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# Characterization of Acid Phosphatase from Carrots

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**Abstract**

**Acid phosphatase (EC 3.1.3.2) from carrots was partially purified by ammonium sulfate fractionation (30% ~80%), Sephacryl S-200 gel filtration, CM-Sepharose CL-6B and DEAE-Sephacel ion exchange chromatography. The optimum pH and temperature of acid phosphatase from carrots were pH 5.5 and 55°C, respectively. The enzyme was most stable at pH 6.0 and relatively unstable below pH 4.0. The activation energy of the enzyme was determined to be 10.6kcal/mole. The enzyme utilized *p*-nitrophenyl phosphate as a substrate among tested possible substrates, whereas it hydrolyzed 5'-IMP and 5'-GMP poorly. The Michaelis-Menten constant ($K_m$) of the enzyme with *p*-nitrophenyl phosphate as a substrate was identified as 0.55mM. Among tested metal ions and inhibitors, $Al^{+++}$, $Zn^{++}$, $Cu^{++}$, fluoride, metavanadate and molybdate ions inhibited the enzyme activity drastically.**

**Key words** : carrot, acid phosphatase, purification, enzyme characterization

## INTRODUCTION

Acid phosphatases can hydrolyze various orthophosphoric monoesters at acidic pH[1]. Acid phosphatases can hydrolyze a group of taste substances in foods, such as 5'-IMP and 5'-GMP, to nucleosides and inorganic phosphates, which cause deterioration in foods[2]. Therefore it would be important to characterize the enzymatic properties of acidic phosphatases from various plant sources which are utilized as plant food materials. So far acidic phosphatase has been reported in Japanese rodishes[2], wheat germ[3], and papayas[4]. Carrots have been used in many food preparations, but no attempt has been made to isolate and characterize the acid phosphatase yet. Therefore, this paper describes properties of partially purified acid phosphatase from carrots.

## MATERIALS AND METHODS

### Materials

5'-AMP, 3'-AMP, 5'-IMP, 5'-GMP, *p*-nitrophenyl phosphate, Na-phytate, *β*-glycerophosphate, Na-metavanadate and *p*-methylaminophenol were obtained from Sigma Chemical Co. DEAE-Sephacel, CM-Sepharose CL-6B and Sephacryl S-200 were purchased from Pharmacia Fine Chemicals. All other reagents were of analytical grade.

### Crude enzyme preparation

A carrot (100g) was cut into small pieces, and was homogenated with 200ml of 50mM Tris-HCl buffer (pH 7.0) containing 0.1M NaCl, in mixer for 3min, and then in bead beater for 3min. The crude homogenate was centrifuged at 10,000rpm for 30min and the supernatant was used as a crude enzyme preparation.

### Enzyme assay

The reaction mixture consisted of 0.35ml of 0.2M Na-acetate buffer (pH 5.5), 0.1ml of 10mM various nucleotides or *p*-nitrophenyl phosphate, and 0.05ml of enzyme. The enzyme reaction was initiated by addition of 0.05ml of enzyme and incubated at 55°C for 15min. The liberated inorganic phosphate was measured by the method of Fiske-Subbarow[5] with

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modification. The released *p*-nitrophenol was measured at 410nm. The amount of released inorganic phosphate was calculated by using phosphate standard curve with potassium dihydrogen phosphate as a standard. The molar extinction coefficient of *p*-nitrophenol is $19.5mM^{-1}cm^{-1}$ at 410nm[6], which was used to calculate the amount of released *p*-nitrophenol. One unit of enzyme activity is defined as the amounts in $\mu$mole of released inorganic phosphate or *p*-nitrophenol per min under the defined conditions.

### Determination of protein concentration

Protein concentration was measured by the methods of Lowry *et al.*[7], using bovine serum albumin as a standard protein.

