Studies on Amino Acids. V.

Studies on the Enzymatic Resolution (IV). Enzymatic Resolution of DL-Methionine (2)*

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The enzymatic resolution of acetyl-DL-methionine previously reported was studied further, in detail. As a result, it was found that metal ion plays an important role on the asymmetric hydrolysis of acyl-DL-methionines by the enzyme preparations of *Aspergillus oryzae*.

In the previous paper¹⁾, the authors reported the enzymatic resolution of DL-methionine through the asymmetric hydrolysis of acetyl-DL-methionine by the enzyme preparations obtained from *Aspergillus oryzae* and pancreas. For the purpose to obtain further advantageous conditions of the resolution process, a study on the more detail of the enzymatic reaction was carried out and is presented in this paper. Especially, the influence of metal ion toward the enzyme prepared from *Aspergillus oryzae* was investigated and compared with that of renal acylase.

MATERIALS AND METHODS

The substrate, acetyl-DL-methionine was prepared by acetylation of DL-methionine with acetic anhydride in acetic acid, and chloroacetyl-L-methionine was prepared by the Schotten-Baumann reaction.

The extent of the enzymatic hydrolysis was determined by measurement of the liberated amino acid with the ninhydrin method or the van Slyke procedure. For the comparison of enzyme activity, the measurement was carried out when the susceptible substrate was from 10 to 35 per cent hydrolyzed.

EXPERIMENT AND RESULTS

"Influence of Metal Ions"

1) *Effect to the Pancreas Enzyme* In the previous

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1) S. Yamada, I. Chibata and S. Yamada, *J. Pharm. Soc. Jap.*, **75**, 113 (1955).

paper, the authors reported that pancreatic enzyme preparations such as pancreatin and prot-amylase could hydrolyze acetyl-DL-methionine, asymmetrically. In this enzymatic hydrolysis, the influence of metal ions was investigated by the addition of metal chlorides to the reaction mixture and the results are listed in Table I.

As shown in the Table, Zn^{++} showed apparent inhibitory effect and Mn^{++} was also slightly inhibitory. There was no ion capable of accelerating the enzyme reaction significantly.

2) *Effect to the Aspergillus Enzyme (Mold Acylase)* Effect of metal ions towards the hydrolytic action of the enzyme produced by the *Aspergillus oryzae* such as Taka-diestase extract and the partially purified preparation, was investigated with the addition of metal chlorides. The partially purified preparation employed for this study was prepared as follows: The extract of Taka-diestase was brought to 0.6 saturation with ammonium sulfate at pH 6.0 and centrifuged. The precipitate was dissolved in water and dialyzed for 70 hours against cold flowing water. To the dialyzed solution acetone was added (60% by volume) at $-3 \sim -4^\circ$ and the precipitate was collected and dried.

TABLE I.
EFFECT OF METAL IONS ON THE HYDROLYSIS OF
ACETYL-DL-METHIONINE BY PANCREATIN⁺

Activity in the Presence of the Ions									
Metal	None	Cu	Mg	Ca	Zn	Mn	Co	Ni	
Activity ⁺⁺	100	108	111	114	48	75	105	111	

⁺A solution containing 0.1M acetyl-DL-methionine, pancreatin extract and $10^{-3}M$ metal chloride was incubated at 37° and pH 6.8, for 24 hours.

⁺⁺Activity expressed as per cent of control.

TABLE II
EFFECT OF METAL IONS ON THE HYDROLYSIS BY *ASPERGILLUS* ENZYME

Exp. No.	Enzyme	Activity in Presence of the Ions								
		None	Cu	Mg	Ca	Zn	Cd	Mn	Co	Ni
1*	Taka-diestase extract	100	9	101	80	16	27	29	181	71
2**	Partially purified prep.	100	0	100	97	39	19	85	150	82

*A solution containing 0.1M acetyl-DL-methionine, Taka-diestase extract and 10^{-5} M metal chloride was incubated at 37° and pH 6.8 for 24 hours.

**A mixture of 1ml of 0.05M acetyl-DL-methionine, 1ml of the enzyme solution, metal chloride (10^{-3} final concentration) and 1ml of Veronal buffer was incubated at 37° and pH 7.0, for 1.5 hours.

This powdered preparation, about 10-fold purified, was dissolved in water and used for the experiment.

The results of the study, which are shown in Table II, disclosed that Cu^{++} , Cd^{++} and Zn^{++} were inhibitory with both preparations. Although Mn^{++} was apparently inhibitory in prolonged incubation with the crude extract, (Table II, exp. 1), the ion showed no decrease of activity under the conditions employed in exp. 2. Besides exp. 1, shown in the Table, another experiment employing phosphate buffer was also carried out, and the only difference observed was the lack of inhibitory effect of Zn^{++} , while, activation by the addition of Co^{++} was evident when either the crude extract or dialyzed preparation was employed in this enzymatic hydrolysis.

"Inactivation by EDTA and Reactivation by Metal Ions"

1) *Effect of EDTA* By the above described experiment, it was disclosed that Co^{++} enhance the

FIG. 1. Effect of EDTA on the Enzymatic Hydrolysis.

