Enzymatic Properties of Polymannuronic Acid 5-Epimerase in Brown Algae

Masahiro ISHIKAWA*¹ and Kazutosi NISIZAWA*²

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The general enzymatic properties of algal polymannuronic acid 5-epimerase were investigated using *Ishige okamurai*, *I. sinicola*, and *Hizikia fusiformis* as starting materials. Its activity was optimal at pH 7.7 and at 30°C. The stability to pH was relatively high, while the enzyme was rather unstable to heat. The activity decreased gradually during storage at 4°C and even at -45°C. The enzyme showed its activity to a polymannuronate and to an alginate rich in mannuronic acid, but no activity to a polyguluronate. It was activated by Ca²⁺, Mn²⁺, and particularly by Mg²⁺, and strongly inhibited by Zn²⁺, Fe³⁺, and *p*-chloromercuribenzoate. The K_m for polymannuronate was estimated to be 7.3×10^{-4} and the molecular weight to be 62,000.

In a previous paper¹⁾, we demonstrated the presence of polymannuronic acid 5-epimerase (PM-5ase) in five selected species of brown algae using in general the method of carbazole-H₂SO₄²⁾. In addition, the enzyme activity in *Ishige sinicola* was confirmed by gas liquid chromatography in which changes in the ratios of mannuronic acid residue (M) to guluronic acid one (G) of substrate were determined during incubation by the estimation of peak areas corresponding to M and G in chromatogram¹⁾.

However, the enzymatic properties of PM-5ase have not been investigated in that work and in addition there are no such investigation so far in literatures except for the work of MADGWICK *et al.*³⁾ who investigated the effect of various metal ions on the PM-5ase of *Pelvetia canaliculata*.

Thus, we studied the general enzymatic properties of PM-5ase in detail, using from several brown algae as experimental material.

Materials and Methods

Algal Materials

All algae used in this work were harvested from Nabeta cove and its neighborhood in the Shimoda Bay.

Substrates

An alginate fragment, SM which consists virtually only of M, and M-rich alginate whose M/G ratio is 19.3 which were the same substrates used in a pervious work¹⁾ were also employed in this

study. In addition, another alginate fragment, SG which consists virtually only of G was prepared from commercial alginate by the similar method to that has been reported in our previous paper⁴⁾.

Preparation of Enzyme Solution and Reaction Mixture

These were made in entirely the same way as described in a previous paper¹⁾.

Enzyme Assays

PM-5ase activity was measured by carbazole-H₂SO₄ method²⁾ and alginate lyase (ALase) activity was assayed by thiobarbituric acid (TBA) method⁵⁾. Protein in enzyme solutions was determined by the method of LOWRY-FOLIN⁶⁾. Reference proteins used for the molecular weight determination of PM-5ase were determined by the absorbance at 260 nm. The enzyme activity was expressed by A and SA according to the calculation described in the preceeding work¹⁾.

Results

Optimal pH

The fronds of *Hizikia fusiformis* (500 g) harvested on October, 1978, and stored at -20°C for several days, were homogenized and an enzyme solution (50 ml) containing 33.0 mg of protein was obtained. Reaction mixtures containing SM and buffers of different pH values were prepared. After incubation at 30°C for 24 h, increases in

*¹ Present address: Hayashibara Company Ltd., 2-3, Shimoishii-1, Okayama 700 (石河正裕: 林原株式会社, 岡山市下石井 1-2-3).

*² Department of Fisheries, College of Agriculture and Veterinary Medicine, Nihon University, Shimouma-3, Setagaya-ku, Tokyo 154 (西澤一俊: 日本大学農獣医学部水産学科, 世田谷区下馬 3-34-1).

the absorbances at 530 nm were measured. A slightly positive ALase activity was detected during incubation, but it does not seem to interfere PM-5ase activities when expressed by A and SA. The maximal activity was found at pH 7.7 as shown in Fig. 1.

Fig. 1. Optimum pH of PM-5ase in an extract from frozen fronds of *H. fusiformis*.

pH Stability

From the fronds of *Ishige okamura* harvested on June 1, 1979 and stored at -20°C for several days, a PM-5ase solution (44 ml) was prepared in the same way as above. It contained 91.5 mg of protein. A series of given small volumes of enzyme solutions were adjusted to the given pH values from 3.5 to 11.5 by the addition of 2 N HCl or 2 N NaOH and stored at 4°C for 24 h. Then parts of these solutions were mixed with SM and buffer (pH 7.7) to make standard reaction mixtures. They were incubated at 30°C for 24 h and their enzyme activities were measured in the same way. Virtually no ALase activity was detected in these reaction mixture. As shown in Fig. 2, PM-5ase activities were fairly stable against pH values from 4 to 10.

Fig. 2. pH stability of PM-5ase in an extract from frozen fronds of *I. okamura*.