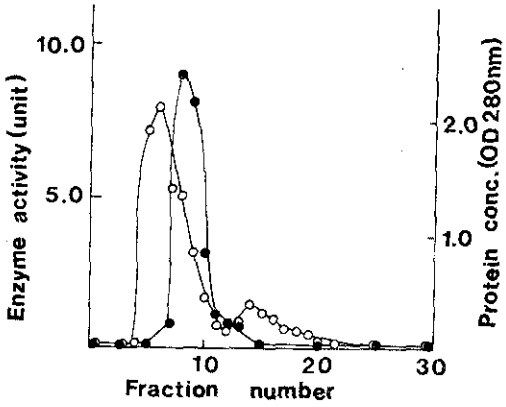


**Fig. 1. Sephacryl S-200 gel filtration of acid phosphatase from carrots.**
Enzyme activity (●—●), Protein(○—○)

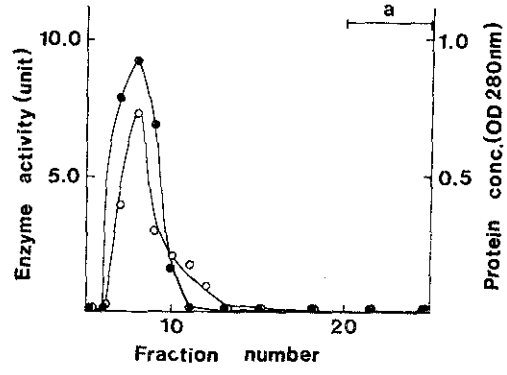


**Fig. 2. CM-Sepharose CL-6B ion exchange chromatography of acid phosphatase from carrots.**
Enzyme activity (●—●), Protein(○—○)
a : 1M NaCl

## RESULTS AND DISCUSSION

### Enzyme purification

200ml of crude enzyme (218.5unit) was initially treated by ammonium sulfate fractionation (30%~80 %), and the precipitate obtained from the fractionation was dissolved in 5ml of 10mM Tris-HCl buffer (pH 7.0). It was loaded to Sephacryl S-200 column (2.5 × 58cm), 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 13. 0ml fraction (Fig. 1). The active fractions (41.1ml) after gel filtration were collected, and loaded to CM-Sepharose CL-6B ion exchange column (2.5 × 10cm), which was equilibrated with the buffer. The column was washed with the buffer, and eluted with 600ml of linear gradient of 0~0.5M NaCl in the buffer. Each fraction of 13.0ml was collected (Fig. 2). The active fractions after CM-Sepharose CL-6B column step were collected (51.0ml), and loaded to DEAE-Sephacel ion exchange column which was preequilibrated with the equilibration buffer. The acid phosphatase activity was eluted mainly in linear gradient of 0~0. 5M NaCl as shown in Fig. 3. The active fractions after DEAE-Sephacel step were collected (117.0ml). The purification procedures of the enzyme is summarized in Table 1 The enzyme was partially purified about 7.1-fold with overall yield of 33.1%. The enzyme after DEAE-Sephacel step was lyophilized for further use.

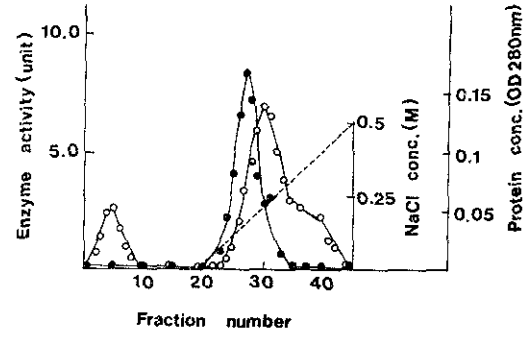


**Fig. 3. DEAE-Sephacel ion exchange chromatography of acid phosphatase from carrots.**
Enzyme activity (●—●), Protein(○—○),
NaCl conc. (- - - - -)

**Table 1. Summary of purification steps**

| Fraction | Total activity (unit) | Total protein (mg) | Specific activity (unit/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude | 218.3 | 225.0 | 0.9 | 100.0 | 1.0 |
| Ammonium sulfate fraction (30%~80%) | 89.1 | 82.4 | 1.1 | 40.8 | 1.2 |
| Sephacryl S-200 | 88.0 | 21.2 | 4.2 | 40.3 | 4.7 |
| CM-Sepharose CL-6B | 81.4 | 18.9 | 4.3 | 37.3 | 4.8 |
| DEAE-Sephacel | 72.2 | 11.3 | 6.4 | 33.1 | 7.1 |

