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An adenylate deaminase from *Porphyra yezoensis* UEDA[1)]

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5'-AMP deaminase was extracted from *Porphyra yezoensis* UEDA f. *narawaensis* MIURA and partially purified to investigate some enzymatic properties. The enzyme was extremely unstable against heat, and remarkably activated by alkaline earth metal ions, particularly by Ca^{2+}. The deaminase was inhibited not only by Fe^{2+} and Zn^{2+} but also by *p*-mercuribenzoate almost to a similar extent. The Km values for AMP, ADP, ATP and adenosine were 5.3×10^{-5}, 6.6×10^{-5}, 11×10^{-5} and 19×10^{-5} M, respectively. The enzyme was slightly activated by Mn^{2+}, but the effect was influenced by pH of reaction mixture and the activity was conspicuously inhibited on alkaline side.

Key Index Words: *Adenylate deaminase*; *Km value*; *optimal pH*, Porphyra yezoensis; *substrate specificity*; *thermal stability*.

It is well known that the laver contains inosinic acid (IMP), though in a smaller amount than other nucleotides in it, and that the acid constitutes one of the flavors characteristic of laver (SU *et al.* 1962, FUJII *et al.* 1967, OOYAMA *et al.* 1968, NAKAMURA *et al.* 1968, NODA *et al.* 1975). Of these investigations, NAKAMURA *et al.* have found an interesting fact that *Porphyra tenera* contains a fairly high amount of IMP while none of it can be detected in other seaweeds such as *Enteromorpha* as well as several brown algae commonly used as the food in Japan. Thus, the confined occurrence of IMP in the laver seemed to be due to possibly higher activity of 5'-adenylic acid (AMP) deaminase in *P. tenera*.

However, no work has been repoted at that time for algal AMP deaminase except that from *Porphyra crispata* by SU *et al.* (1966). SU and LU (1972) have thereafter purified the AMP deaminase of this laver and found that the enzyme consists of 4 subunits as found recently with the AMP deaminase of mammalian origin (OGASAWARA *et al.* 1977).

We have also been interested in this enzyme in laver, and made similar investigation on *Porphyra yezoensis*, the representative laver of Japanese "Nori" at present. Another reason for our investigation lied in that no such investigation has been performed on this important laver.

Although we used partially purified enzyme preparation, a considerably high AMP deaminase activity was detected and con-

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firmed that the deaminase we obtained may be available for the rudimentary investigation of its enzymatic properties. Thus, we obtained some additional information about them, and we report the results in this paper.

Materials and Methods

1. *Material*: *Porphyra yezoensis* UEDA f. *narawaensis* MIURA, which was cultivated in the Tokyo Bay, harvested in winter season in 1979 and stored at −20°C, was used as the experimental material.

2. *Substrates*: AMP, ADP, ATP, adenosine, NAD and adenine used as substrate and IMP used as marker were purchased from Wako Chemical Industry, Ltd.

3. *Enzyme assay*: The enzyme reaction was carried out in the cell of a Shimazu model-UV 200 S spectrophotometer in a reaction mixture containing 2.8 m*l* of 0.05 M Tris-acetate buffer, pH 6.5, 0.1 m*l* of 3 mM of AMP and 0.1 m*l* of enzyme solution.

The enzyme activity was measured by decrease in the absorbance at 265 nm at temperature between 20°C and 25°C except otherwise stated.

4. *Analyses*: Ammonia formed in reaction mixture was determined by the phenol method (CHANEY *et al.* 1962), and protein in enzyme solution was measured by the LOWRY-FOLIN's method (1951).

5. *Paper chromatography*: After an appropriate incubation period, remaining AMP and IMP produced were absorbed together on activated charcoal. The charcoal mixture was collected by filtration and extracted with an ammoniacal ethanol (ammonia : water : ethanol = 5 : 45 : 50, v/v). The extract was concentrated and a small aliquot of it was applied on Whatman No. 1 filter paper using the solvent system (n-propanol : conc. NH_4OH : water=6 : 3 : 1, v/v) as developing agent. The spots together with those of authentic AMP and IMP obtained by the ascending technique at 20°C for 20-hr run were detected under UV-ray at 253 nm wave length and identified them on the basis of Rf values.

