Properties of Fish Gastric Hyaluronidase

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The properties of hyaluronidase prepared from gastric mucosa of mackerel were studied. Determination of the activity in a 1:2 dilution of McILVAINE buffer revealed an optimum pH of 4.8, which shifted to 2.8 with the addition of NaCl up to a concentration of 0.15 M. Various chlorides such as NaCl, KCl and $MgCl_2$ enhanced the activity, and their effect might be attributed to Cl^- . At a concentration of 10^{-4} M Zn^{++} enhanced the activity, although Ag^+ and Cu^{++} scarcely affected and Hg^{++} inhibited it. Polycation increased the activity; on the contrary, polyanion inhibited it. The maximum activity was found at approximately 50°C under the conditions tested. The MICHAELIS constant for potassium hyaluronate was 0.50 mg per ml. The molecular weight was estimated to be approximately 56,000 by the gel filtration method.

In the previous papers¹⁻³⁾ the authors reported the presence of hyaluronidase which acts as a N-acetylhexosaminidase in the stomach of certain fish. Because of an interest in its peculiar origin, properties of the hyaluronidase are investigated in the present work.

Materials

Fish gastric hyaluronidase. The electrophoretically homogeneous enzyme³⁾ (10,000 Turbidity Reducing Unit of TOLKSDORF⁴⁾/mg protein) prepared from gastric mucosa of mackerel, *Scomber japonicus*, was used.

Reagents. Crude potassium hyaluronate of human umbilical cord was kindly supplied by Sansei Seiyaku Co. Ltd. (Japan) and purified by the method of MATHEWS⁵⁾. Analysis of the purified hyaluronate gave the following values: hexosamine, 36.5% (GARDELL's method⁶⁾); uronic acid, 40.5% (carbazole method⁷⁾); sulfate 0.1% (benzidine method⁸⁾). Super special grade of chondroitin sulfates A, C and D were purchased from Seikagaku Kogyo Co. Ltd. (Japan). Keratosulfate was prepared from bovine cornea by the procedure of MEYER *et al*⁹⁾. Other reagents used were purchased commercially.

Experimental Methods

Substrate specificity of hyaluronidase. Corroboration of the degradation of mucopolysaccharides was performed as follows; a test tube containing 1 ml of gastric hyaluronidase solution (1,000 TRU/ml of deionized water) and 1 ml of mucopolysaccharide solution (2.4 mg/ml of 0.1 M acetate buffer of pH 4.8) was kept at 37°C for 12 hours. After stopping the reaction by heating the tube in a boiling water bath for 3 minutes,

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the solution was determined for reducing power by the method of NELSON-SOMOGYI¹⁰. In control experiment, enzyme and substrate were incubated separately and put together just before the heating.

Determination of hyaluronidase activity. Unless otherwise stated, a mixture of 0.25 ml of gastric hyaluronidase solution suitably diluted with deionized water and 0.25 ml of potassium hyaluronate solution (2.4 mg/ml of 0.1 M acetate buffer of pH 4.8 or MCILVAINE buffer of pH 2.8) was incubated at 37°C for 20 minutes. After the reaction was stopped by heating, N-acetylhexosamine end group released was determined as follows: to the solution was added 0.1 ml of 0.8 M potassium tetraborate solution, and the whole was heated for 3 minutes in a boiling water bath, followed by cooling, mixing with 3 ml of *p*-dimethylaminobenzaldehyde reagent of REISSIG *et al.*¹¹, and measurement for color development at 544 m μ . In the case of the reaction carried out in more acidic condition than pH 3.0, a small quantity of 0.5 N sodium hydroxide solution was added to neutralize the reaction mixture before the determination of N-acetylhexosamine end group. The additive such as salt was added, if necessary, into the buffer solution.

Results and Discussion

Substrate specificity. Gastric hyaluronidase degraded hyaluronic acid, chondroitin sulfates A, C and D as shown in Table 1. No activity was detected toward heparin, keratosulfate and chitin. It seems that the hyaluronidase exhibits the same substrate specificity as bovine testicular enzyme in consideration of the properties of hyaluronidases obtained from various origins¹².

