Studies on the Rainbow Trout Aldolase III. Enzymic properties of the aldolase from rainbow trout muscle

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III. Enzymic properties of the aldolase from rainbow trout muscle

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Summary

1) The enzymic properties of rainbow trout muscle aldolase were investigated.

2) The rainbow trout muscle aldolase is the most active at 30°C which is over 10°C lower than that of rabbit, carp and tuna muscle aldolases.

3) Optimum pH of the fish enzme is variable with buffer systems or assay systems. It was found at pH 9.5 in glycine buffer and at pH 9.0 in tris-HCl buffer by cyanide trapping method. In hydrazine sulfate trapping method, however, it was the most active at pH 7.1. The pH profiles of the reactions catalyzed by this enzyme resemble those of the rabbit, carp and tuna muscle enzymes.

4) The Michaelis constant, Km, is found to be 1.67×10^{-3} M/L at pH 9.0 and 0.80×10^{-3} M/L at pH 7.4.

5) The activation energy of the reaction is 3.3×10^{3} cal/mole at pH 9.0.

6) The activity of the aldolase from rainbow trout muscle is inhibited by divalent metal ions and cysteine. The effect of mercuric ion on it was the most powerful among the metal ions tested.

7) The aldolase from rainbow trout muscle becomes almost inactive after 15 weeks kept at −10°C and after 30 weeks at −25°C, respectively.

As introduced previously, the investigations on the enzymic properties of fish aldolase are very little in comparison with those of animals. In 1958, Shibata (1) reported on the properties of partially purified aldolase from carp muscle. After that the tuna muscle aldolase was studied by Kwon and Olcott (2). Recently it was reported that Komatsu and Feeney (3) and Lai and Chen (4) crystallized the muscle aldolase from cold-adapted antarctic fish and cod, respectively, and studied them. On the other hand, Connell (5) and Shibata et al. (6) investigated the changes of the aldolase activity of fish muscle during cold-storage. It was found that the fish aldolase was generally unstable by comparison with the animal enzyme.

Previously we have reported a new method for the crystallization of the aldolase from rainbow trout muscle (7) and its physico-chemical properties (8).

In this paper, we describe the enzymic nature of the rainbow trout muscle aldolase. The experimental data also indicated that this fish muscle aldolase was more susceptible than that of animals'.

Experimental and Results

Preparation of Crystalline Aldolase

Preparation of the aldolase from rainbow trout muscle was made as reported in a previous paper (7) and the crystalline aldolase was purified three or four times by recrystalization.

Aldolase Assay

Aldolase activity was assayed by two methods using cyanide and hydrazine sulfate as trapping agent. In the former, the method of Taylor et al. (9) was used. In the hydrazine assay system, a slight modification of the method of Swenson and Boyer (10) was introduced as follows:

0.1 ml of aldolase solution containing approximately 15 μg of aldolase was added to the mixture of 0.2 ml of 0.1 M fructose-1, 6-diphosphate and 0.7 ml of 0.5 M hydrazine sulfate that was brought in advance to 30°C. After incubating for 10 minutes at 30°C, the reaction was stopped with addition of 3 ml of 10 per cent trichloroacetic acid. 1 ml of the reaction mixture was transfered to each of tubes. 1 ml of 0.75 N sodium hydroxide was added to each tube and the mixture was allowed to stand for 10 minutes at room temperature. Then 1 ml of 0.005 M 2, 4-dinitrophenylhydrazine in 2 N HCl was added to it respectively and the tubes were placed in water bath at 30°C for 10 minutes. Thereafter each solution was filled up with 0.75 N NaOH to make a total volume of 10 ml.

The protein concentration (mg/ml) of the aldolase solution was calculated from measurement of the absorption at 280 mμ using 0.90 of extinction coefficient for 0.1 per cent of protein concentration.

Effect of Enzyme Concentration on the Reaction Velocity

The reaction velocity of the rainbow trout aldolase increased in proportion to the protein concentration within a range of experiments (Fig. 1). 15–40 μg of enzyme by the cyanide trapping method, and 10–20 μg by the hydrazine sulfate method were made available for the determination of the aldolase activity.