To the dialyzed extract of Taka-diestase treated with various concentrations of EDTA, 0.4M neutralized acetyl-DL-methionine was added and incubated at 37° and pH 6.8, for 2.5 hours. Activity of the enzyme solution without EDTA treatment, taken as 100.

hydrolytic action of the enzyme produced by *Aspergillus oryzae*. So, in order to find out whether Co^{++} is the mere activator or the enzyme requires essentially the metal ion for the hydrolytic action, the effect of metal chelating agent, EDTA, was investigated.

Namely, the extract of Taka-diestase was dialyzed against cold water for 2 days. No decrease of activity of the enzyme was observed during the dialysis. The dialyzed enzyme solution was treated with EDTA in concentrations ranging from 10^{-2} to 10^{-8} M, at 0° and pH 7 for 1 hour, then hydrolytic activity was determined. As shown in Fig. 1, the enzyme was markedly inactivated from the 10^{-4} M concentration of EDTA.

2) *Reactivation by Metal Ions* For further study of the relation between the enzyme action and metal ion, along with the experiment on the effect of metal ions to the partially purified preparation of the *Aspergillus* enzyme described in the previous chapter, the experiment was simultaneously carried out with the EDTA treated enzyme so as to investigate the reactivation by metal ions. The enzyme solution was treated with a 10^{-3} M concentration of EDTA at room temperature and pH 7 for 1 hour, then dialyzed against cold distilled water for 2 days. This solution was preincubated with metal chloride and activity was determined. The activity was represented as per cent of control without EDTA treatment for comparison and is shown in Table III.

The treatment with EDTA abolished almost completely the enzymatic hydrolysis of acetyl-DL-methionine. Although this inhibition could be reversed only partially, by the addition of Zn^{++} and other ions, reactivation by Co^{++} proved to be most remarkable among the metal ions investigated. (When reactivated with Co^{++} , the EDTA treated-enzyme showed activity even higher than that of those treated. This may be interpreted that the inhibitory substance of the preparation is removed by EDTA treatment and activation of Co^{++} becomes more effective).

TABLE III
REACTIVATION OF INACTIVATED ENZYME BY METAL IONS

EDTA treatment	Activity in the Presence of the Ions							
	None	Cu	Mg	Ca	Zn	Cd	Mn	Co Ni
None	100	0	104	97	45	19	100	146 86
Treated	5	0	9	19	60	25	7	176 46

A mixture of 1ml of enzyme solution, metal chloride (final concentration, $10^{-3}M$) and 1ml of Veronal buffer was pre-incubated at 37° , for 1 hour. Then 1ml of $0.05M$ acetyl-DL-methionine was added and incubated at 37° and pH 7.0, for 1.5 hours.

These results suggest that the enzyme is a metal enzyme, that is, the metal ion is not separated by dialysis, however, the enzyme is apparently inactivated by EDTA and reactivated by Co^{++} . Although the decision as to whether or not Co^{++} is an actual component of the enzyme must necessarily wait, until the enzyme has been isolated in the pure form.

"Activation by Cobalt and its Practical Application" The fact that Co^{++} activates the enzymatic hydrolysis of acetyl-DL-methionine, should be applied advantageously in the practical process of enzymatic resolution of DL-methionine. So, studies on the optimal concentration of Co^{++} and its practical resolution in the presence of metal ion were carried out.

1) *Effect of Cobalt Ion Concentration* The effect of various concentrations of Co^{++} on the enzymatic hydrolysis of acetyl-DL-methionine were investigated. The result, shown in Fig. 2, indicated that the optimal concentration for activation was between $10^{-4}M$ to 10^{-3} , concentration higher than $10^{-2}M$ decreased activation under the conditions employed by the authors. However, it may be possible that the optimal concentration for the activation somewhat various accord-

FIG. 2. Effect of Co^{++} Concentration on the Enzymatic Hydrolysis.

A solution containing $0.1M$ acetyl-DL-methionine, Taka-diestase extract and various concentrations of cobalt chloride was incubated at 37° and pH 6.8, for 21 hours.

ing to the purity and concentration of the enzyme employed.

2) *Practical Application* It is evident from the above experiments that Co^{++} activates the enzymatic hydrolysis. Although, in the practical resolution, it is desirable to employ a higher substrate concentration and a less amount of enzyme preparation. So, in order to confirm whether or not the activated enzyme with the addition of Co^{++} advantageously accomplishes, without denaturation, the asymmetric hydrolysis of the susceptible component of the substrate than that of without activation, practical resolution process was carried out with and without the addition of Co^{++} . The reaction mixture consisting of $0.6M$ acetyl-DL-methionine, extract of Taka-diestase (corresponding to 1/20 and 1/40-weight of the substrate) and $6 \times 10^{-4}M$ Co^{++} was adjusted to pH 6.8 and incubated at 37° . The rate of hydrolysis was compared with the control run without the addition of Co^{++} .

