Studies on Amino Acids. VI.

Studies on the Enzymatic Resolution (V). Enzymatic Resolution of DL-Lysine (2)*

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The advantageous resolution procedure of DL-lysine previously reported, consisting of the enzymatic hydrolysis of ϵ -benzoyl- α -acetyl-DL-lysine with mold enzyme preparation was innovated. Susceptibility of ϵ -benzoyl- α -acetyl-DL-lysine towards various enzyme preparations such as kidney, pancreas extracts, renal acylase and carboxy-peptidase, was investigated. As a result, it was found that although the substrate was readily hydrolyzed by the mold enzyme preparations, the other enzyme preparations showed, almost no, if any, hydrolytic activity.

Since there had not been any procedure completely satisfactory for the optical resolution of DL-lysine, the authors studied the enzymatic resolution of DL-lysine, such as the asymmetric synthesis of acyl L-lysine anilide and asymmetric hydrolysis of acyl DL-lysines. As a result, the procedure consisting of the enzymatic hydrolysis of ϵ -benzoyl- α -acetyl-DL-lysine was found to be an advantageous method for the resolution of DL-lysine.

Namely, the advantages of this procedure is as follows: a) The substrate can be easily prepared from the intermediate of the synthesis of DL-lysine; b) the substrate can be readily asymmetrically hydrolyzed by mold enzyme; c) the separation of the hydrolyzed antipode is very simple, for benzoyl-L-lysine crystallizes out during the incubation procedure. These results were reported in our previous paper¹⁾.

The present paper delivers the results obtained by the further study of this enzymatic hydrolysis of the acyl DL-lysine such as metal activation and susceptibility of the substrate to several enzyme preparations.

EXPERIMENT AND RESULTS

Effect of pH

The effect of the pH to the asymmetric hydrolysis of ϵ -benzoyl- α -acetyl-DL-lysine by the enzyme preparation produced by *Aspergillus oryzae* was investigated. Namely, to the neutralized substrate solution, Taka-diestase extract and phosphate buffer of various pH were added and incubated. The liberated amino-N was determined by the ninhydrin colorimetric method

FIG. 1. Effect of pH on the Enzymatic Hydrolysis of ϵ -Benzoyl- α -acetyl-DL-lysine.

A mixture of 2ml of 0.025M neutralized substrate solution, 2ml of M/15 phosphate buffer and 1ml of Taka-diestase extract (corresponding to one-tenth weight of the substrate) was incubated at 37°, for one hour.

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1) I. Chibata, S. Yamada and S. Yamada, This Bulletin, 20, 174 (1956).

and the result was expressed as percent activity of the control at the optimal pH (Fig. 1). As shown in the Figure, the optimal pH for the enzymatic hydrolysis was found to be in the range 7.0 to 7.5.

Effect of Metal Ions

In a previous paper²⁾ the effect of various metal ions to the hydrolysis of acetyl-DL-methionine by the enzyme produced by *Aspergillus oryzae* was studied. Since it is possible that the influence of metal ions varies with difference of the substrates, so the experiments employing ϵ -benzoyl- α -acetyl-DL-lysine as the substrate were carried out. Although both shorter incubation by the addition of 10^{-4} M metal chlorides and prolonged incubation in the presence of 10^{-3} M concentration were performed, no different influence was observed. As listed in Table I, Mn^{++} showed an inhibitory effect while Co^{++} apparently enhanced the enzymatic hydrolysis. The inhibitory effect of Zn^{++} observed in the case of acetyl-DL-methionine was not evident under the conditions employed in this experiment. Presence of Co^{++} activated the enzyme action from 1.8 to 2 times that of the controls, in both substrates.

TABLE I
EFFECT OF METAL IONS ON THE ENZYMATIC
HYDROLYSIS OF ϵ -BENZOYL- α -ACETYL-
DL-LYSINE

Exp. No.	Activity in the Presence of the Ions							
	None	Mg	Ca	Zn	Sr	Mn	Co	Ni
1*	100	105	104	98	100	60	185	86
2**	100	107	102	102	104	22	178	91

*A solution containing 0.01M neutralized substrate, Taka-diestase extract and 10^{-4} metal chloride was incubated at 37° and pH 7.5, for 1 hour.