Result

1. *Preparation of enzyme*: Laver fronds (50 g) frozen at −20°C were ground with 15 m*l* of 0.05 M Tris-HCl buffer, pH 7.5, containing 1 mM mercaptoethanol in an ice-cooled mortar in the presence of a small amount of quartz sand. The homogenate was centrifuged at 10,000×g for 30 min and precipitate formed from the supernatant by salting out on a saturation between 45% and 70% of ammoniumsulfate was recovered by centrifugation at 10,000×g for 30 min. The precipitate was dissolved in 10 m*l* of 0.05 M Tris-acetic acid buffer, pH 6.5 and dialyzed in a cellophan bag against the same buffer overnight. The dialyzed solution was used as the enzyme throughout this work.

2. *Examination of enzyme activity*: Reaction mixtures containing various concentrations of enzyme were prepared and decreases in the absorbance at 265 nm per min were determined. The results were shown in Fig. 1. The enzyme activity was enhanced in proportion to the enzyme

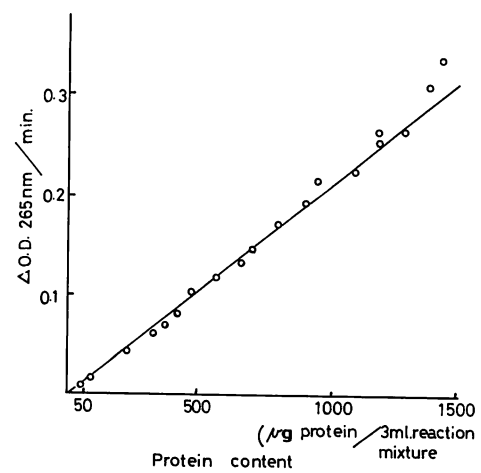

Fig. 1. Relationship between amounts of enzyme and its activity.

concentration expressed as LOWRY-protein in the range of approximately 50 μg to 1300 μg in 3 m*l* of reaction mixture.

3. *Time course of enzyme activity*: Three kinds of reaction mixtures containing increasing enzyme concentrations were incubated and the time course of each one was followed. The results were shown in Fig. 2. They pictured patterns commonly found with most of enzyme reactions.

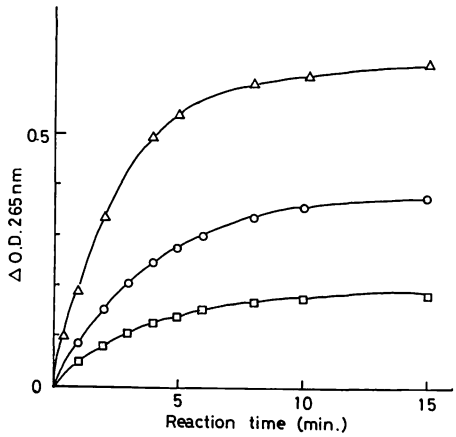

Fig. 2. Time course of adenylate deaminase activities at different concentrations expressed by protein contents in 3 m*l* of reaction mixture.
—△— 982 μg, —○— 491 μg, —□— 245.5 μg.

4. *Optimum pH*: The enzyme reactions were carried out in 0.05 M NaCl-containing 0.05 M Tris-acetic acid buffers of different pH values from 4.0 to 9.0. An optimum activity area was found at pH's from 6.0 to 6.5, as shown in Fig. 3.

5. *Thermal stability*: A series of reaction mixtures was first heated at every 5°C-raising temperatures from 25°C to 70°C for 5 min and for 20 min each, and then enzymic activities of them were measured after they were cooled to room temperature. The results were shown in Fig. 4. Heating at 45°C even for 5 min decreased the activity almost to a half extent. The enzyme was remarkably unstable to heat.

