Histidine Metabolism in Fish—III. Purification and Some Properties of Histidine Deaminase from Mackerel Muscle

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In a previous paper, we have reported that the urocanic acid pathway possibly occurs in fish tissues¹⁾ and that the enzyme, histidine deaminase, catalyzing the initial step of the pathway appears in the livers of all fish but in the muscle of only a few kinds of fish²⁾.

This enzyme activity has been reported to be found in vertebrate liver³⁾, human epiderma⁴⁾ and bacteria⁵⁾. The properties of the enzyme⁶⁾ and the mechanism of action⁷⁾ were examined by the use of mammalian liver and *Pseudomonas fluorescens*, respectively.

The present paper deals with the partial purification of histidine deaminase and some properties of the enzyme isolated from fresh mackerel muscle.

Some differences in the properties were observed between the present enzyme and the enzymes of liver and of bacteria. SH compounds and the metal chelator, EDTA**, which have been reported to influence on the catalytic activities of the last two enzymes, did not significantly affect the activity of the muscle enzyme.

Materials and Methods

Materials. The muscle in a *rigor mortis* of mackerel, *Scomber japonicus*, was used for the experiments.

Enzyme assay. Enzyme activity was assayed by the method of TABOR and MEHLER⁸⁾ with a slight modification. The standard assay mixture contained 2 ml of 0.25 M Tris-phosphate-HCl buffer (pH 9.0), 10 μ moles of histidine and 0.5 ml of the enzyme solution in a total volume of 3.0 ml. The mixture was incubated at 37°C and the reaction was stopped by the addition of 0.5 ml of 30% perchloric acid, the precipitated proteins were removed by filtration. The increase in UV absorption of the filtrate, which is due to the formation of urocanic acid, was measured at 277 m μ , pH 7.5. The enzymatic unit was expressed as micromoles of urocanic acid formed per minute.

Protein determination. Protein was determined by the method of LOWRY *et al.*⁹⁾

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** Abbreviations used are: EDTA, ethylenediaminetetraacetate; GSH, glutathione; pCMB, *p*-chloromercuribenzoate.

Results and Discussion

Purification of the enzyme. Fresh muscle (2.4 kg), which was completely free of bloody muscle, was homogenized with 3-fold volumes of 1% KCl. After centrifugation at $10,000\times g$ for 20 minutes, the supernatant (*Crude extract*) served as the starting material for purification of the enzyme.

To the crude extract, solid ammonium sulfate was added to 70% saturation, the pH being kept at 7.0 with solid K_2HPO_4 . After standing for 24 hours, the precipitate was collected by centrifugation, dissolved in 0.02 M phosphate buffer (pH 6.8), and the solution was subjected to dialysis against 5 liters of the same buffer. The resulting insoluble material was sedimented by centrifugation.

To the supernatant (*Fraction I*), L-histidine·HCl·H₂O was added to a concentration of 0.1 M. This solution was adjusted to pH 8.0 with 0.5 M Tris (hydroxymethyl) aminomethane, and heated at 55°C for 15 minutes, followed by centrifugation at $10,000\times g$ for 20 minutes.

The supernatant solution (*Fraction II*) was subsequently fractionated with $(NH_4)_2SO_4$ between 35% and 65% of saturation. The precipitate was dialyzed against 0.02 M phosphate buffer (pH 8.0) for 12 hours.

The dialyzed solution (*Fraction III*) was applied to a column of DEAE-Sephadex A-50 (4.8×50 cm) which was previously equilibrated with 0.02 M phosphate buffer, pH 6.8; then the protein was eluted with a phosphate buffer gradient from 0.02 M to 0.20 M (pH 6.8). The enzyme was usually recovered between fractions No. 100 and No. 140 (10 ml/fraction). This enzyme was not absorbed by the column of DEAE-cellulose which PETERKOFISKY⁷⁾ used for purification of the bacterial histidine deaminase.

After the fractions containing the activities were pooled, concentrated and dialyzed in the same manner, the dialyzed solution (*Fraction IV*) was chromatographed again on the second DEAE-Sephadex A-50 column (2.6×50 cm) with a gradient elution from 0.020 M to 0.075 M phosphate (pH 6.8). The elution pattern is shown in Fig. 1. The enzyme fraction recovered between fractions No. 48 and No. 60 was combined, concentrated and dialyzed in the same way. At this step the specific activity of enzyme is approximately 100 times as high as that of the crude extract.

