzyme among tested substrates.

Nucleotidase can hydrolyze nucleotide into nucleoside and inorganic phosphate. The hydrolyzed nucleoside can be transformed inoto nucleotide by specific kinase. Therefore, nucleotidase and kinase form a substrate cycle (1). The two enzyme activities are important to determine the anabolic or catabolic state of cell (2).

Several studies have been performed on phosphatases of *Aspergillus niger* (3-6). The phosphatases are grouped as acidic or alkaline phosphatase depending on pH optima. In addition to *p*-nitrophenyl phosphate, the phosphatase can hydrolyze nucleotides, sugar phosphates and inorganic polyphosphates. The fungal phosphatase has a preference toward *p*-nitrophenyl phosphate as a substrate. During studies on culture of *Aspergillus niger*, an acidic nucleotidase has been identified, which shows slight different properties compared to phosphatase from the fungus. It shows higher substrate efficiency (V_{max}/K_m) toward 3'-AMP. This paper describes the partial purification and properties of acidic nucleotidase from *Aspergillus niger*.

Key words: Acidic nucleotidase, *Aspergillus niger*
***Corresponding author**

Materials and Methods

Materials

5'-AMP, 3'-AMP, *p*-nitrophenyl phosphate, Na-phytate, β-glycerophosphate, α-D-glucose-1-phosphate, Na-metavanadate, and *p*-methylaminophenol were obtained from Sigma Chemical Co. DEAE-Sephacel and Sepharose CL-6B were from Pharmacia Fine Chemicals. All other reagents used were of analytical grade.

Microorganism and cultivation

Organism used was *Aspergillus niger* which was isolated from soil (7). The solid medium consisted of wheat bran (25g) and water (25 ml), and cultivation was carried out by addition of seed culture (inoculum size: 1%) to the culture vessel (250 ml Erlemeyer flask) and incubated for 7 days at 35℃.

Wort broth was used in case of liquid culture (8). The medium contained (g/liter) malt extract,

15; peptone, 0.78; maltose, 12.75; dextrin, 2.75; glycerol, 2.35; dipotassium hydrogen phosphate, 1.0; and ammonium chloride, 1.0. The final pH was 4.8.

Crude enzyme preparation

250 m*l* of distilled water was added to wheat bran koji and mixture was stirred with a glass rod to extract the enzyme completely. The mixture was kept at 4℃ overnight and filtered through Toyo No. 2 filter paper. The filtrate was centrifuged at 3,000 rpm for 30 min. The supernatant was used as a crude enzyme solution. In case of liquid culture, the culture broth itself was used as a crude enzyme preparation.

Enzyme assay

The reaction mixture consisted of 0.35 m*l* of 0.2 M Na-acetate buffer (pH 4.5), 0.1 m*l* of 10 mM 5'-AMP, 3'-AMP or *p*-nitrophenyl phosphate, and 0.05 m*l* of enzyme. The enzyme reaction was initiated by addition of 0.05 m*l* of enzyme and incubated at 55℃ for 20 min. The liberated inorganic phosphate was measured by the method of Fiske-Sabburow (9) with modification. The released *p*-nitrophenol was measured at 410 nm. The amount of released inorganic phosphate was calculated by using phosphate standard curve with potassium dihydrogen phosphate as a standard. The molar extincition coefficient of *p*-nitrophenol is 19.5 $mM^{-1} cm^{-1}$ at 410 nm (10), which was used to calculate the amount of released *p*-nitrophenol. One unit of enzyme activity is defined as the amounts in μmole of released inorganic phosphate or *p*-nitrophenol per min under the defined conditions.

Determination of protein concentration

Protein concentration was measured by the method of Lowry *et al.* (11) using bovine serum albumin as a standard protein.

Results and Discussion

Enzyme purification

7.5 m*l* of crude enzyme was loaded to Sepharose CL-6B column (2.5×58 cm), which was equilibrated with buffer (10 mM Tris-HCl buffer, pH 7.0). Elution was carried out with same buffer and the elute was collected in 5.7 m*l* fraction (Fig. 1). The active fractions (17.1 m*l*) after gel filtration were collected, and loaded to DEAE-Sephacel ion exchange chromatography column (2.5×15 cm), which was preequilibrated with the buffer. The column was washed with the same buffer and eluted with 600 m*l* of linear gradient of 0~0.5 M NaCl in the buffer. Each fraction of 5.7 m*l* was collected (Fig. 2). The purification procedures of the enzyme is summerized in Table 1. The enzyme was partially purified about 10.3-fold with an overall yield of 54.6%. The elution pattern and purification fold are same when 3'-AMP or *p*-nitrophenyl phosphate was used as a substrate.

