Studies on Amino Acids. IV. Studies on the Enzymatic Resolution (III): Enzymatic Resolution of DL-Tryptophan.*

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To find out a convenient method for the resolution of DL-tryptophan, asymmetric synthesis of acyl L-tryptophan anilides, and asymmetric hydrolysis of DL-tryptophan esters and DL-tryptophan amide were investigated. As a result, the enzymatic hydrolysis of DL-tryptophan amide was found to be an advantageous procedure for the resolution of DL-tryptophan.

As a part of the studies on amino acids, the authors have been studying the enzymatic resolution of racemic amino acids, and the results of the investigations on the enzymatic resolution of DL-methionine¹⁾ and DL-lysine²⁾ have been already reported by the authors. This paper presents the results of the study on the resolution of DL-tryptophan.

Several reports on the asymmetric synthesis of acyl L-tryptophan anilide from acyl DL-tryptophan and aniline by papain have been already published (Table I). The authors carried out the experiment by modifying the method reported by Albertson. As a result, asymmetric synthesis was attained and acetyl L-tryptophan anilide was obtained in an almost quantitative yield. However, the following hydrolysis of acetyl L-tryptophan anilide to L-tryptophan was unsuccessful under the conditions employed by the authors. So, it may be possible to say that the resolution procedure through the enzymatic anilide synthesis is not adequate

for the preparation of L-tryptophan.

On the enzymatic resolution of tryptophan esters, Brenner⁶⁾ studied the hydrolysis of the methyl ester and reported that pure L-tryptophan was obtained when crystalline chymotrypsin was employed. As the employment of amorphous chymotrypsin or pancreatin resulted the product of optically lower purity, so the purification procedure through naphthalene- β -sulfonates of the crude isomers was necessary to obtain a pure product. The authors also investigated the hydrolysis of the methyl ester by dissolving the ester in benzene so as to prevent chemical hydrolysis during the enzymatic reaction, and the benzene layer was contacted with an aqueous extract of pancreas enzyme preparation with stirring. However, the optical purity of the hydrolyzed product was somewhat low, namely about 80%. Whereas, on the other hand, Ravinovich⁷⁾ reported the resolution through the enzymatic hydrolysis of the isopropyl ester. The isopropyl ester was therefore hydrolyzed as in the case of the methyl ester. Although the product obtained by this procedure was optically pure, the yield of purified L-tryptophan was not satisfactory. These results are listed in Table II.

As the above described procedures were

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TABLE I
ASYMMETRIC SYNTHESIS OF ACYL L-TRYPTOPHAN ANILIDES

Acyl	L-Anilide (yield)	Ref.
Acetyl	90%	3
Acetyl	77%	4
Acetyl	92%	*
Carbobenzoxy	100%	5

* Result obtained by the authors.

TABLE II
ASYMMETRIC HYDROLYSIS OF DL-TRYPTOPHAN ESTERS

Ester	Enzyme Preparation	L-Tryptophan yield (%)	$[\alpha]_D$	Ref.
Methyl	Cryst. Chymotrypsin	crude 96%	$-30 \pm 2^\circ$	6
"	Pancreas Enz. (naph- β -sulf.)†	crude 90%	-23°	"
"	"	51%	$-32.5 \pm 1^\circ$	"
"	Pancreatin	crude 68%		*
		recryst. 52%	-25.9°	
i-Propyl	Pancreatin	90%	-31.6°	7
"	"	crude 60.3%		*
		recryst. 39.6%	-32.5°	

* Result obtained by the authors.

† Purified as naphthalene- β -sulfonate.

not so satisfactory for the resolution of DL-tryptophan, the authors synthesized a new tryptophan derivative, DL-tryptophan amide, and studied the enzymatic hydrolysis of this substrate. The substrate was prepared from DL-tryptophan by esterification and subsequent amination as shown in Table III. For the hydrolysis of the substrate, enzyme preparation such as pancreatic enzyme, "Protamylase", *Aspergillus* enzyme "Taka-diastrase" and papain were employed and their susceptibility and the optimal pH were investigated. In this experiment, the extent of hydrolysis was estimated by the titration of liberated carboxyl groups according to the Grassman-Hyde⁸⁾ procedure. As a result, it was found

that the amide was asymmetrically hydrolyzed most rapidly by pancreas enzyme, and the optimal pH for Protamylase, papain and Taka-diastrase was approximately 5.5 and 7, respectively. So, the practical resolution of DL-tryptophan amide was carried out by the employment of pancreas enzyme. After the completion of enzymatic reaction, organic solvent-soluble D-tryptophan ester was separated from insoluble L-tryptophan by extraction. The L-tryptophan obtained by this procedure showed high optical purity, and furthermore, the yield was satisfactory. The influence of several metal ions to this enzyme reaction was also studied. The study revealed that, Mn^{++} and Mg^{++} activate the enzyme, while Co^{++} retards the reaction. This metal activation is useful for the practical resolution in

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TABLE III
 SYNTHETIC ROUTE OF DL-TRYPTOPHAN AMIDE

the shortening of incubation and the curtail of enzyme preparation.