The solution after dialysis (*Fraction V*) was subjected to chromatography on a hydroxylapatite column (5.5×7.5 cm), which was previously equilibrated with 0.1 M phosphate buffer, pH 6.8. The proteins were eluted by the buffer increasing the concentration stepwise from 0.1 M to 0.2 M via 0.15 M, followed by a gradient from 0.2 M to 0.4 M (pH 6.8), as shown in Fig. 2. Considerable amounts of proteins which contained no enzyme activity were found in the eluates of both 0.10 M and 0.15 M phosphate.

The fraction from No. 100 to No. 165 were combined and concentrated as above (*Fraction VI*) and applied to a column of Sephadex G-200 (3×65 cm), previously washed

Fig. 1. The second DEAE-Sephadex chromatography of muscle histidine deaminase.

A linear gradient from 0.020 M to 0.075 M (pH 6.8) of phosphate was applied for elution of the proteins. Fractions within the horizontal arrow were combined (= *Fraction V*).

●—●, activity; ○—○, protein; ----, phosphate

Fig. 2. Chromatography of muscle histidine deaminase on hydroxylapatite.

A gradient from 0.2M to 0.4M of phosphate was applied after the elution of a large portion of the enzyme fraction. Fractions within the horizontal arrow were combined (= *Fraction VI*).

●—●, activity; ○—○, protein

Fig. 3. Gel-filtration of muscle histidine deaminase on Sephadex G-200.
Fractions within the horizontal arrow were combined (= *Fraction VII*).
●—●, activity; ○—○, protein

Table 1. Purification of histidine deaminase from mackerel muscle.

Fraction	Procedure	Total volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (10^{-3} unit/mg)
<i>Crude extract*</i>	—	7,300	160,600	14.5	0.09
I	First $(\text{NH}_4)_2\text{SO}_4$ -precipitation (0~0.7 saturation)	1,490	74,700	10.9	0.14
II*	Heat-treatment	1,310	17,200	8.3	0.48
III	Second $(\text{NH}_4)_2\text{SO}_4$ -precipitation (0.35~0.65 saturation)	350	10,100	7.5	0.74
IV	First DEAE-Sephadex chromatography	700	6,700	5.4	0.81
V	Second DEAE-Sephadex chromatography	350	343.0	3.6	10.5
VI	Hydroxylapatite chromatography	75	203.1	2.9	14.3
VII	Sephadex G-200 gel-filtration	60	56.5	2.1	37.2

* In order to remove excess amount of free histidine, dialyzed against 0.1 M phosphate buffer, pH 6.8 for 24 hours before assay.

with 0.1M phosphate buffer (pH 6.8). The proteins were eluted with phosphate buffer as shown in Fig. 3.

The fractions between No. 39 and No. 50 (*Fraction VII*), containing high enzymatic activity, were pooled and stored at 5°C with little loss of the activity over a period of several weeks after concentration with $(\text{NH}_4)_2\text{SO}_4$.

An outline of the purification procedures is presented in Table 1. The material in Fraction VII was used to investigate the properties of the enzyme.

Stability of the enzyme. Stability of the muscle enzyme was examined at pH 8.0 at various temperature. As shown in Fig. 4, this enzyme was relatively stable below 55°C but unstable above this temperature. The same test was also carried out using the enzyme in Fraction I prior to heat-treatment and almost the same results were obtained as with the purified enzyme.

Some workers have shown the availability of heat-treatment for purification of the enzyme from liver⁶⁾ and bacteria¹⁰⁾, and therefore, histidine deaminase seems to be a relatively heat-stable enzyme.

A pH stability test was carried out at the range of pH 4.0~10.0 at 37°C. The muscle enzyme was found to be stable between pH 6.2~10.0, but significantly unstable below pH 6.2.

Fig. 4. Heat-stability of muscle histidine deaminase.

The enzyme (154 μg) was incubated in 0.05 M Tris-acetate buffer (pH 8.0) for 15 min. at various temperatures, then rapidly cooled in an ice bath. The reaction mixture contained 1 ml of the enzyme solution (154 μg), 300 μ moles of phosphate buffer and 10 μ moles of histidine in total volumes of 4.5 ml. After the mixture was incubated at 37°C, the reaction was stopped by adding 60% perchloric acid. With an aliquot of the filtrate, the urocanic acid formed was estimated spectrophotometrically at 277 $m\mu$ (pH 7.3).