### Effect of pH on activity and stability

The effect of pH on activity of the enzyme was investigated in range of pH 3.0 to 10.0. The enzyme activity was maximal at pH 5.5 with *p*-nitrophenyl phosphate as a substrate (Fig. 4). The optimal pH of 5.5 was slightly lower than those of papaya and Japanese radish phosphatase which were reported as pH 6.0[2,4]. Since there was no enzyme activity at alkaline pH, the phosphatase from the carrot seems to be acid phosphatase. The pH stability of the enzyme was measured by standard assay conditions after preincubation of the enzyme at given pH values at 30° C for 24 hrs. The enzyme was most stable at pH 6.0 as shown in Fig. 5. In case of wheat germ acid phosphatase the pH stability was pH 4~7[3]. The buffers (0.1M) used was as follows : pH 3.0~5.5, Na-acetate ; pH 6.0~8.0, Tris-HCl ; pH 8.5~10.0, Na-borate.

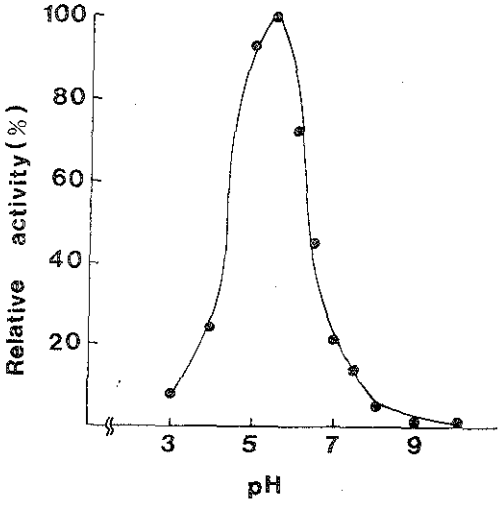


Fig. 4. Effect of pH on activity of acid phospatase from carrots.

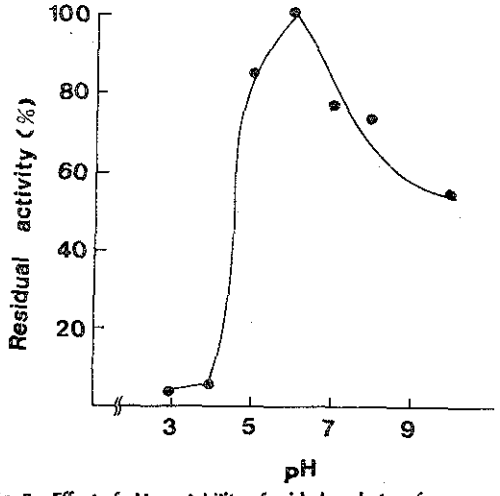


Fig. 5. Effect of pH on stability of acid phosphatase from carrots. (30° C, 24hr incubation)

### Effect of temperature

The effect of temperature on activity of the enzyme was examined at various temperatures ranged from

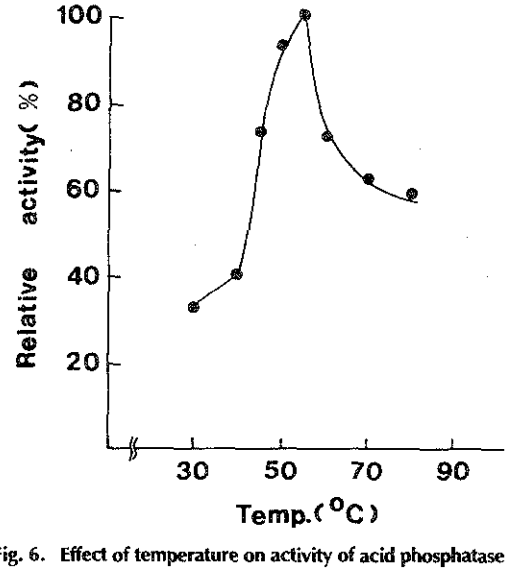


Fig. 6. Effect of temperature on activity of acid phosphatase from carrots.