Time Course of Reaction Velocity

In order to know the optimum reaction time and reaction velocity constant of the fish enzyme, its activity was determined by both methods using cyanide and hydrazine sulfate. Figure 2a and 2b show that the enzyme activity reduced after 6 minutes and 35 minutes, respectively. Therefore the reaction time of 5 minutes in the cyanide method and 10 minutes in the hydazine sulfate method

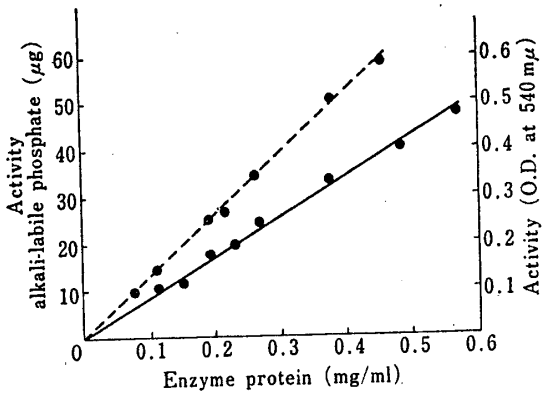
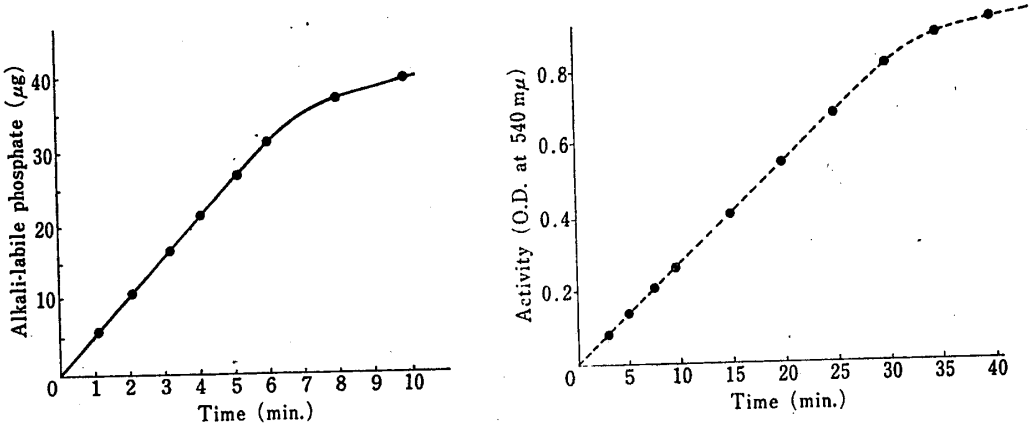


FIG 1. Linearity of response with rainbow trout muscle aldolase concentration.
—— incubated for 5 min. at 30°C, by cyanide trapping method
---- incubated for 10 min. at 30°C, by hydrazine sulfate trapping method



(a) (b)

FIG 2a. Time course of rainbow trout aldolase activity by cyanide trapping method (incubated at 30°C).
FIG 2b. Time course of rainbow trout aldolase activity by hydrazine sulfate method.

was employed. The reaction velocity constant was found to be 5.41×10^{-5}M/min. by cyanide method.

Effect of Temperature

The effect of temperature on the activity of the rainbow trout aldolase is shown in Fig. 3. It was the most active at about 30°C on both methods and was greatly retarded at 40°C and completely inactivated at 55°C. It is reported that the temperature optimum for the aldolase activity of carp (1) and tuna (2) are from 42° to 45°C by the cyanide trapping method. On the other hand, Herbert et al. (11) found that the rabbit aldolase remained active even at 60°C. As to the influence of low temperature on the enzyme activity, it was found that the aldolases from rabbit, carp and tuna muscles demonstrated little activity at below 20°C, but the rainbow trout enzyme had two-thirds of the highest value of the activity at the same temperature. These results coincided with those of Komatsu