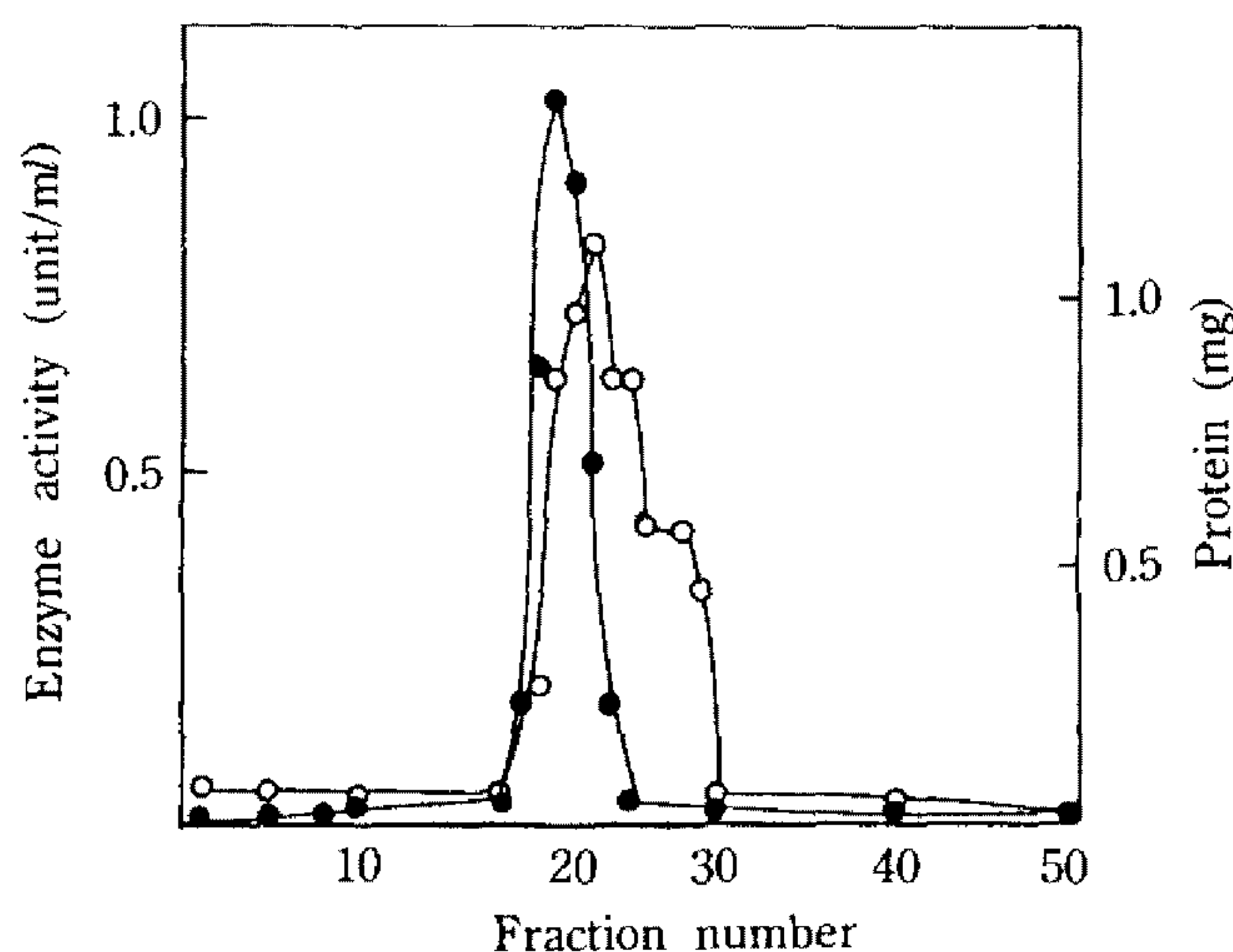

Fig. 1. Sepharose CL-6B gel filtration of acidic nucleotidase.
5'-AMP was used as a substrate.
Enzyme activity, (●); Protein (○).