**A solution containing 0.1M neutralized substrate, Veronal buffer, Taka-diestase extract and 10^{-3} metal chloride was incubated at 37° and pH 7.5, for 24 hours.

Effect of Cobalt Ion Concentration

Since the results of the above experiments indicate that Co^{++} accelerates the enzyme reaction, the effect of various concentrations on the enzymatic hydrolysis by a dialyzed extract of Taka-diestase was investigated. The results, summarized in Fig. 2, disclosed that the optimal concentration for the activation was in the range of 10^{-4} M to 10^{-3} M, and concentration higher than 10^{-2} M retarded the reaction. This observation coincides with the results observed in the enzymatic hydrolysis of acetyl-DL-methionine with a Taka-diestase extract.

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

A solution containing 0.01M ϵ -benzoyl- α -acetyl-DL-lysine, dialyzed extract of Taka-diestase and various concentrations of cobalt chloride was incubated at 37° and pH 7.0, for 2 hours.

Practical Resolution Procedure

It is always desirable from the economical standpoint to obtain a maximum yield of product in a minimum incubation time for the practical resolution of racemic amino acids. So, the previously reported procedure of enzymatic hydrolysis of ϵ -benzoyl- α -acetyl-DL-lysine was modified and carried out in the presence of Co^{++} . To a neutralized solution of the substrate (final concentration, 0.2M), an enzyme solution (corresponding to one-fortieth weight of Taka-diestase to the substrate) and cobalt chloride (final concentration, 10^{-3} M) were added. The digestion mixture was adjusted to pH 7.0, if necessary, and incubated at 37°. A simultaneous run for the determination of the progress of the reaction indicates that the reaction completed within forty hours. The crystals of ϵ -benzoyl-L-lysine separated during the incubation were collected by filtration; yield was over 90%. The chemical hydrolysis with hydrochloric acid and subsequent treatment with ion exchange resin as described in the previous paper¹⁾ gave L-lysine monohydrochloride; yield around 85%, $[\alpha]_D^{25} = +20.6$ (C. 3.5N-HCl). Anal. Found: C, 39.00; H, 8.62; N, 15.12. Calcd. for $C_6H_{15}N_2O_2Cl$: C, 39.46; H, 8.28; N, 15.34.

The analysis of the product showed high optical purity and no contamination with Co^{++} . As in the case of the enzymatic resolution of acetyl-DL-methionine, the addition of Co^{++} is an advantageous innovation of the preparative purpose.

Susceptibility to the Pancreatic and Renal Enzymes

Apart from the authors experiments with mold

2) I. Chibara, A. Watanabe and S. Yamada, This Bulletin, 21, 291 (1957).

enzyme, the attempt by Utzino and Yoneya³⁾ with hog kidney extract has been the only known report on the enzymatic hydrolysis of the ϵ -benzoyl- α -acetyl-DL-lysine. According to their report, the substrate was completely resistant to the hog kidney enzyme. Therefore the authors reinvestigated their experiments and also performed the comparative study of the hydrolytic activity of the pancreatic, renal and mold enzyme preparations to the substrate.

i) *Hydrolysis by Hog Kidney Extract* At first, following the report by Utzino and Yoneya, the hydrolysis by hog kidney extract was determined by the formol titration.

Fresh frozen hog kidney was ground with acetone, filtered and dried. The powder was then stirred for 30 minutes with 7 times its weight of water, and filtered. The filtrate was used as the hog kidney extract. The digestion mixture of the neutralized substrate solution and the extract was incubated and the reaction mixture was titrated at proper intervals. A simultaneous run with Taka-diestase extract was performed for the sake of comparison.

The results are shown in Table II, which indicates that the hydrolysis did not proceed by hog kidney extract as reported, although the coloring of the digestion mixture made the end point of titration indistinct.