30° C to 80° C (Fig. 6). The enzyme exhibited maximal activity at 55° C when assayed using *p*-nitrophenyl phosphate as a substrate. The optimal temperature of papaya acid phosphatase was reported to be 37° C[4] and that of Japanese radish enzyme was reported to be 45° C[2]. Therefore the optimum temperature of carrot acid phosphatase was higher than those of papaya and Japanese radish acid phosphatases. The activation energy of the enzyme was measured by replotting Fig. 6 as log velocity *versus* reciprocal absolute temperature. From the slope obtained (Fig. 7), the activation energy of the enzyme was calculated to 10.6 kcal/mole. It is similar to that of wheat germ acid phosphatase, which is reported to be 12.3kcal/mole[3] and that of papaya acid phosphatase, which was reported to be 10.9kcal/mole[4]. The thermoinactivation of the enzyme was determined by assaying residual enzyme activity at standard assay conditions after incubation of the enzyme at various temperatures ranging from 30° C to 80° C for 20min (Fig. 8). The enzyme was relatively stable below 50° C, but it was rapidly inactivated above that temperature.

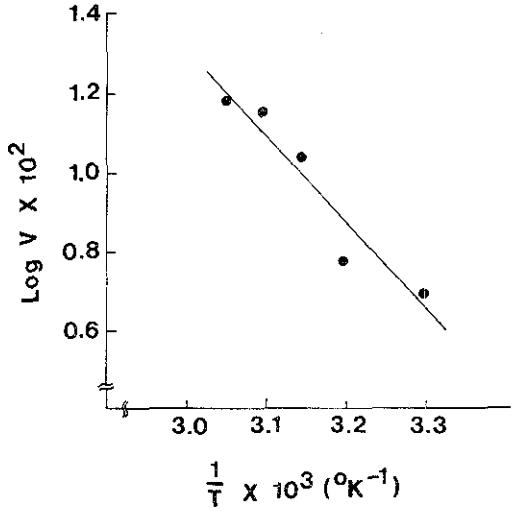


**Fig. 7. Arrhenius plot of acid phosphatase from carrots.**

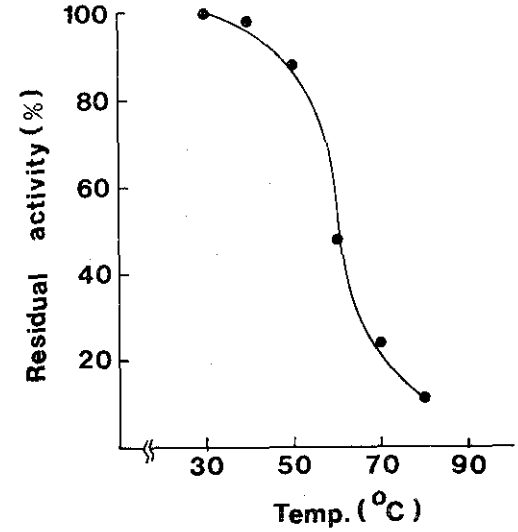


**Fig. 8. Thermoinactivation of acid phosphatase from carrots.**

### Substrate specificity

The relative activities of the partially purified acid phosphatase to a number of phosphorylated compounds are summarized in Table 2. The enzyme from carrot found to be non-specific, hydrolyzing synthetic substrate, sugar phosphates, and nucleotides. However, it was most active against *p*-nitrophenyl phosphate. The enzyme can hydrolyze 5′-IMP and 5′-GMP poorly which are taste compounds in foods.

### Kinetic properties

Fig. 9 exhibits the Lineweaver-Burk plot of carrot acid phosphatase with *p*-nitrophenyl phosphate as a substrate. The Michaelis-Menten constant ($K_m$) was measured by varying *p*-nitrophenyl phosphate concentration from 0.25mM to 3.0mM, and it was determined to be 0.55mM. The $K_m$ values of acid phosphatase from Japanese radish[2] and papaya[4] were 0.31 mM and 1.0mM, respectively. Therefore the $K_m$ value of acid phosphatase from carrot was higher than that of Japanese radish, but lower than that of papaya.