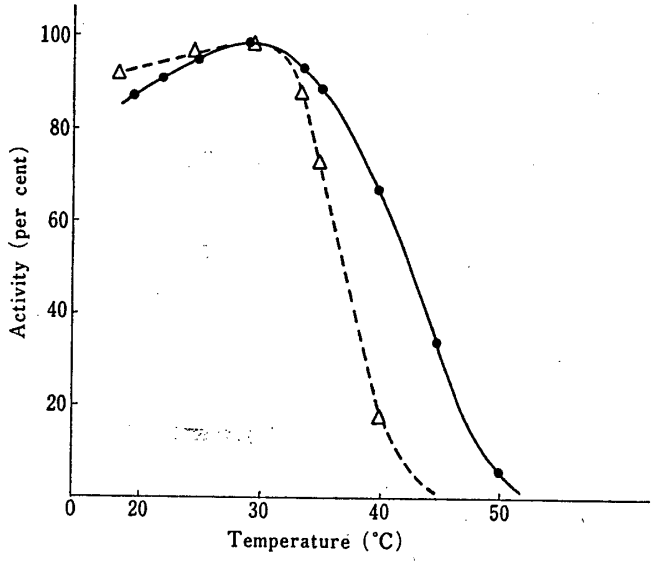


FIG 3. Effect of temperature on the reaction velocity of rainbow trout muscle aldolase.
——•—— incubated for 5 min. by KCN trapping method
----▵---- incubated for 10 min. by hydrazine sulfate trapping method

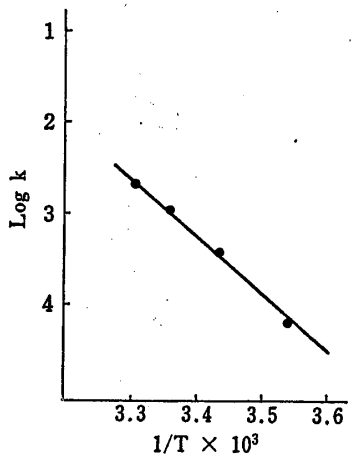


FIG 4. Influence of temperature on reaction velocity. Reaction velocity was computed from the first-order equation.

and Feeney (3) on the two species of cold-adapted antarctic fishes.

The effect of temperature on the reaction velocity constant, based on the ascending branch of the curve in Fig. 3, is shown in Fig. 4. From the slope of the line obtained, the activation energy was calculated at 3.3×10^3 calories per mole. This value agrees with that of tuna which is about one-third of that of the rabbit muscle aldolase.

Effect of pH

The effect of pH on the activity of the rainbow trout aldolase is shown in Fig. 5. The optimum pH was variable depending on the buffer systems and methods. It was the most active at about pH 9.5 in the glycine buffer and at pH 9.0 in the

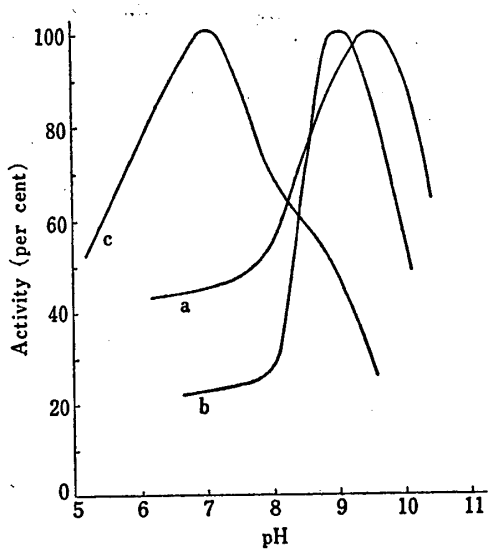


FIG 5. Effect of pH on the reaction velocity
a) incubated for 5 min. at 30°C in glycine buffer
b) incubated for 5 min. at 30°C in tris-HCl buffer
c) incubated for 10 min. at 30°C in hydrazine sulfate buffer

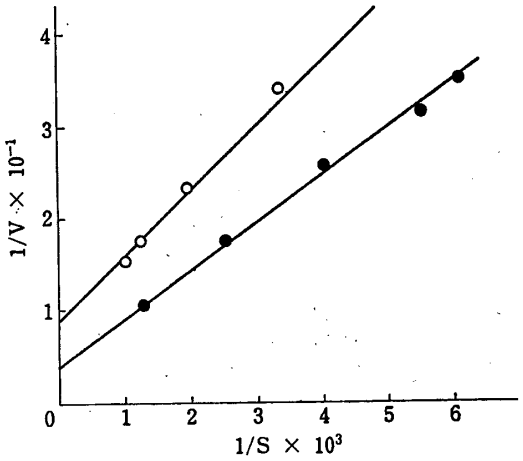


FIG 6. Effect of substrate concentration on the reaction velocity.
——•—— incubated for 5 min. at 30°C by KCN trapping method (pH 9.0), Km= 1.67×10^{-3} M/L
——○—— incubated for 10 min. at 30°C by hydrazine sulfate trapping method (pH 7.4), Km=0.80×10^{-3} M/L

tris-HCl buffer by the cyanide trapping method, and was at pH 7.1 by the hydrazine sulfate method.