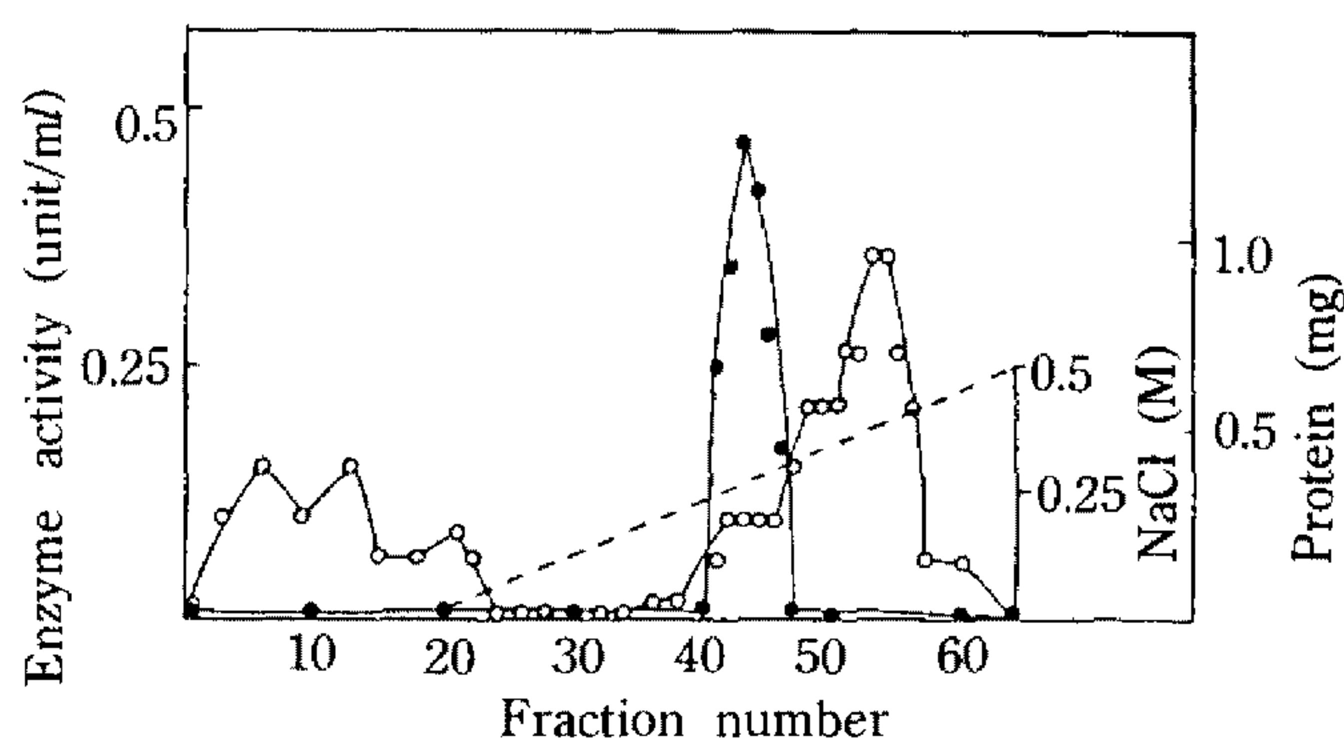

Fig. 2. DEAE-Sephacel ion exchange chromatography of acidic nucleotidase.
The active fractions after Sepharose CL-6B column were pooled, and loaded to DEAE-Sephacel ion exchange column. 5'-AMP was used as a substrate.
Enzyme activity, (●); Protein (○); NaCl conc. (----).

Table 1. Summary of enzyme purification procedure

Step	Volume (ml)	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude extract	7.5	3.48	15.7	0.22	100.0	1.0
Sepharose CL-6B gel filtration	17.1	2.6	4.55	0.57	74.7	2.59
DEAE-Sephacel ion exchange chromatography	34.2	1.9	0.84	2.26	54.6	10.3