**Table 2. Substrate specificity of acid phosphatase from carrot**

| Substrate (2mM) | Relative activity (%) |
|---|---|
| *p*-Nitrophenyl phosphate | 100.0 |
| 5′-AMP | 5.9 |
| 3′-AMP | 4.7 |
| 5′-ADP | 26.0 |
| 5′-ATP | 33.5 |
| Phytic acid | 0.0 |
| *β*-Glycerophosphate | 32.3 |
| *α*-D-Glucose-1-phosphate | 1.5 |
| 5-GMP | 4.7 |

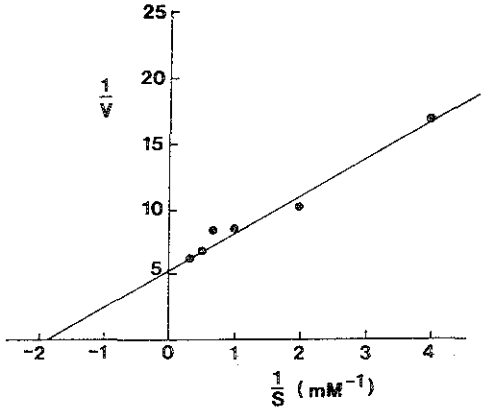


**Fig. 9. Lineweaver-Burk plot of acid phosphatase with *p*-nitrophenyl phosphate as a substrate.**

**Table 3. Effect of metal ions on activity of acid phosphatase from carrots**

| Metal ions (10mM) | Relative activity (%) |
|---|---|
| Control | 100.0 |
| $NiCl_2$ | 91.2 |
| $AlCl_3$ | 5.2 |
| $CaCl_2$ | 45.6 |
| $MnCl_2$ | 66.6 |
| $CuCl_2$ | 17.5 |
| $ZnCl_2$ | 7.0 |
| $BaCl_2$ | 92.9 |
| $CrCl_3$ | 50.8 |
| $HgCl_2$ | 3.5 |
| $CoCl_2$ | 84.2 |
| $MgCl_2$ | 43.8 |

**Table 4. Effect of various inhibitors on activity of acid phosphatase from carrots**

| Inhibitor (1mM) | Relative activity (%) |
|---|---|
| Control | 100.0 |
| NaF | 17.5 |
| Na citrate | 96.5 |
| Na tartarate | 94.7 |
| Na-metavanadate | 3.5 |
| L-phenylalanine | 14.0 |
| Ammonium molybdate | 3.5 |
| EDTA | 84.2 |

### Effect of added metal ions and inhibitors

The effect of various metal ions and various inhibitors on the enzyme activity was determined by adding metal ions and various inhibitors to the standard assay mixture (Table 3, 4). The enzyme activity was drastically inhibited by metal ions such as $Al^{+++}$, $Zn^{++}$ and $Hg^{++}$ ions. The enzyme activity was also inhibited by various compounds such as fluoride, metavanadate, molybdate ions and L-phenylalanine.

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# 당근 Acid Phosphatase의 특성

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## 요 약

당근으로부터 acid phosphatase를 유안염석 (30%~80%), Sephacryl S-200gel 여과, CM-Sepharose CL-6B 양이온 교환수지, 그리고 DEAE-Sephacel 음이온 교환수지를 이용하여 부분정제하였다. *p*-nitrophenyl phosphate를 기질로 사용했을 경우에 최적 pH는 5.5, 그리고 최적온도는 55°C였다. 효소의 활성화에너지는 10.3kcal/mole였다. 효소는 pH 6.0에서 가장 안정하였으며, 50°C이하에서 대체로 안정하였다. 효소는 *p*-nitrophenyl phosphate를 기질로 가장 잘 이용하였으며, 5′-IMP 와 5′-GMP는 기질로 거의 이용하지 못하였다. 효소는 *p*-nitrophenyl phosphate를 기질로 했을 경우에 $K_m$값이 0.55mM 이었다. Aluminium, zinc, mercury 이온은 효소의 활성을 저해하였으며, 또한 fluoride, metavanadate, molybdate 이온과 L-phenylalanine도 효소의 활성을 저해하였다.