The results, summarized in Fig. 3, show the advantage of the addition of Co^{++} . The practical

FIG. 3. Asymmetric Hydrolysis of Acetyl-DL-methionine in the Presence and Absence of Co^{++} .

Solutions containing $0.6M$ neutralized acetyl-DL-methionine and an extract of Taka-diestase (1/20 and 1/40-weight of the substrate) with and without the addition of Co^{++} were incubated at 37° , and pH 6.8.

isolation of L-methionine was also carried out by the method as described in the previous paper. The L-methionine, obtained in the same yield as by the previous method, was optically pure and showed no contamination with Co^{++} , $[\alpha]_D^{25} = +23.0^\circ$ (C 3, N·HCl). Anal. Found: C, 40.01; H, 7.23; N, 9.48. Calcd. for $\text{C}_5\text{H}_{11}\text{NO}_2\text{S}$: C, 40.25; H, 7.43; N, 9.39. So the enhancement of enzymatic hydrolysis by Co^{++} can be said to be a very useful procedure for the shortening of incubation-time and the curtailment of enzyme preparation in the resolution of acetyl-DL-methionine for preparative purpose.

"Comparison of Renal and Mold Acylases in Metal Activation"

Greenstein and his coworkers have made extensive studies on the hog kidney enzyme which hydrolyzes acyl amino acids and named the purified enzyme acylase I. They investigated the effect of Co^{++} towards the hydrolysis of chloroacetyl and glycyl-amino acids with this enzyme²⁾, and reported that the hydrolytic rate of chloroacetyl-L-methionine was inhibited by all concentrations of Co^{++} applied namely, from 10^{-2} to 10^{-4}M (percent decrease -62 to -15). The authors recognized that similarly with renal acylase, the enzyme produced by mold is capable of hydrolyzing acyl amino acid, although the hydrolysis of acetyl-DL-methionine by the enzyme was markedly accelerated by the presence of Co^{++} .

In order to ascertain the question whether this discrepancy between renal and mold acylases with regard to the effect of Co^{++} , is due to the difference of the characters of both enzymes or either due to the dif-

the hydrolysis of acetyl-DL-methionine and chloroacetyl-DL-methionine by employing hog kidney acylase and mold acylase under the same conditions which were employed by Greenstein.

As for mold acylase, the partially purified preparation described in the previous chapter and an extract of wheat bran cultivated with *Aspergillus oryzae* No 9³⁾ were employed; Renal acylase I., was prepared from hog kidney⁴⁾. The enzyme solution and Veronal buffer were preincubated with cobalt chloride (final concentrations, 10^{-3} and 10^{-4}M) at 37° , for 5 minutes before adding the substrate.

The neutralized substrate solution, composed of 0.05M acetyl-DL-methionine and 0.025M chloroacetyl-L-methionine, was added and it was incubated from 1 to 2 hours. Simultaneously, the enzyme reactions in the absence of Co^{++} were performed for the controls. The data are shown as percent increase or decrease in their rates and are summarized in Table IV.

With acylase I, the hydrolysis of chloroacetyl-L-methionine was apparently inhibited by Co^{++} , which coincides with the report of Greenstein; although, in the case of acetyl-DL-methionine, the inhibitory effect of Co^{++} was found to be less than that of the former substrate. While, in the case of mold acylase, the hydrolytic rates of the both substrates were markedly accelerated by either partially purified preparation or crude extract in the presence of Co^{++} . It is of interest to note that, renal and mold acylases, which hydrolyze acyl amino acid, behave differently towards Co^{++} in the hydrolysis of chloroacetyl-L-methionine under the conditions employed. The authors are now study-

TABLE IV
EFFECT OF Co^{++} ON THE RENAL AND MOLD ACYLASES

Substrate	Concentration of Co^{++} (M)	Renal Acylase I	Mold	
			Extract	Purified prep.
Acetyl-DL-methionine	0	100	100	100
	10^{-3}	90	123	155
	10^{-4}	97	122	145
Chloroacetyl-DL-methionine	0	100(332*)	100(173*)	100(215*)
	10^{-3}	70	126	141
	10^{-4}	75	115	120

After 1ml of enzyme solution, 0.7ml of Veronal buffer and 0.3ml of cobalt chloride (final concentrations, 10^{-3} and 10^{-4}M) were preincubated at 37° for 5 minutes, 1ml of the neutralized substrate (0.05M acetyl-DL-methionine and 0.025M chloroacetyl-L-methionine) was added and incubated at 37° and pH 7, for 2 hours.

* Values are expressed as per cent of acetyl-DL-methionine.

ference of the substrates, the authors have investigated

2) K.R. Rao, S.M. Birnbaum, R.B. Kingsley, and J.P. Greenstein, *J. Biol. Chem.*, **198**, 507 (1952).

3) Preparation of the extract will be described in the successive report.

4) S. M. Birnbaum, L. Levintow, R. B. Kingsley, and J. P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952).

ing the enzyme properties of mold acylase, especially of substrate specificity aiming to compare them with other acyl amino acid hydrolyzing enzymes such as renal acylase and carboxypeptidase. These results will be published in the near future.

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