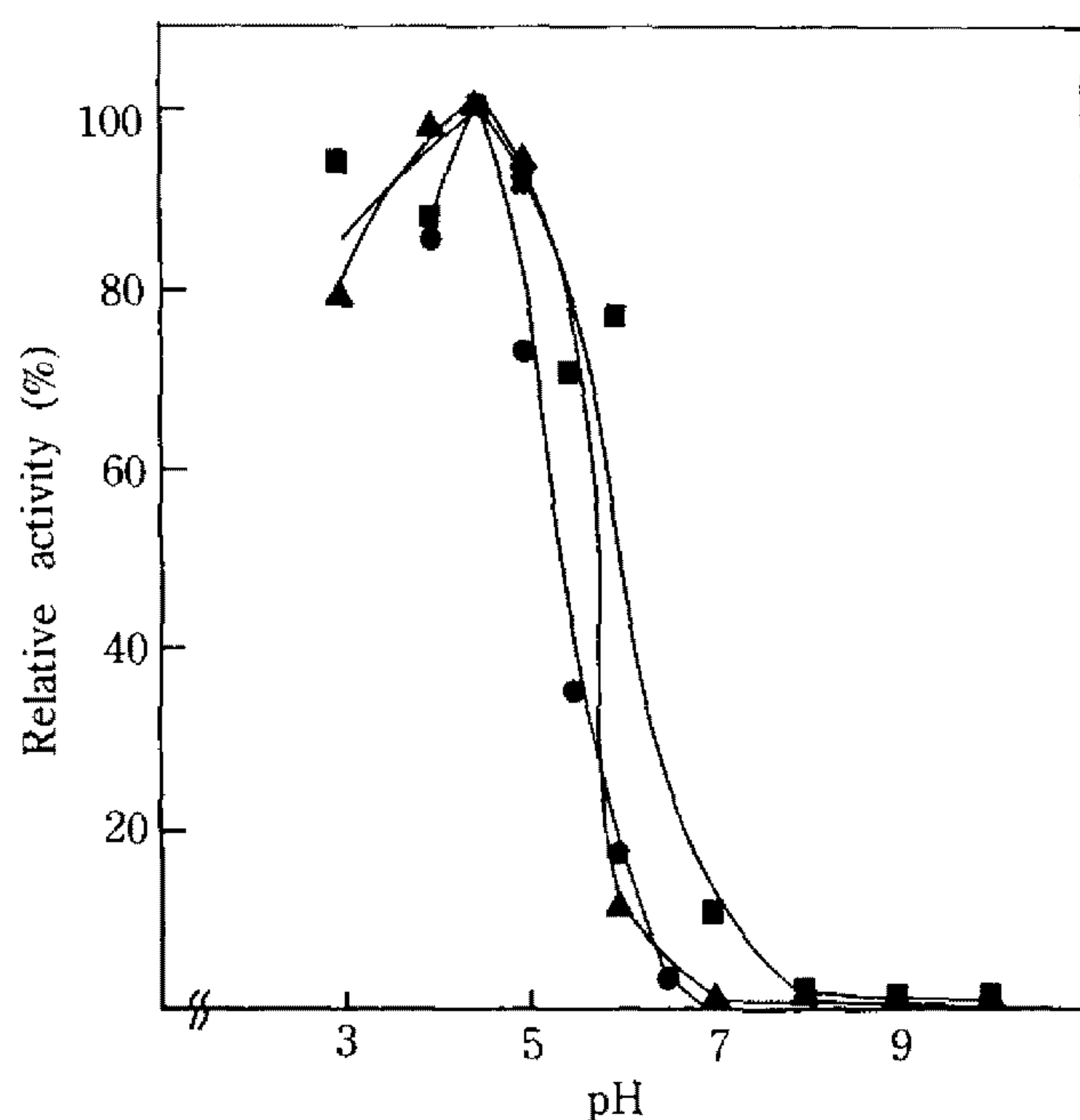

Fig. 3. Effect of pH on activity of acidic nucleotidase. The assay conditions were same as described in Methods except pH of the reaction. The buffers (0.14 M) used were as follow: pH 3.0~6.5, Na-acetate; pH 7.0~8.0, Tris/HCl; pH 9.0~10.0, boric acid.
3'-AMP (■), 5'-AMP (●), *p*-nitrophenyl phosphate (▲).