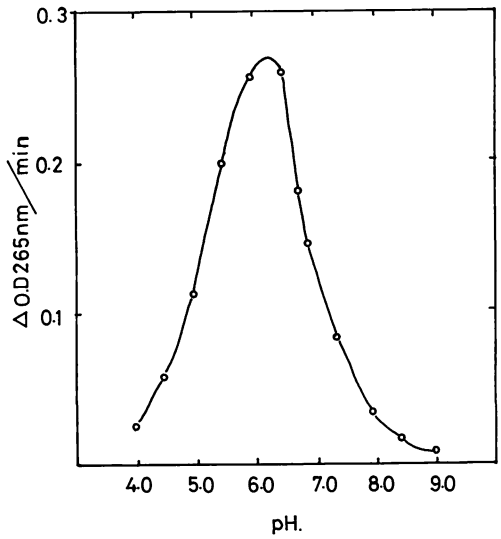

Fig. 3. Optimum pH of adenylate deaminase activity.

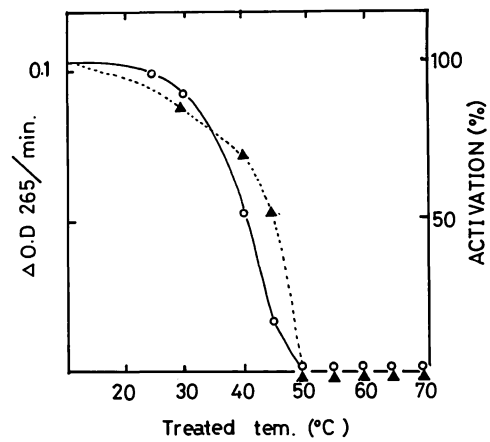

Fig. 4. Thermal stability of adenylate deaminase activity.
--▲-- 5 min., —○— 20 min.

6. *Effects of various metal ions on enzyme activity*: The effects of Ca^{2+}, Ba^{2+}, Mg^{2+}, Li^{+}, K^{+}, Mn^{2+} and Cd^{2+} on enzyme activity were investigated using their chloride salts. The results were shown in Figs. 5 and 6. All ions acted more or less as activator, but particularly higher with Ca^{2+} and the effects were in the order of $Ca^{2+} \gg Ba^{2+} > Mg^{2+} > Li^{+} \fallingdotseq K^{+}$ (Fig. 5). In contrast, effects of Mn^{2+} and Cd^{2+} were particular in that they effected in an enhancing way at lower concentrations while the effect was gradu-

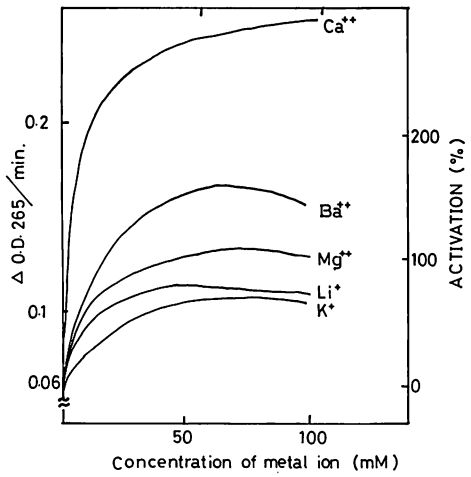

Fig. 5. Effect of various concentrations of metal ions on adenylate deaminase activity.

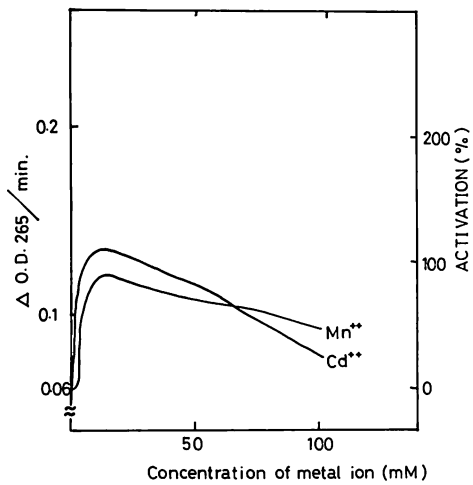

Fig. 6. Effect of various concentrations of Mn^{2+} and Cd^{2+} on adenylate deaminase activity.

ally decreased at higher concentrations (Fig. 6).

