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Carbonic Anhydrase in Pearl Oyster

I. Distribution and Some Properties of the Enzyme*

By

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Introduction

Carbonic anhydrase, which catalyses the reaction $CO_2 + H_2O \rightleftarrows H_2CO_3$, plays an important role in the transport of respiratory carbon dioxide in the blood of higher vertebrates (12). This enzyme is also known to concern the regulation of the acid-base equilibrium of the body in association with the production of hydrochloric acid in the gastric mucosa (3). It is further possible that the enzyme has a function in the calcification of bone and in the formation of egg-shells and other calcareous structures (6, 13). In molluscs, carbonic anhydrase has been found in the mantle tissue, and suggested to concern with the shell formation (17).

The pearl has the chemical constitution similar to the shell, and it is surmised that the pearl formation is of the same mechanism as the shell formation from the biochemical point of view. Although several investigations on the pearl formation have been made from various aspects (10, 14, 15), we have no information from an enzymological angle.

As the first step of my approach toward this problem, carbonic anhydrase activity of various tissues of pearl oyster (*Pinctada martensii*) was examined. Further studies were made on some properties of the the molluscan carbonic anhydrase, e. g. its stability to temperature and pH, and the effect of inhibitors.

Experimental

Methods. The determination of enzyme activity was made by the rapid manometric method of Meldrum & Roughton (11) at 10°C. One of the depressions of a reaction vessel contained 2.0 ml. of 0.186 M. $NaHCO_3$ dissolved in 0.038 M. NaOH, while the other contained 1.0 ml. of 0.4 M. phosphate buffer solution of pH 6.8

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(2), and the final fluid volume was made up to 4.0 ml. by adding the enzyme solution or distilled water.

The vessel was shaken at a rate of 360 oscilations per minute without shaking the manometer. For this purpose, the stopper of reaction vessel was jointed to the manometer with a thick rubber tube, and a shaking rod was connected to the upper part of the stopper. Manometric readings were taken every ten seconds for one minute, beginning immediately after the start of the shaking. The enzyme activity was calculated by the formula of Meldrum & Roughton based on the amount of carbon dioxide evolved in the second interval (in μl. per 10 sec.). To avoid the effect of diffusion, the concentration of the enzyme solution was always adjusted within the range there were strict linear relations between the amount of enzyme added to the reaction vessels and the observed catalytic effect.

Materials. The specimens of pearl oyster used in this study were two-year-old and obtained from the Matoya Farm of the Nippon Pearl Co. at Matoya Bay, Mie Prefecture. The tissues were dissected out, blotted with the filter-paper, and then weighed. After grinding with a small amount of quartz sand, they were suspended in distilled water in the proportion of 1 ml. to each 25 mg. of tissue and left stand for 12 hours at about 10°C. The suspensions thus prepared were used for studying the distribution of the enzyme in various tissues. The mucus secreted from the mantle epithelium facing the inner surface of the shell was gathered and diluted three to ten times its volume with distilled water.

The enzyme solution for the test of stability to pH and temperature as well as of inhibitors was prepared in the following manner. The gill tissue, which has the highest enzyme activity among many tissues as will be described later, was homogenized as 100 mg. per ml. in distilled water. This tissue suspension was autolysed in the above mentioned condition, and after centrifuging, the supernatant fluid was used for the experiment.

Distribution of the enzyme. The materials for this study were collected in October, 1952. The mean weight of whole body with shell was 29 g., and that of shell was 15 g. The enzyme activity of various tissues is given in table 1, where each figure is expressed in Roughton & Meldrum unit per 50 mg. fresh tissue.

The enzyme is present in many tissues of the pearl oyster, but is absent from the mucus which is secreted by the mantle epithelium. The presence of a large amount of carbonic anhydrase in the gill is in good agreement with the results found in fresh-water bivalves (4, 7), octopus and cuttle-fish (16), and certain crustaceans (5). This fact suggests that the enzyme should play an important role in the elimination of respiratory carbon dioxide in the gill tissue.

The secreting mechanism of shell-forming materials from the mantle is not yet well known. However, from our knowledge of the growth and structure of

Table 1. Carbonic anhydrase activity of various tissues.*

Tissues	Number of animals	Enzyme units per 50 mg. wet tissue	
		Range	Mean
Gill	11	6.6—10.8	9.3
Mantle edge	11	2.6— 5.6	4.0
Epidermis of mantle**	11	1.8— 3.7	2.2
Gonad	10	1.2— 3.8	2.2
Digestive diverticula	10	1.1— 3.6	2.0
Adductor muscle	10	0.3— 1.7	0.7
Mucus from mantle	9	Nil	

* In another experiment, it has been found that the enzyme is absent from the blood of the pearl oyster.
** The epidermis of the mantle facing the inner surface of the shell.

shell in the pearl oyster, it is surmised that the ability of secreting shell materials should be considerably larger in the edge than in other parts of the mantle. In the present examination, a considerable amount of cardonic anhydrase was found in this locus of the mantle. This fact may be a support to an assumption that the enzyme is an active agent in the formation of calcium carbonate. In this study, the so-called pearl sac within which the pearl is formed, was not examined, because the tissue is too small to obtain a sufficient amount for the experiment. However, the experiment on the pearl sac will be carried out before long.