The procedure above described through the enzymatic hydrolysis of DL-tryptophan amide can be said to be an advantageous method for the preparation of L-tryptophan. That is, the substrate is readily hydrolyzed by a small amount of easily available pancreas enzyme preparation and optically pure L-tryptophan is obtained in a good yield.

EXPERIMENTAL

Asymmetric Synthesis of Acetyl-L-Tryptophan Anilide. In an Erlenmeyer flask was placed 4.92 g of acetyl-DL-tryptophan, 20 ml of N-NaOH, 2 ml of aniline and 20 ml of citrate buffer (pH 4). The enzyme solution, prepared by extracting 1.2 g of papain with 15 ml of distilled water containing a small amount of potassium cyanide, was then added. The pH was brought up to 4.5 if necessary, and the flask was stoppered and placed in an incubator at 37° for five days. After cooling the mixture, the separated anilide was filtered and washed with water; yield 2.92 g (92%), m.p. 196-199°, $[\alpha]_D^{25} + 66.9^\circ$ (5% in acetic acid).

No way could be found to prepare L-tryptophan from

this compound by acid hydrolysis without extensive destruction.

Asymmetric Hydrolysis of DL-Tryptophan Esters. i) *Enzymatic Hydrolysis of the Methyl Ester.* To a solution of 2.7 g of DL-tryptophan methyl ester dissolved in 20 ml of benzene, 5 ml of 5% pancreatin extract was added and it was then stirred at 37° for 7 hours. After the reaction, the separated crystals were collected by filtration and washed with alcohol; yield 0.85 g (67.2%). Recrystallization of the crude L-tryptophan from aqueous alcohol gave the product, m.p. 272-275° (dec.), $[\alpha]_D^{30} - 25.9^\circ$ (1% in water).

ii) *Enzymatic Hydrolysis of the Isopropyl Ester.* A mixture of 4.1 g of DL-tryptophan and 30 ml of isopropyl alcohol containing 15% hydrogen chloride was refluxed for one hour. After cooling, the separated crystals of DL-tryptophan isopropyl ester hydrochloride were filtered and washed with ether; yield 4.0 g (70.5%), m.p. 227-228° (dec.). A mixture of DL-tryptophan isopropyl ester hydrochloride dissolved in 10 ml of water and 30 ml of ether was cooled to -10°, and aqueous ammonium hydroxide was added under stirring until it became alkaline to phenolphthalein. The ethereal layer was separated and the aqueous layer was repeatedly extracted with ether. The combined ether extracts were dried over anhydrous sodium sulfate. Evaporation of the solvent left the isopropyl

ester, yellow oil; yield 1.4 g (80.5%).

The ester thus obtained, was dissolved in 20 ml of benzene and 5 ml of 5% extract of pancreatin was added. The reaction mixture was stirred at 37° for 8 hours. After completion of the reaction, the separated crude L-tryptophan was collected; yield 0.35 g (60.3%). Recrystallization from aqueous alcohol yielded a pure product, 0.23 g (39.6%), m.p. 277–278° (dec.), $[\alpha]_D^{20}$ –32.5° (1% in water).

Asymmetric Hydrolysis of DL-Tryptophan Amide. i) *Preparation of DL-Tryptophan Amide.* To a stirred suspension of 5 g of DL-tryptophan methyl ester hydrochloride in 80 ml of ether, aqueous solution containing 5.4 g of potassium carbonate was added under cooling in an ice-salt bath. The ethereal layer and the ether extract of the aqueous layer were combined, washed with water and dried over anhydrous sodium sulfate. Evaporation of the solvent left 4 g of the oily free ester. The ester was dissolved in 60 ml of methanol saturated with ammonia and allowed to stand for several days in a stoppered pressure bottle at room temperature. Concentration of the reaction mixture and recrystallizations from methanol-chloroform and water gave DL-tryptophan amide, colorless pillars, m.p. 127–128°, yield 2.8 g (71.5%).