7. *Relationship between effects of Mn^{2+} and pH of reaction mixture*: The effect of Mn^{2+} was particular as described above, so its dependence upon the pH of reaction mixture was investigated. As shown in Fig. 7, the enzyme activity once enhanced by 50 mM of Mn^{2+} at around optimum pH's was lowered gradually toward acidic side whereas rapidly dropped toward alkaline side

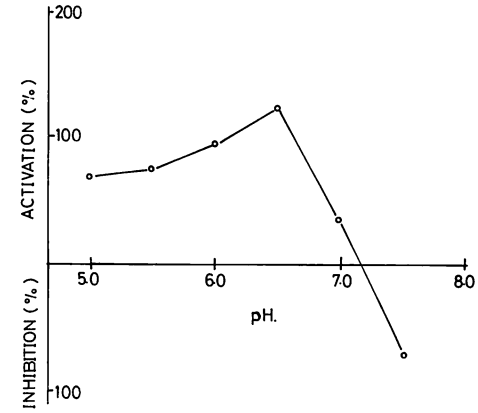

Fig. 7. Effect of 50 mM of Mn^{2+} as a function of pH.

and an inhibition began to occur at pH values slightly higher than 7.0. The inhibition was further enhanced as the pH value becomes higher.

Although not shown here, Na^+ showed 26% as less effective as that of K^+ while even 0.1 mM of Fe^{2+} inhibited the deaminase activity down to 50% of that in the absence of metal ion, and Zn^{2+} also inhibited remarkably. Addition of 1 mM of this ion decreased 85% of the activity of that without metal ion. *p*-CMB also showed as potent inhibition as that of Fe^{2+}. Thus, of various metal ions tested, the two ions of Fe^{2+} and Zn^{2+} acted as inhibitor.

8. *Substrate specificity*: A given portion of the same enzyme solution was each incubated with ADP, ATP, adenosin, NAD and adenine in addition to AMP in the standard reaction mixture except for 0.1 mM of substrates, then decrease in the absorbance at 265 nm was measured. The activity for adenine was not detected under this condition, but the activities for others were measured to be such ratios in strength as 100 : 74 : 17 : 81 : 5, respectively, when the activity for AMP was taken as 100.

9. *Km values for substrates*: According to LINEWEAVER-BURK's treatment, Km values for AMP, ADP, ATP and adenosine were measured under the standard reaction condition except for varying substrate

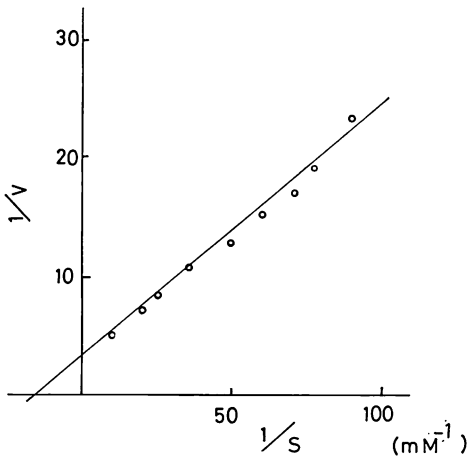

Fig. 8. Lineweaver-Burk plots of adenylate deaminase activity.

concentrations. Representing these results, that for AMP only was shown in Fig. 8. The values were estimated to be 5.3×10^{-5}, 6.6×10^{-5}, 11×10^{-5}, and 19×10^{-5} M, respectively. Thus, affinities of the deaminase seemed heigher for AMP and ADP than those for ATP and adenosine, and they seemed independent of the number of phosphate group of the substrates.

10. *Determination of ammonia produced in reaction mixture*: A reaction mixture containing 0.1 m*l* of 3 mM AMP, 2.8 m*l* of 0.05 M succinate buffer, pH 6.5 and 0.1 m*l* of an enzyme solution was incubated at 30°C for 60 min. The ammonia formed from AMP was determined to be 2.1×10^{-4} μM as an average value from 4 times repeating independent experiments.