Optimal Temperature

The fronds of *Ishige sinicola* (900g) harvested on January 29, 1979 and stored at -20°C for several days were used as enzyme source. The enzyme solution (60 ml) thus obtained contained 25.8 mg of protein and showed virtually no ALase activity at least for 24 h. A series of standard reaction mixtures was incubated at every 10°C -raising temperatures from 10°C to 40°C for 24 h, and PM-5ase activities were determined in the same way as above. As shown in Fig. 3, the highest activity was found at 30°C .

Fig. 3. Optimum temperature of PM-5ase in an extract from frozen fronds of *I. sinicola*.

Thermal Stability

1. Treatment at temperatures above 30°C : A series of enzyme solutions same as those of the optimal temperature experiment was left at every 10°C -raising temperatures from 30°C to 60°C for 10 min and immediately cooled in ice-water. Then standard reaction mixtures were prepared, using these heat-treated enzyme solutions and SM as substrate. They were incubated at 30°C for 24 h and PM-5ase activity was measured. As shown in Fig. 4, in which remaining enzyme activities are expressed as relative values to the remaining activity on treatment at 30°C , PM-5ase activity decreased rapidly by treatment at temperatures above 50°C .

Fig. 4. Thermal stability of PM-5ase in an extract from frozen fronds of *I. sinicola*.

2. Treatment at lower temperatures: Parts of the enzyme solution used in the experiment (1) were left individually at 4°C and -45°C for 15 days. Aliquots of these enzyme solutions, after thawing when stored at -45°C, were removed at intervals and PM-5ase activities were determined. The results are shown in Fig. 5. PM-5ase activity stored at -45°C was more stable than that at 4°C.

Fig. 5. Time course curves of PM-5ase activity in an extract from *I. sinicola* fronds during storage in lower temperatures.

▲—▲, frozen at -45°C; ●—●, stored at 4°C.

Substrate Specificity

An enzyme solution (27 ml) containing 16.7 mg protein was prepared from frozen fronds of *Hizikia fusiformis* harvested on October 6, 1978. Using SM, M-rich alginate and SG, PM-5ase activities were measured after 24 h incubation at 30°C. Virtually negligible ALase was detected during this incubation. The PMase activities are shown in Table 1. PM-5ase activity for M-rich alginate was about 1.5 times higher than that for SM. The fact suggests that the chain length of substrate may be related to the enzyme activity. In contrast, PM-5ase showed no activity toward SG at least under the experimental conditions used here.

Table 1. Substrate specificity of PM-5ase activity in an extract from frozen fronds of *Hizikia fusiformis*

Substrate	Abs. ⁵³⁰ ₂₄ -Abs. ⁵³⁰ ₀	A	SA
M-rich alginate	0.070	2.03	8.17
SM	0.055	1.31	5.28
SG	0.003	—*	—*

* Negligible.

Effect of Metal Ions

An enzyme solution (30 ml) containing 68.1 mg of protein was prepared from frozen fronds of *Ishige sinicola* harvested on January 29, 1979. Using SM as substrate, various standard reaction mixtures except for containing each different metal ion at a 10 mM concentration. After 24 h incubation at 30°C, PM-5ase activities of these mixtures were measured. ALase activity measured simultaneously was not detected. Upon addition of FeCl₄ to the reaction mixture, no visible precipitate was formed in contrast to result from using alginate as substrate. The results are shown in Table 2. Fe³⁺ and particularly Zn²⁺ showed remarkably high inhibition while the PM-5ase activity was more or less enhanced by such ions as Mg²⁺, Mn²⁺ and Ca²⁺. The enhancement by Mg²⁺ was slightly higher than Mn²⁺.

Effect of *p*-Chloromercuribenzoic Acid (*p*-CMB)

Enzyme solution used in the pH stability experiment was employed for this experiment. Using SM as substrate, standard reaction mixtures except for containing different concentrations of *p*-CMB were incubated at 30°C for 24 h. As shown in Table 3, *p*-CMB inhibited PM-5ase in a way seeming competitive.

Michaelis Constant

The same enzyme solution as used in the pH stability experiment was used. Using SM as sub-

Table 2. Effects of various metal ions on PM-5ase activity in an extract from frozen fronds of *I. sinicola*

Compounds	Concentration (mM)	Abs. ⁵³⁰ ₂₄ -Abs. ⁵³⁰ ₀	A	SA	Relative activity (%)
Buffer* ¹	—	0.073	2.06	2.26	100
Standard* ²	—	0.085	2.43	2.67	118
FeCl ₃	10	0.050	0.78	0.86	38
ZnCl ₂	10	0.007	0.20	0.22	10
CaCl ₂	10	0.084	2.37	2.60	115
MgCl ₂	10	0.085	2.64	2.91	129
MnCl ₂	10	0.095	2.41	2.66	118

*¹ 0.1 M Tris-HCl buffer (pH 7.7).