Anal. Found: C, 65.46; H, 6.46; N, 20.65. Calcd. for $C_{11}H_{13}ON_2$: C, 65.00; H, 6.45; N, 20.68.

The amide was dissolved in methanol and dry hydrogen chloride was introduced. Concentration and recrystallization from methanol-chloroform gave the hydrochloride, m.p. 231°.

Anal. Found: C, 54.70; H, 5.48; N, 18.01. Calcd. for $C_{11}H_{14}ON_2Cl$: C, 55.11; H, 5.89; N, 17.53.

The hydrochloride of DL-tryptophan amide was also synthesized from the ester hydrochloride as follows: 5 g of DL-tryptophan methyl ester hydrochloride was dissolved in 70 ml of methanol saturated with ammonia. The reaction mixture was allowed to stand for several days in a stoppered pressure bottle at room temperature. Then the mixture was concentrated in vacuo, the residue recrystallized from methanol-chloroform yielding DL-tryptophan amide hydrochloride; yield 3.4 g (72.0%), m.p. 230–233°. A mixed melting point determination with the sample obtained from the former method showed no depression.

ii) *Optimal pH and Enzyme Activity.* Susceptibility of the substrate and the optimal pH to the various enzyme preparations such as Protamylase (Teikoku Hormone Mfg. Co.), Taka-diestase (Sankyo Co. Ltd.) and Papain (E. Merck Darmstadt) were investigated

under comparable conditions. Namely, to 2 ml of 0.25 M neutralized DL-tryptophan amide hydrochloride solution, 7.5 ml of Veronal buffer in the range of pH 4–9 and 0.5 ml of 10% extract of the enzyme preparation were added. The digestion solution was incubated at 37° for 20 hours and the extent of the reaction was assayed by titration. The results are shown in Fig. 1.

Although all the enzymes tested could hydrolyze the substrate, pancreatic enzyme, Protamylase, was most active. The optimal pH for the action of Protamylase, papain and Taka-diestase was found to be about 8.0, 5.5 and 7.0, respectively. In a longer incubation of the digestion mixture of Protamylase, hydrolysis did not proceed over 100% (50% of the racemic substrate), which indicates the action of pancreas enzyme to the substrate is asymmetric.

FIG. 1. Enzymatic Hydrolysis of Tryptophan Amide.

○—○ Protamylase. ●—● Papain.
×—× Taka-diestase.

iii) *Isolation of L-Tryptophan.* To a solution containing 2.4 g of DL-tryptophan amide hydrochloride, 10 ml of 5% extract of Protamylase was added. The solution was adjusted to pH 8 and filled up to 100 ml with water and/or buffer solution. Incubation was carried out at 37° and the extent of hydrolysis was checked during the reaction. The reaction was usually attained within 70 hours. After the reaction, the incubation mixture was made up to pH 5 with acetic acid and boiled for a while to coagulate the enzyme protein. The separated precipitate was removed and the filtrate was concentrated in vacuo. To the residue 100 ml of alcohol was added and the mixture was allowed to stand overnight in a refrigerator. The separated crude L-tryptophan was collected by filtration and recrystallized from aqueous alcohol; it yielded

FIG. 2. Effect of Metal Ions to the Enzymatic Hydrolysis of Tryptophan Amide.

○—○ Control (no addition). ×—× Mn⁺⁺
 ●—● Mg⁺⁺ △—△ Co⁺⁺

colorless scales of L-tryptophan; yield 0.72 g (70.2%), m.p. 284–287°, $[\alpha]_D^{25} -32.0^\circ$ (0.5% in water).

Anal. Found: C, 64.72; H, 6.03; N, 13.46. Calcd. for $C_{11}H_{12}ON_2$: C, 64.69; H, 5.92; N, 13.73.

iv) *Effect of Metals.* The effect of certain divalent metal ions was studied by the addition to the reaction solution. Each reaction solution consisted of 2 ml of 0.25 M neutralized DL-tryptophan amide hydrochloride solution, 0.5 ml of 2.5% extract of Protamylase, 4.5 ml of distilled water, 1 ml of 0.005 M metal salt solution, and 2 ml of Veronal buffer at pH 8.0. Ions of manganese and magnesium were found to stimulate the enzyme reaction (Fig. 2). Besides these ions, salts of cobalt and strontium were also tested and it was proved that cobalt was inhibitory while strontium showed no effect toward the enzyme. It was found that by adding the proper metal ion to activate the enzyme reaction, incubation of the practical resolution procedure is either shortened or quantity of the enzyme preparation employed can be decreased.

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