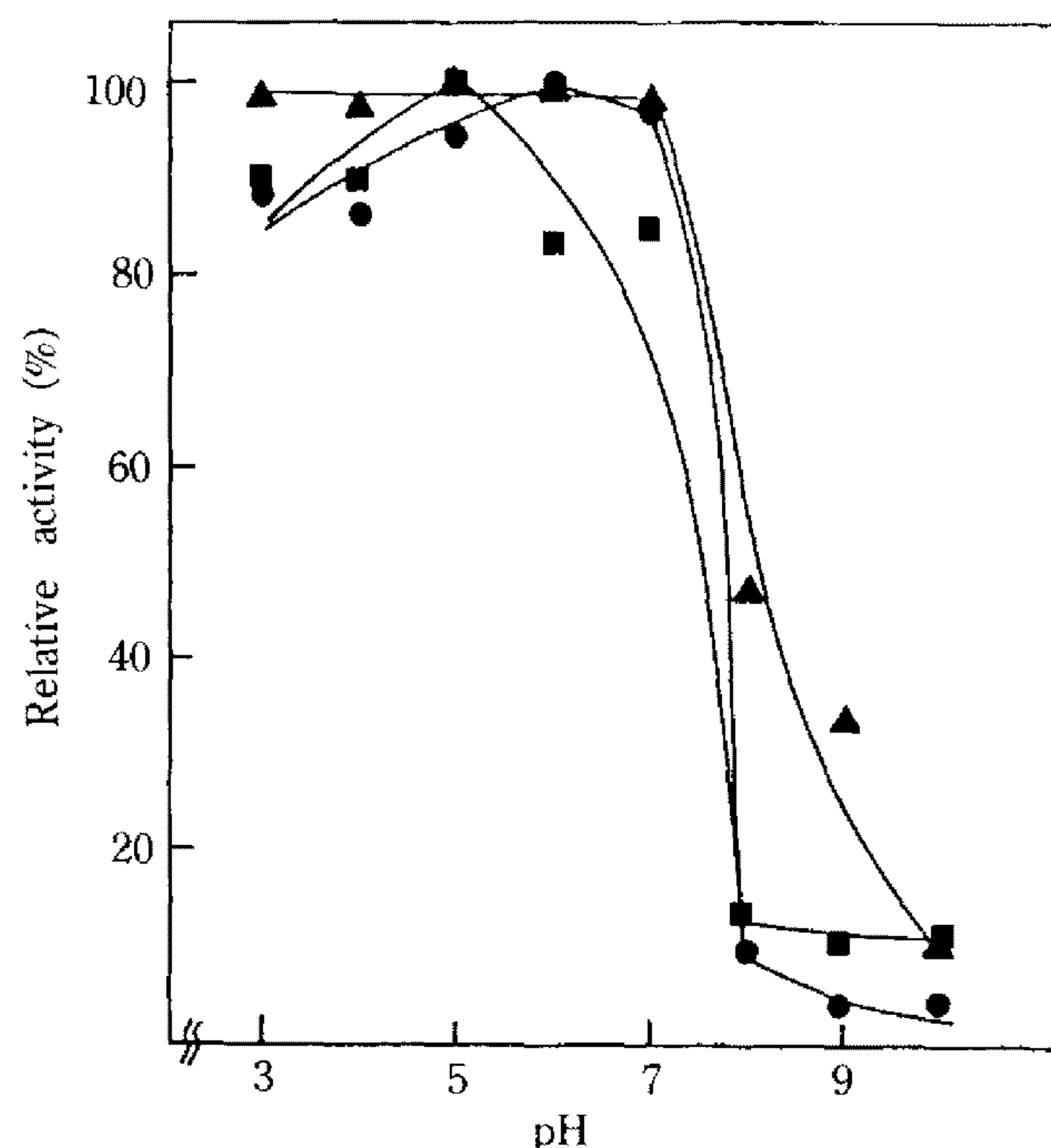

Fig. 4. Effect of pH on stability of acidic nucleotidase. The enzymes of various pH values were preincubated for 18 hrs at 30℃, and the residual activities were assayed under the standard assay conditions.
3'-AMP (■), 5'-AMP (●), *p*-nitrophenyl phosphate (▲).

Effect of pH on activity and stability

The effect of pH on activity of the enzyme was examined over pH 3.0 to 9.0. Regardless of substrates used the enzyme showed maximal activity at pH 4.5 as shown in Fig. 3. Therefore, it seems to be acidic nucleotidase. The pH stability of the enzyme was measured by standard assay conditions after preincubation of the enzyme at a given pH values at 30℃ for 18 hrs. The enzyme was stable at acidic pH, and the stability of the enzyme was decreased sharply at alkaline pH. As shown in Fig. 4, the profile for activity and stability of the enzyme is similar when 3'-AMP, 5'-AMP or *p*-nitrophenyl-phosphate was used as a substrate.