Effect of Substrate Concentration

The Michaelis constant, Km, for the reaction is also dependent on the assay system. That is 1.67×10^{-3} at pH 9.0 and 0.8×10^{-3} at pH 7.4 for the rainbow trout muscle aldolase (Fig. 6). Shibata (1), and Kwon and Olcott (2) reported

TABLE 1. *Effect of metal ions on rainbow trout muscle aldolase*

Metal ion	Final concentration (Mol)	Inhibition (%)
Hg^{++}	1×10^{-4}	100
	5×10^{-5}	56
	1×10^{-5}	30
	5×10^{-6}	7
Cu^{++}	5×10^{-4}	100
	1×10^{-4}	73
	5×10^{-5}	27
	1×10^{-5}	0
Mg^{++}	5×10^{-3}	57
	1×10^{-3}	40
	5×10^{-4}	0
Fe^{++}	5×10^{-3}	100
	5×10^{-4}	47
	1×10^{-4}	10
Zn^{++}	5×10^{-3}	100
	1×10^{-3}	40
	5×10^{-4}	0

that it was 1.4×10^{-3} at pH 9.0 and 4.5×10^{-4} at pH 7.5 for the carp muscle aldolase and was 2×10^{-3} for the tuna muscle aldolase, respectively. As mentioned above, the Km value for the rainbow trout muscle aldolase is close to those reported for the carp and tuna aldolases. On the contrast, it was found to be 1×10^{-5} to 6×10^{-5} for the animal enzyme by the optical method. It is suggested that the Michaelis constant for the fish aldolase is generally higher than that of animals.

Effect of Metal Ions on the Aldolase Activity

It is known that the class II aldolase demands divalent metal ion on the activation, while class I aldolase does not. In the present studies, the inorganic compounds such as $HgCl_2$, $CuCl_2$, $ZnSO_2$, $MgCl_2$, and $FeSO_4(NH_4)_2\ SO_4$ were disssolved in glycine buffer at pH 9.0 and their effects were examined on the aldolase activity.

As pointed out earlier, the activity of the rainbow trout muscle aldolase was inhibited by the divalent metal ions which contaminated the reagents used (Table 1). Among them the inhibitory action of Hg^{++} was the most powerful. The inhibition of other metal ions appeared at 10^{-3} or 10^{-4}M of the ions which were lower than their concentration to make inactive the activity of carp and rabbit aldolases. It is noticeable that the rainbow trout aldolase is more susceptible to divalent metal ions than that from different sources. Although Shibata reported that the carp enzyme was not inactivated by EDTA, the rainbow trout aldolase reduced the activity by 20 per cent of it at 5×10^{-2} M. Considering that the fish

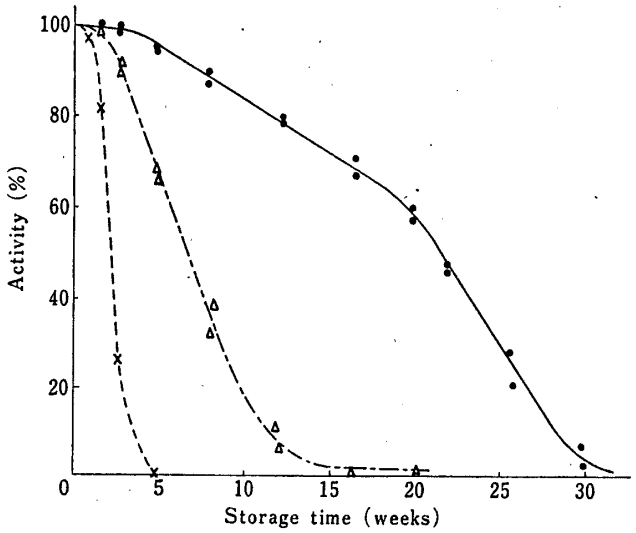


FIG 7. Changes in the aldolase activity of rainbow trout flesh during storage at various temperature.