Table 1. Substrate specificity of mackerel gastric hyaluronidase.

Mucopolysaccharides	Degradation
Hyaluronic acid	+
Chondroitin sulfate A	+
Chondroitin sulfate C	+
Chondroitin sulfate D	+
Heparin	—
Keratosulfate	—
Chitin	—

pH optimum. MCILVAINE buffer indicating various pH was used instead of the buffer described in the experimental methods. The maximum activity of gastric hyaluronidase for hyaluronate occurred at pH 4.8 in the absence of NaCl as shown in Fig. 1, while at pH 2.8 in the presence of 0.15 M NaCl. The shift of optimum pH from 4.6 to 3.0 in the absence or presence of 0.15 M NaCl was observed by using chondroitin sulfate C as a substrate. MADINAVEITIA and QUIBELL¹³, and ALBURN and WHITLEY¹⁴ have reported similar shift of optimum pH toward bovine testicular hyaluronidase.

Fig. 1. pH-dependence of the activity of mackerel gastric hyaluronidase at 37°C.

Fig. 2. Effect of salts on the activity of mackerel gastric hyaluronidase at pH 2.8.

Effect of salts. Effect of various salts except those of heavy metals on the activity of gastric hyaluronidase was determined at pH 4.8. As shown in Table 2, various chlo-

Table 2. Effect of salts on the activity of mackerel gastric hyaluronidase at pH 4.8.

Salts	Concentration of salts	
	0.01 M	0.05 M
	Relative activity (%)	
NaCl	238	214
KCl	212	213
NH ₄ Cl	179	159
CH ₃ COONa	94	78
Na ₂ SO ₄	113	82
MgSO ₄	125	97
MgCl ₂	144	116
None	100	100

rides enhanced the activity. The effect of chlorides was conspicuous at pH 2.8 as shown in Fig. 2. It is reasonable to assume that the enhancing effect of chlorides on the activity of gastric hyaluronidase is attributed to Cl⁻, since the enzyme activity was vigorously inhibited by 5×10^{-3} M HgCl₂ as shown in Table 3, but the masking of Hg⁺⁺ with ethylenediaminetetraacetic acid was found to increase the activity. On the one hand, effect of Ag⁺, Zn⁺⁺, Cu⁺⁺ and Hg⁺⁺ was determined (these heavy metals were tested as acetate). The result in Fig. 3 showed that Hg⁺⁺ at a concentration of 10^{-4} M inhibited the activity

Table 3. Effect of mercuric chloride and ethylenediaminetetraacetic acid on the activity of mackerel gastric hyaluronidase at pH 2.8.

HgCl ₂ (M)	EDTA (M)	Relative activity(%)
0	0	100
0.5×10^{-3}	0	10
0	0.5×10^{-2}	122
0.5×10^{-3}	0.5×10^{-2}	429

Fig. 3. Effect of heavy metal ions on the activity of mackerel gastric hyaluronidase at pH 4.8.

Fig. 4. Effect of macroions on the activity of mackerel gastric hyaluronidase at pH 4.8.

of gastric hyaluronidase to approximately 40% and at 10^{-3} M almost completely, although Ag^+ and Cu^{++} scarcely affected the activity at 10^{-4} M and Zn^{++} accelerated it at the concentrations tested. MEYER and RAPPORT¹⁵⁾ have reported that Ag^+ , Zn^{++} and Cu^{++} inhibited the activity of bovine testicular hyaluronidase at 10^{-4} M by reversible combination with the enzyme protein. From the above experiments, it is probable to recognize that fish gastric hyaluronidase is more stable than bovine testicular enzyme toward those heavy metal ions.