Effect of temperature

The effect of temperature on activity of the enzyme was investigated at various temperatures ranged from 30℃ to 80℃ (Fig. 5). The enzyme exhibited the maximal activity at 55℃ when assayed using 5'-AMP or 3'-AMP as a substrate. But optimal temperature became 70℃ when *p*-nitrophenyl phosphate was used as a substrate.

The activation energy of the enzyme was measured by replotting Fig. 5 as log velocity *versus* reciprocal absolute temperature. From the slope obtained, the activation energy of the enzyme was calculated to 6.95 kcal/mole for 5'-AMP, 4.76 kcal/mole

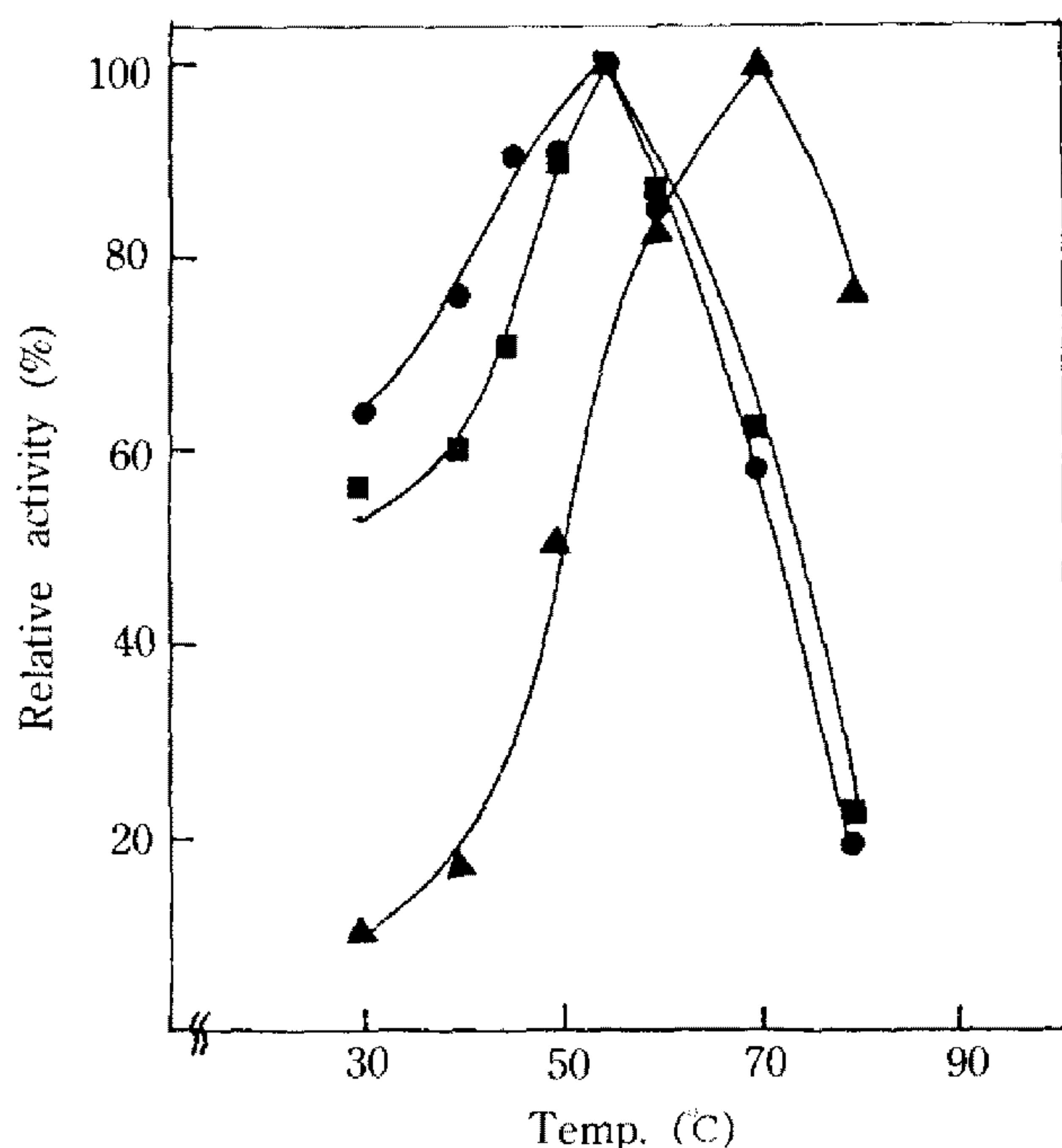

Fig. 5. Effect of reaction temperature on acidic nucleotidase.
The assay conditions were as described in Methods except reaction temperature.
3′-AMP (■), 5′-AMP (●), *p*-nitrophenyl phosphate (▲).