*² 0.1 M Tris-HCl buffer, 3.4 mM CaCl₂, 0.1 mM DTT.

Table 3. Effect of *p*-CMB on PM-5ase activity in an extract from frozen fronds of *I. okamurai*

<i>p</i> -CMB (mM)	Abs. ₂₄ ⁵³⁰ -Abs. ₀ ⁵³⁰	A	SA	Relative activity (%)
0	0.103	2.11	2.54	100
0.01	0.085	1.78	2.14	84
1	0.058	1.18	1.42	56

strate, *K_m* value of PM-5ase was estimated by Lineweaver-Burk's procedure. As shown in Fig. 6, a *K_m* value of 7.3×10^{-4} was obtained. Since a remarkable inhibition was observed at a concentration of 11.4 mM of SM, the value at this point was excluded from the calculation of the *K_m*.

Fig. 6. Lineweaver-Burk plots for SM by PM-5ase from frozen fronds of *I. okamurai*.

Molecular Weight

An enzyme solution (16 ml) containing 37.4 mg of protein was obtained from frozen fronds of tip areas of *Ishige okamurai* collected on June 1, 1979. The enzyme solution showed a PM-5ase activity of 2.77 as SA value while its ALase activity was negligibly low.

On the other hand, to a Sephadex G-100 (Superfine) column (20 × 800 mm) equilibrated with the same buffer as used for the dialysis of PM-5ase solution¹⁾, were successively applied the following reference proteins at 4°C; horse heart cytochrome C (mol. wt., 12,500), chymotrypsinogen A of bovine pancreas (mol. wt., 25,000), egg albumin (mol. wt., 45,000), bovine serum albumin (mol. wt., 68,000) and aldolase of rabbit muscle (mol. wt., 158,000), all of which were manufactured by Boehringer Mannheim GmbH, Mannheim, West Germany. The column was eluted with a buffer, pH 7.7, consisting of 0.02 M Tris-HCl, 0.1 mM

EDTA, 10 mM CaCl₂, 1 mM dithiothreitol, 2.17 M glycerol, at a flow rate of 40 ml per day, and every 2 ml of eluates was collected. Thus, the elution volumes of these proteins measured by UV absorption were plotted in a form of *V_e/V₀* on abscissa, and their molecular weights were on ordinate at a logarithmic scale in order to make a standard curve.

To the same Sephadex column, was applied the PM-5ase solution obtained above, and eluted in the same way. Protein in each fraction was determined by LOWRY-FOLIN's method and PM-5ase activities were measured by carbazole-H₂SO₄ method using SM as substrate. On the basis of the elution volume containing PM-5ase activity, the molecular weight of the enzyme was estimated to be 62,000 as shown in Fig. 7.

Fig. 7. Determination of molecular weight of PM-5ase from frozen fronds of *I. okamurai*.

Discussion

The algal PM-5ase studied in this work was stable in such a wide pH range as from 4 to 10 while it was relatively sensitive to heat as seen in the facts that nearly one third of its activity was lost by heating at 50°C for 10 min and the inactivation gradually proceeded for storage even at -45°C. Since the results were obtained from the experiments using crude enzyme solution, the reason for them was not clear. Possibly, freezing and thawing procedure might cause such gradual inactivation in the latter case. However, it seemed desirable that attention must be paid rather to temperature than to pH of extraction medium upon the preparation of PM-5ase from algal material.

It has been reported³⁾ that *Pelvetia* PM-5ase

was markedly activated by Mn^{2+} , but our *Ishige* PM-5ase activity was slightly more enhanced by Mg^{2+} rather than Mn^{2+} . It was common, however, that both PM-5ases were strongly inhibited by Zn^{2+} while Ca^{2+} protected these PM-5ases in a similar fashion. The fact that Ca^{2+} needed to the full activity of algal PM-5ases resembles entirely the property of PMase obtained from *Azotobacter vinelandii*⁷⁾.

The algal PM-5ase showed the activities for SM and M-rich alginate while almost no activity was detected to SG. The fact suggests that equilibrium of the conversion of M to G is shifted far and away toward G side. This assumption is compatible with the fact that both *Ishige* and *Pelvetia*³⁾ PM-5ases possess high affinities for their substrates so as to be inhibited strongly at higher substrate concentrations. In this respect, it seemed correct to call this enzyme PM-5ase rather alginate 5-epimerase which also implies the capacity to convert G to M.

In the present work, the molecular weight of *Ishige* PM-5ase was estimated to be 62,000. The value does not seem to represent the molecular weights of general PM-5ases in brown algae, but it seems significant to be on the order of an average

of those of hydrolases in general.

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