Stability to temperature. One ml. of the enzyme solution (17 E. U. per ml.), prepared from the gill tissue as previously described, was mixed with 1.0 ml. of 0.1 M. phosphate buffer solution (pH 7.0). One drop of toluene was added to

Table 2. Stability to temperature.

Temperature °C.	Survived enzyme activity (%)		
	After 10 min.	After 60 min.	After 12 hours
10	—	100	100
25	—	100	100
35	—	100	89
50	—	40	0
65	31	0	—

the mixture. The mixed solutions were exposed to various temperature for the time ranging from ten minutes to twelve hours. After this treatment the survived enzyme activity was determined. From the results summarized in table 2, it may be seen that the crude enzyme is stable at below 25° C., unstable at above 35° C. and destroyed completely within a short time at above 50° C.

In this series of experiments, two other tests were carried out. In the first test, tissues were placed in glass bottles, kept frozen for several days and then extracted by the method of the distribution study. In the second test, the aqueous enzyme solution were kept for several days in Dewar's vessel refrigated at 0° C. with cracked ice. In both cases, the anzyme activity did not decreased during the stored period.

Stability to pH. One ml. of the enzyme solution (15 E. U. per ml.) was mixed with 2.0 ml. of buffer solution having various pH values. One drop of toluene was added to the mixture. The solutions were kept for 12 hours at room temperature, and then determined the survived enzyme activity. Table 3 presents the results.

Table 3. Stability to pH.

pH	2.0	3.4	4.5—8.0	10	11	13
Buffer soln. (O. 1 M.)	Citrate-HCl		Phosphate	Glycine-NaOH		0.1 N. NaOH
Survived enzyme activity (%)	0	38	100	100	73	0

The crude enzyme is stable at pH 4.5−10, but is destroyed at below pH 3.4 or above pH 11.

Effect of inhibitors. One ml. of enzyme-buffer solution (10 E. U. per ml.), prepared by mixing one part of the enzyme solution with one part of 0.2 M. buffer solution of phosphate or veronal-HCl (pH 7.0), was kept for one hour at room temperature affer adding 1.0 ml. of aqueous solution of various chemical sudstances (prepared to give final concentration ranging from 10^{-2} M. to 10^{-4} M.). After this treatment the survived enzyme activity was determined. Table 4 shows the results. The degree of inhibition was expressed in per cent.

The enzyme is inhibited strongly by Hg and weakly by Zn among metal salts, though not affected by Mn and Cd. The metal inhibitors, e. g., cyanide sulfide and thiocyanide bring a heavy inactivation on the enzyme. These inhibitions suggest the presence of some metal element in the active radical of the molluscan carbonic anhydrase. Sulphanilamide, a specific poison for carbonic

anhydrase of higher vertebrates (9), destroys also completely the molluscan carbonic anhydrase in concentration of 10^{-3} M.

Table 4. Inhibition of molluscan carbonic anhydrase by chemical compounds.

Chemical compound	Buffer soln.	Concentration	Inhibition (%)
$MnCl_2$	Without	10^{-2} M.	0
$CdSO_4$	Veronal	10^{-2} M.	0
$ZnSO_4$	〃	10^{-2} M.	15
$HgCl_2$	Phosphate	10^{-2} M.	83
〃	〃	10^{-3} M.	32
KCN	Phosphate	10^{-2} M.	100
〃	〃	10^{-3} M.	60
KSCN	〃	10^{-2} M.	53
Na_2S	〃	10^{-2} M.	83
Sulphanil-amide	Phosphate	10^{-3} M.	100
〃	〃	10^{-4} M.	54

From results of these tests of inhibitions as well as the stability to temperature and pH, the properties of the molluscan carbonic anhpdrase seem quite similar to those of higher animal's carbonic anhydrase which is a zinc protein (13), but are very different from the properties (1, 8) of plant carbonic anhydrase.

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Summary

Carbonic anhydrase in pearl oyster, *Pinctada martensii*, was studied with references to its distribution in various tissues, stability to temperature and pH, and the inhibitory action of various chemical substances.

It was confirmed that the enzyme content was the largest in the gill tissue, considerably more in the mantle edge as compared with other parts of the mantle,

and that the enzyme was absent from the mucus secreted from the mantle epithelium facing the inner surface of the shell.

The crude enzyme was stable in temperature lower than 25° C. and in pH 4.5−10.0. The molluscan carbonic anhydrase was inhibited strongly by metal inhibitors, e. g., cyanide, thiocyanide and sulfide, and also by sulphanilamide which had been known as a specific poison for carbonic anhydrase of higher animals.

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