--×--×-- storaged at 0°C —△—△— storaged at -10°C

—•—•— storaged at -25°C

enzyme was very sensitive to a small amount of EDTA or a trace of divalent metal ions, it may be due to the purity of the enzyme concerned.

Furthermore Shibata found that the activity of the carp aldolase was lessened by 8.5 per cent at 5×10^{-3} M cysteine, while the rainbow trout aldolase was reduced to about 50 per cent by cysteine at 1×10^{-3} M. Thus Shibata postulated that the SH group also takes part in the activity of the enzyme.

Changes in Aldolase Activity during Frozen Storage

Rainbow trout, which was 600 to 800 g in body weight, was stunned by a blow on the head, decapitated, skinned and filleted. The dark muscle located under the lateral line was removed, and then the fillet was wrapped in polyethylene pouch, and stored at 0°C, -10°C and -25°C, respectively. The aldolase activity of the muscle was determined as follows: 5 g of muscle cut off from the central part of the fillet was ground with sand and 10 ml of tris-HCl buffer (pH 7.3) at 2°C. in the mill. The homogenate was centrifuged for 30 minutes at 12,000 g and passed through a filter paper (Toyo filter paper No 4). The filtrate was diluted with 50–60 volumes of cold water. Aldolase activity of it was assayed by the cyanide trapping method and the enzyme concentration was determined by the Biuret method. The results are shown in Fig. 7.

The enzyme activity of the rainbow trout muscle stored at 0°C (unfrozen) was completely reduced to zero after 5 weeks. In case of the sample stored at -10°C, it was inactivated after 15 weeks. However, the muscle stored at -25°C kept its activity at the maximum until 4 weeks. After that it gradually depressed but

remained at 60 per cent of its maximum activity till 20 weeks. Thus the changes of the aldolase activity of the rainbow trout muscle during storage is very slight when stored at −25°C.

Connell (5) reported that the cod and haddock muscles stored at −14°C demonstrated that aldolase activity of about one-fourth of the maximum even after 60 weeks. Furthermore he suggested that the cod muscle was more affected by freezing and immediate thawing than haddock muscle. It is seen that the rainbow trout muscle aldolase was more affected by freezing and immediate thawing than cod muscle.

Discussion

The enzymic properties of the rainbow trout muscle aldolase are different from those of mammalian enzyme. Especially the thermal optima for the maximum velocity and the temperature of heat denaturation of the fish aldolase were lowered by over 10°C than those of the rabbit muscle aldolase. It is said that the rainbow trout aldolse is thermo-labile. This enzyme, however, demonstrates a high activity at below 20°C as compared with the aldolase of animals and other fishes such as carp and tuna. Furthermore it is very interesting to note that the aldolase of tuna and carp which are subtropical or warm water fishes are less stable than those of the rabbit, but more stable than those of the rainbow trout. The thermostability of this enzyme may be dependent on the habitat temperature of the species concerned.

The pH profiles and Michaelis constant of the reaction catalyzed by the rainbow trout muscle aldolase resemble those of other aldolases. Although the effect of temperature on Michaelis constant (Km) was not investigated in the present studies, it is known that the minimum of Michaelis constant for lactic dehydrogenase (12) and pyruvic phospho-kinase (13) was found at the environmental temperature of the fish studied. Komatsu and Feeney (3) reported that, when the Km values were determined for the different muscle aldolases, its minimum point was not found at the environmental temperature of the cold-adapted fish.

Activation energy of the rainbow trout aldolase is also lower than that of other aldolases, although it is said that the activation energy does not correlate with the environmental temperature by Hochachka and Somero (13).

The activity of this fish enzyme was greatly inhibited by divalent metal ions. It is known that class I aldolase is present in higher animals, higher plants, protozoas, and green algae and is inhibited by divalent metal ions, and that class II aldolase is present in bacteria, yeast and blue green algae and demands metal ions. Therefore rainbow trout muscle aldolase belongs in the former type. On the contrast, carp or tuna muscle aldolase was not inhibited by EDTA, while the rainbow trout aldolase was done by the same agent. Shibata suggested that EDTA protected the enzyme against being tainted with metal ions. In our ex-

periments it is suggested that EDTA is not only chelating substance but also a kind of inhibitor for the aldolase.

It was demonstrated that the aldolase activity of rainbow trout muscle was decreased during storage. Thus this enzyme in storing muscle was more susceptible than the other aldolase from different sources in the same manner.

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