Table 2. Substrate specificity of the enzyme

Substrate (2 mM)	Enzyme activity (Unit/ml)
ATP	0.09
5′-ADP	0.22
5′-AMP	0.62
3′-AMP	0.83
PNPP	0.12
Phytic acid	0.04
β-Glycerophosphate	0.24
α-D-Glucose-1-phosphate	0.21

for 3′-AMP as a substrate. When *p*-nitrophenyl phosphate was used as a substrate, the activation energy became 11.82 kcal/mole. It is similar to that of wheat germ acidic phosphatase, which is reported to be 12.3 kcal/mole at pH 5 (12). This result shows the 3′-AMP is the prefered substrate among them.

Substrate specificity of the enzyme

The relative activities of the partially purified acidic nucleotidase to a number of phosphorylated compounds are summarized in Table 2. The enzyme found to be non-specific, hydrolyzing synthetic substrate, sugar phosphates, nucleotides. However, it is most active against 3′-AMP. The substrate specificity of the purified enzyme is completely different from recently reported 4-nitrophenylphosphatase from *Aspergillus niger* (13), but it is similar to 5′(3′)-nucleotidase from human placenta (2).

Table 3. Kinetic parameters using variable substrates

	K_m (mM)	V_{max} (Unit/ml)	V_{max}/K_m
3′-AMP	1.39	1.15	0.83
5′-AMP	1.5	0.83	0.55
PNPP	5.77	0.66	0.11

Table 4. Effect of metal ions on activity of acidic nucleotidase

Metal ions (20 mM)	Relative activity (%)
Control	100
$NiCl_2$	97.7
$AlCl_3$	50.0
$CaCl_2$	97.7
$MnCl_2$	97.7
$CuCl_2$	90.9
$ZnCl_2$	100.0
$BaCl_2$	72.7
$HgCl_2$	95.4
$CoCl_2$	97.7
$FeCl_3$	6.8
$MgCl_2$	100.0
EDTA	102.2
EDTA + $MnCl_2$	93.1
$NaVO_3$ (1 mM)	46.0
$(NH_4)_6Mo_7O_{24}$ (0.1 mM)	0.0

3′-AMP was used as a substrate.

Kinetic properties

Table 3 shows the effect of varying substrate on kinetic parameter of acidic nucleotidase. From double reciprocal plots for 3′-AMP, 5′-AMP and *p*-nitrophenyl phosphate, the K_m and V_{max} values of the enzyme for the substrates were measured at pH 4.5 and 55℃. When *p*-nitrophenyl phosphate was used as a substrate, the K_m values of wheat germ acidic phosphatase, 4-nitrophenyl phosphatase of *Aspergillus niger* and 5′(3′)-nucleotidase of human placenta were 0.99 mM (12), 37 μM (13), and 1.3 mM (2), re-

Table 5. Effect of various nucleotides, nucleosides, bases and inorganic polyphosphates on the activity of acidic nucleotidase

	Relative activity (%)	
	0.1 mM	0.5 mM
Control	100	100
ATP	109	100
5′-ADP	109	96
Adenosine	97	100
Adenine	102	100
Ribose	93	104
Inosine	97	104
P_i	115	96
PP_i	105	108
PPP_i	89	104

3′-AMP was used as a substrate.

spectively. The K_m value of the purified acidic nucleotidase with *p*-nitrophenyl phosphate exhibited higher K_m value than those of other enzymes. The enzyme shows the highest substrate efficiency (V_{max}/K_m) toward 3′-AMP, which demonstrates that 3′-AMP is the prefered substrate.

Effect of metal ions

The effect of various metal ions on the enzyme activity was determined by adding metal ions to the standard assay mxiture (Table 4). The enzyme activity seems not to be affected by divalent cations and EDTA, but inhibited by metal ions such as ferric, aluminium, vanadate and molybdate ions. The enzyme was strongly inhibited by molybdate ion, which is regarded as transition state analog.