Response to pH. Response of the enzyme activity to pH is illustrated in Fig. 5. The enzyme was observed to be relatively active under alkaline condition and the maximum activity occurred at pH 9.0. This value is in accord with

Fig. 5. The effect of pH on the activity of histidine deaminase from the muscle.

●—●, phosphate buffer (0.1 M)
○—○, carbonate buffer (0.1 M)

Table 2. Effect of various chemicals on the histidine deaminase activity of mackerel muscle.

Chemicals	Concentration (M)	Relative activity (%)
None	—	100
Cu ⁺⁺	1 × 10 ⁻³	86
Zn ⁺⁺	1 × 10 ⁻⁴	100
Cd ⁺⁺	1 × 10 ⁻³	1
Co ⁺⁺	1 × 10 ⁻⁴	89
EDTA	1 × 10 ⁻³	100
Cysteine	1 × 10 ⁻³	66
Cysteine	1 × 10 ⁻⁵	100
GSH	1 × 10 ⁻³	94
GSH	1 × 10 ⁻⁴	96
Thioglycol	1 × 10 ⁻³	90
Indoacetamide	1 × 10 ⁻³	90
Indoacetamide	1 × 10 ⁻⁴	95
pCMB	1 × 10 ⁻⁴	13
pCMB	1 × 10 ⁻⁴	66
+cysteine	1 × 10 ⁻³	

Prior to the assay, the standard assay mixtures without histidine containing 77 μ g of the Fraction VII enzyme were incubated with the various chemicals for 5 min. Metal ions were added as CuCl₂, ZnCl₂, cadmium acetate, and cobalt acetate.

that reported by TABOR and MEHLER⁸⁾.

Effect of inhibitors on enzyme activity. As shown in Table 2, Zn⁺⁺ (10⁻⁴ M) and the metal chelator, EDTA (10⁻³ M) did not affect the enzyme activity. Iodoacetamide, GSH, cysteine, thioglycol, Cu⁺⁺, and Co⁺⁺ showed a slight inhibition, while pCMB and Cd⁺⁺ markedly inhibited the enzyme activity. Inhibition by pCMB (10⁻⁴ M) was reversed by cysteine to the extent of the action of itself (66% relative activity).

Since EDTA did not act as an inhibitor by chelating metal ions, it appears that the enzyme has no requirement for co-factors. The metal ions, such as Zn⁺⁺, Co⁺⁺, and Cd⁺⁺, have been known as activators for the liver and the bacterial enzymes^{8,11)}, however, these ions did not stimulate the reaction catalyzed by the muscle enzyme.

PETERKOFISKY and MEHLER¹²⁾ found the inhibition of the bacterial enzyme by cysteine and its derivatives as well as EDTA and demonstrated that the inhibition was all due to metal chelation. However, TABOR and MEHLER⁸⁾ and PETERKOFISKY⁷⁾ had described the activation by SH compounds such as thioglycolate or thioglycol. All the SH compounds investigated here rather inhibited than stimulated the activity of the muscle enzyme, indicating that cysteine and GSH do not act as metal chelators but behave as inhibitors with SH groups to the enzyme. The evidence thus obtained further provides a hypothesis that the muscle enzyme has a site which is easily affected by the SH compounds, in addition to an active site containing SH groups. The fact that there was no EDTA-inhibition and no thioglycol-activation, appears to show that the enzyme is a slightly different type of histidine deaminase from that of the liver and Pseudomonads.

The effects of activators and the comparison of the affinity to histidine between the muscle enzyme and the liver enzyme obtained from mackerel, will be reported in the next paper.

Summary

1. A procedure for obtaining a partially purified preparation of histidine deaminase from mackerel muscle was developed and some properties of the enzyme were examined.
2. The deaminase was purified from mackerel muscle about 380-fold using a method of fractionation employing ammonium sulfate, heat-treatment, DEAE-Sephadex chromatography, hydroxylapatite chromatography and Sephadex G-200 gel-filtration.
3. The enzyme was relatively stable at the temperature below 55°C, at the alkaline condition.
4. The pH optimum of the enzyme was found at 9.0.
5. Metals, with the exception of Zn and *p*CMB showed slight or strong inhibitory effects on the enzyme action, but EDTA did not cause an inhibition. SH compounds did not activate the enzyme reaction, however, the *p*CMB-inactivation was recovered by the addition of cysteine.

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