Effect of macroions. Effect of macroions on the activity of gastric hyaluronidase is shown in Fig. 4. Heparin sodium which acts as a polyanion inhibited the activity, on the contrary protamine sulfate which does as a polycation enhanced it. BERNFELD *et al.*¹⁶⁾ have reported similar relation between macroions and bovine testicular hyaluronidase.

Temperature-dependence. Activity of gastric hyaluronidase was determined at

various temperatures, and the result obtained is shown in Fig. 5. The maximum activity is found at approximately 50°C.

Fig. 5. Temperature-dependence of the activity of mackerel gastric hyaluronidase at pH 4.8.

Fig. 6. Stability of mackerel gastric hyaluronidase against heating at pH 4.8.

Fig. 7. LINEWEAVER-BURK's plots of mackerel gastric hyaluronidase for potassium hyaluronate at pH 4.8. v : μ mole of N-acetylglucosamine per ml of enzyme solution per minute.

Fig. 8. Molecular weight of mackerel gastric hyaluronidase.

Stability for heating. Gastric hyaluronidase solution was preincubated with varying temperature and time, and the remaining activity was determined. The result obtained is shown in Fig. 6. The activity was completely extinguished by the preincubation at 60°C for 5 minutes.

MICHAELIS constant. A solution of gastric hyaluronidase (600 TRU/ml) and 0.1 M acetate buffer of pH 4.8 were used. Activity of gastric hyaluronidase was determined with varying the concentration of potassium hyaluronate. The MICHAELIS constant was 0.50 mg per ml as shown in Fig. 7.

Molecular weight. The molecular weight of gastric hyaluronidase was estimated by the gel filtration method of ANDREWS.¹⁷⁾ From the point of elution position to the logarithm of molecular weight in Fig. 8, the molecular weight of the hyaluronidase was estimated to be approximately 56,000.

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References

- 1) M. KITAMIKADO and H. YAMAMOTO: This Bull., **35**, 466-470 (1969).
- 2) M. KITAMIKADO and H. YAMAMOTO: *ibid.*, **36**, 385-390 (1970).
- 3) H. YAMAMOTO and M. KITAMIKADO: *ibid.*, **37**, 621-625 (1971).
- 4) S. TOLKSDORF: *Method Biochem. Analysis*, **1**, 429-457 (1954).
- 5) M. B. MATHEWS: in "Methods in Enzymology" (E. F. NEUFELD and V. GINSBURG ed.), Vol. 8, 657-658, Academic Press, New York and London (1966).
- 6) S. GARDELL: *Acta Chem. Scand.*, **7**, 207-215 (1953).
- 7) T. BITTER and H. M. MUIR: *Anal. Biochem.*, **4**, 330-334 (1962).
- 8) C. A. ANTONOPOULOS: *Acta Chem. Scand.*, **16**, 1521-1522 (1962).
- 9) K. MEYER, A. LINKER, E. A. DAVIDSON, and B. WEISSMANN: *J. Biol. Chem.*, **205**, 611-616 (1953).
- 10) M. SOMOGYI: *ibid.*, **195**, 19-25 (1952).
- 11) J. L. REISSIG, J. L. STROMINGER, and L. F. LOLOIR: *ibid.*, **217**, 959-966 (1955).
- 12) K. MEYER, A. LINKER, P. HOFFMAN, and E. D. KORN: Proc. Intern. Symp. Enzyme Chem. Tokyo Kyoto (1957) p. 132, Pergamon Press, England (1959).
- 13) J. MADINAVEITIA and T. H. H. QUIBELL: *Biochem. J.*, **35**, 456-460 (1941).
- 14) H. E. ALBURN and R. W. WHITLEY: *J. Biol. Chem.*, **192**, 379-393 (1951).
- 15) K. MEYER and M. M. RAPPORT: *ibid.*, **188**, 485-490 (1951).
- 16) P. BERNFELD, L. P. TUTTLE, and R. W. HUBBARD: *Arch. Biochem Biophys.*, **92**, 232-239 (1961).
- 17) P. ANDREWS: *Biochem. J.*, **91**, 222-233 (1964).