Regulation of acidic nucleotidase

Many enzymes of nucleotide metabolism are subject to activation or inhibition by nucleotides, nucleosides or inorganic phosphates. Therefore, effect of various nucleotides, nucleosides, bases and inorganic polyphosphates on the activity of acidic nucleotidase was investigated. As shown in Table 5, the enzyme activity is not inhibited or activated by various tested compounds. Therefore, the acidic nucleotidase from *Aspergillus niger* is not regulated by nucleotides, nucleosides, bases and inorganic polyphosphates. Therefore, it seems to be no allosteric effectors for the enzyme. This result is similar to that of 5′(3′)-nucleotidase from human placenta (2), but different from 4-nitrophenyl phosphate from *Aspergillus niger* (12).

요 약

*Aspergillus niger*로부터 acidic nucleotidase를 Sepharose CL-6B gel 여과와 DEAE-Sephacel 이온교환수지를 이용하여 부분정제하였다. 5′-AMP와 3′-AMP를 기질로 사용했을 경우에 효소의 최적 pH는 4.5, 그리고 최적온도는 55℃였다. 그러나, *p*-nitrophenyl phosphate를 기질로 사용했을 경우에는 최적 pH는 변화가 없었으나, 최적온도는 70℃였다. 효소의 활성화에너지는 3′-AMP, 5′-AMP 그리고 *p*-nitrophenyl phosphate를 기질로 사용했을 경우에 각각 4.76 kcal/mole, 6.95 kcal/mole 그리고 11.82 kcal/mole였다. 본 효소는 산성 pH에서 안정하였다. 효소의 활성은 nucleotide, nucleoside 및 무기 인산을 첨가하였을 경우에 변화가 거의 없었다. Ferric, aluminium, vanadate 그리고 molybdate 이온은 효소의 활성을 저해하였다. 효소는 3′-AMP, 5′-AMP 및 *p*-nitrophenyl phosphate에 대해서 K_m값이 각각 1.39 mM, 1.5 mM 및 5.77 mM였다. 본 효소의 substrate efficiency (V_{max}/K_m)는 3′-AMP가 가장 높았다.

Acknowledgement

This report is supported by a grant from Korea Research Foundation (1991).

References

1. Richard, P. 1988. Interactions between deoxyribonucleotide and DNA synthesis. *Ann. Rev. Biochem.* **57**: 349-379.
2. Högland, L. and P. Richard. 1990. Cytoplasmic 5′(3′)-nucleotidase from human placenta. *J. Biol. Chem.* **265**: 6589-6595.
3. Komato, T. 1975. Formation of multiple forms of acid and alkaline phosphatases in relation to culture age of *Aspergillus niger*. *Plant. Cell Physiol.* **16**: 643-657.
4. Nagasaki, S. 1968. Physiological aspects of various enzyme activities in relation to culture age of *Aspergillus niger* mycelia. *J. Gen. Appl. Microbiol.*

14: 147-161.

5. Nagasaki, S. 1968. Cytological and physiological studies on phosphatases in developing cultures of *Aspergillus niger*. *J. Gen. Appl. Microbiol.* **14**: 263-277.
6. Rokosu, A. and O. Uadia. 1980. Isolation, purification and partial characterization of alkaline phosphatase from *Aspergillus niger*. *Int. J. Biochem.* **11**: 541-544.
7. Hong, J.M., K.A. Lee, J. Kim, and I. Park. 1990. Production and properties of invertase from *Aspergillus niger*. *J. Kor. Soc. Food Nutr.* **19**: 577-582.
8. Harrigan, W.F. and E.M. Margaret. 1976. Laboratory Methods in Food and Dairy Microbiology, p. 377, Academic Press, London.
9. Fiske, C.H. and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**: 375-400.
10. Hayakawa, T., F. Okada, M. Tsutsui, N. Sato, and I. Igaue. 1991. Effect of phytate on the hydrolysis of *p*-nitrophenyl phosphate with phosphatase from various sources. *Agric. Biol. Chem.* **55**: 651-657.
11. Lowry, O.H., H.J. Roserbrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
12. Waymack, P.P. and R.L. Van Etten. 1991. Isolation and characterization of a homogeneous isozyme of wheat germ acid phosphatase. *Arch. Biochem. Biophys.* **288**: 621-633.
13. Versaw, W.K., M.A. Bevins, and J. Markwell. 1991. Purification and properties of a 4-nitrophenylphosphatase from *Aspergillus niger*. *Arch. Biochem. Biophys.* **287**: 85-90.

(Received November 18, 1991)