11. *Identification of IMP produced in reaction mixture*: Two reaction mixture each containing 2.5 m*l* of 3 mM AMP, 70 m*l* of 0.05 M succinate buffer, pH 6.5 were incubated at 30°C for 60 min. One was heated at 80°C for 20 min soon after mixing and incubated in the same way. They were then treated similarly to prepare samples for paper chromatography. Two kinds of spots were recognized on the paper chromatogram with the non-heated reaction mixture and their Rf values were 0.204 and 0.133 while those of authentic AMP and IMP were 0.231 and 0.144, respectively. A single spot having the Rf of 0.231 was obtained with the reaction mixture heated before starting the reaction. Thus, the Rf values of both spots from the non-heated reaction mixture were slightly different from those of authentic AMP and IMP, but no other spot was found on the paper chromatogram than those obtained above. Therefore, the spots from non-heated reaction mixture must represent AMP and IMP, repectively.

Discussion

The enzyme preparations used in the present work were of dialyzed ones after salting out with ammonium sulfate. They, therefore, were not higher in purity.

However, since the activity measured by the absorbance at 265 nm enhanced linearly with the amounts of enzyme measured by LOWRY-FOLIN's method (1951) and its time course showed a common curve, we used the preparations at this stage of purification in the present work.

Of the results obtained, effects of various metal ions were noticeable. In particular, it was found that Mn^{2+} and Cd^{2+} activate the enzyme more at lower concentrations than at higher ones. Moreover, the effect of Mn^{2+} varied depending on pH of reaction mixture, and it became inhibitory toward alkaline side of optimum pH. The fact was entirely similar to that obtained by SU *et al.* (1966) from *P. crispata*. Possibly, it may be due to the property of substrate which has multiple anionic charges.

We investigated the effects of Fe^{2+} and Zn^{2+} on the enzyme activity, though not tried in the work of Su *et al.* (1966), and we found that these metal ions inhibited the deaminase of *P. yezoensis* remarkably. In contrast, alkaline earth metals such as Ca^{2+} and Ba^{2+} activated remarkably. The fact was entirely similar to the result from *P. crispata*. Similarly, *p*-CMB, an inhibitor of SH-enzyme, inhibited the deaminase of

P. yezoensis, too.

We measured Km values for ADP, ATP and adenosine in addition to AMP for comparison with the result from *P. crispata* (SU *et al.* 1966). These values were almost in the same order except for slight differences for ATP and adenosine. The similarity suggests that a single enzyme reacts with these substrates, as has been demonstrated by SU *et al.* (1966) for *P. crispata* deaminase.

SU and LU (1972) have demonstrated the AMP deaminase from *P. crispata* is a tetramer. Although in the present work, however, we did not investigate in this respect, the AMP deaminase of *P. yezoensis* may be of a similar structure, judging from the fact that the deaminase was extremely unstable against heat.

Acknowledgement

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西澤一俊・岡田往子・久保清明・安斎　寛：　ナラワスサビノリの
アデニル酸デアミナーゼについて

我国で現在浅草海苔として食用にされている海苔の大部分を占めているナラワスサビノリ (*Porphyra yezoensis* UEDA f. *narawaensis* MIURA) に関しては，AMP デアミナーゼの研究は試みられていない。そこで筆者らは，千葉県産のスサビノリを材料として，この酵素を抽出し，部分的に精製した標品を用いて，二・三の酵素実験を行ない，得られた結果を *P. crispata* で得られたものと比較した。その酵素的性質の大部分はよく似ていたが，違っている点もあった。結果を要約すると次のようになる。

この酵素は熱に不安定で，50°C 10 分間加熱で完全に失活する。種々の金属イオンにより賦活されるが，$Ca^{2+} \gg Ba^{2+} > Mg^{2+} > Li^{+} \fallingdotseq K^{+}$ の順で，Mn^{2+} や Cd^{2+} では 10 mM 付近では賦活度は大きいが，100 mM を越えると，賦活度はゼロに近づく。また，Mn^{2+} の影響は明らかに pH に左右され，至適酸度 (pH 6.0～6.5) 付近では，約 2 倍の賦活を示したが，pH 7.0 を越えるとむしろ阻害的に働く。AMP に対する Km は 5.3×10^{-5} M であったが，ADP や ATP にも作用を示し，その Km はそれぞれ 6.6×10^{-5}，19×10^{-5} M であった。(154 東京都世田谷区下馬 3-34-1 日本大学農獣医学部水産学科)