TABLE II
ENZYMATIC HYDROLYSIS OF ϵ -BENZOYL- α -ACETYL-DL-LYSINE

Enzyme	Incubation time		
	20 hrs.	68 hrs.	116 hrs.
Kidney ext.	5.6% ⁺	5.6%	4.2%
Taka-diastase ext.	26.5%	44.0%	72.0%

A mixture of 5ml of 0.1M neutralized substrate solution, 10ml of water and 10ml. of enzyme solution was incubated at pH 7 and 37°. After appropriate incubation, 4ml of the digestion solution was taken and determined by formol titration with N/10 sodium hydroxide.

+ Percentage of hydrolysis of the susceptible antipode.

ii) *Hydrolysis by Renal, Pancreas and Mold Extracts* To obtain further accurate results, the comparative studies were performed by the ninhydrin method.

As animal source enzymes, the above mentioned hog kidney extract prepared from fresh frozen beef pancreas followed by the former preparation were employed. Besides the extract of Taka-diastase, the extracts of *Aspergillus oryzae* No. 9 and *Penicillium*

TABLE III
ENZYMATIC HYDROLYSIS OF ϵ -BENZOYL- α -ACETYL-DL-LYSINE

Enzyme prep.	Incubation time		
	20 hrs.	48 hrs.	96 hrs.
Kidney ext.*	—	—	0.5%
Pancreas ext.*	—	—	0.6%
Taka-diastase ⁺	38%	59%	81 %
<i>Oryzae</i> . No. 9. ⁺	46%	64%	80 %
<i>Pen. vinaceum</i>	41%	63%	78 %

*A mixture of 4ml of 0.1M neutralized substrate solution, 2ml of M/15 phosphate buffer, 1.5ml of water and 0.5ml of enzyme solution was incubated at pH 7, and 37°.

⁺A mixture of 5ml of 0.02M neutralized substrate solution, 2.5 ml of M/15 phosphate buffer and 25ml of enzyme solution was incubated at pH 7.5, and 37°.

vinaceum cultivated on wheat bran were also tested as mold enzyme preparations. (The detail of the preparation of the extracts will be described in the next report of this serial work).

The results, summarized in Table III, show that the substrate is neither hydrolyzed by hog kidney extract nor by beef pancreas extract, although the substrate is readily hydrolyzed by all of the mold preparations employed.

iii) *Susceptibility to Renal Acylase and Carboxypeptidase* From the above experiments, it was disclosed that the ϵ -benzoyl- α -acetyl-DL-lysine was not hydrolyzed by kidney and pancreas extracts. So the behavior of the substrate towards further purified enzymes such as carboxypeptidase and renal acylase, which were known to show hydrolytic activity toward the acyl amino acids, were investigated.

The renal acylase was prepared from fresh hog kidney according to the method described by Greenstein and his co-workers⁴⁾. Carboxypeptidase was prepared from beef pancreas by the method of Anson⁵⁾.

TABLE IV
ENZYMATIC HYDROLYSIS OF ϵ -BENZOYL- α -ACETYL-DL-LYSINE

Enzyme	Incubation time		
	20 hrs.	44 hrs.	72 hrs.
Acylase 1	2.0%	3.7%	7.0%
Carboxypeptidase	0 %	0 %	0 %
Taka-diastase	30.0%	62.0%	85.0%

A mixture of 4ml of 0.02M neutralized substrate solution, 2ml of M/15 phosphate buffer and 2ml of enzyme solution was incubated at pH 7.5 (pH 7.0 for acylase 1).

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

5) M.L. Anson, *J. Gen. Chem.*, **20**, 663, 777 (1937).

3) S. Utzino and T. Yoneya, *Ber.*, **85**, 860 (1952).

The substrate was incubated with these enzyme preparations and the Taka-diastrase extract as the control. The extent of hydrolysis was determined by the ninhydrin method. The results listed in Table IV, indicate that the substrate was completely insensitive to carboxypeptidase and it seemed slightly susceptible to acylase I under those conditions employed by the authors. However, the hydrolysis by renal acylase was significantly lesser than that of the mold enzyme preparation.

It is of interest to note that three enzymes, i.e., carboxypeptidase, renal and mold acylases, which

were known to act on acyl amino acids, were differentiated by their behavior towards β -benzoyl- α -acetyl-DL-lysine.

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