Resolution of Amino Acids. II. Resolution of Racemic ε-Acyllysine and δ-Acylornithine Amides by Leucine Aminopeptidase

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In a previous communication1), it was shown that racemic phenylalanine and γ-phenyl-α-aminobutyric acid amides can be resolved by leucine aminopeptidase. In this paper, a similar method is described, which is based upon the asymmetric hydrolysis of the amides of ε-benzoyl-DL-lysine, δ-benzoyl-DL-ornithine and δ-carbobenzoxy-DL-ornithine by a partially purified leucine aminopeptidase derived from swine kidney2).

For the resolution, the initial substrate concentration was set at 0.05 mol., and the course of hydrolysis was followed measuring evolved ammonia. After analysis reveals the theoretical 50% hydrolysis, ω-acyl-L-amino acid was collected by filtration, and ω-acyl-D-amino acid amide in the filtrate was separated by means of ion-exchange resin. In this way, ε-benzoyl-DL-lysine amide was resolved in a good yield. δ-Benzoyl-DL-ornithine amide was hydrolyzed in the same manner. However, the difference of the solubilities in water of δ-carbobenzoxy-L-ornithine and -D-ornithine amide hydrochloride is bigger than that of the corresponding benzoyl derivatives, and therefore the amide of δ-carbobenzoxy-DL-ornithine may be in some cases more suitable for the purpose.

Experimental

Preparation of Leucine Aminopeptidase. — A partially purified enzyme solution of Step 4 by Smith was prepared as described2). Its activities against ω-acyl-amino acid amides are shown in Table I, the proteolytic coefficient (C_1) of L-leucine amide being included for comparison. The rates of hydrolysis were measured by titration of 0.2 to 0.6 ml. samples with 0.005 N hydrochloric acid by means of the Conway microdiffusion method3).

As shown in Table I, ω-acyl-L-amino acid amides are hydrolyzed at 1/4∼1/7 times the rate

TABLE I. PROTEOLYTIC COEFFICIENT (C_1) OF AMINO ACID AMIDES BY PARTIALLY PURIFIED LEUCINE AMINOPEPTIDASE*

Substrates	C_1 at different initial concn. of substrates**		
	0.01 M	0.025 M	0.05 M
L-Leucine amide	5.6	3.2	2.0
ε-Benzoyl-DL-lysine amide	1.3	0.84	0.49
δ-Benzoyl-DL-ornithine amide	1.1	0.73	0.50
δ-Carbobenzoxy-DL-ornithine amide	0.82	0.49	0.32

* Enzyme was preincubated in 0.1 M Tris buffer of pH 8.0 with 0.008 M manganese chloride for one hour at 38°C. Assays were conducted in 0.1 M Tris buffer of pH 8.0 at 38°C with preincubated enzyme added. $C_1 = \log[100/(100-\%\ \text{hydrolysis})]/(\text{min.} \times \text{mg. protein } N \text{ per ml. test solution})$.

** Concentrations of L-form of substrates.

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1) A. Tanaka and N. Izumiya, This Bulletin, **31**, 529 (1958).

2) E. L. Smith, "Methods in Enzymology", Vol. 2, edited by S. P. Colowick and N. O. Kaplan, Academic Press, Inc., New York (1956), p. 88.

3) R. B. Johnston, M. J. Mycek and J. S. Fruton, *J. Biol. Chem.*, **185**, 629 (1950).

for L-leucine amide, a most sensitive substrate for the enzyme. However, the efficient resolution would be still possible with the susceptibilities of ω-acyl-amino acid amides in the orders described above.

Synthesis of Compounds. — *ε-Benzoyl-*DL*-lysine ethyl ester hydrochloride.* — ε-Benzoyllysine[4,5] (200 g.) was suspended in ethanol (4 l.) and dry hydrogen chloride was introduced to saturation at room temperature. The solution was refluxed for one hour and evaporated in vacuo. The residue was dissolved in ethanol (2 l.), and the same procedure was repeated again. Treatment of the residue with dry ether yielded crystals*. These orystals were recrystallized from ethanol-ether; yield, 236 g. (94%); m. p. 91~94°C.

Anal. Found: N, 8.82. Calcd. for $C_{15}H_{23}O_3N_2Cl$: N, 8.90%.

*ε-Benzoyl-*DL*-lysine amide hydrochloride.* — The above compound (189 g.) was dissolved in methanol (3.4 l.) previously saturated with dry ammonia at 0°C, the solution was kept at room temperature for three days and then concentrated in vacuo to dryness. The residual crystals were collected by the aid of methanol and acetone. This was dissolved in hot water, the solution was concentrated, and additions of methanol and acetone yielded crystals; yield, 108 g. (63%); m. p. 235~237°C (decomp.).

Anal. Found: C, 54.73; H, 6.73; N, 14.46. Calcd. for $C_{13}H_{20}O_2N_3Cl$: C, 54.64; H, 7.05; N, 14.71%.

*δ-Benzoyl-*DL*-ornithine ethyl ester hydrochloride.*— This compound was prepared from δ-benzoylornithine[4-6] (236 g.) in the same way as that for benzoyllysine ethyl ester hydrochloride; yield, 288 g. (96%); m. p. 175~177°C.

Anal. Found: N, 9.33. Calcd. for $C_{14}H_{21}O_3N_2Cl$: N, 9.31%.

*α-Carbobenzoxy-δ-benzoyl-*DL*-ornithine ethyl ester.*— To a suspension of the above compound (9.0 g.) in chloroform (90 ml.) was added triethylamine (9.4 ml.). The compound went into solution immediately, and carbobenzoxy chloride[7] (4.8 ml.) was added to the solution under cooling. The mixture was allowed to react at room temperature overnight, washed with water, dilute bicarbonate solution, dilute hydrochloric acid, and water; the organic layer was dried over sodium sulfate and concentrated in vacuo to yield a syrup which was crystallized by the addition of petroleum ether. This was recrystallized from ethyl acetate-petroleum ether; yield, 10.3 g. (86%); m. p. 110°C.

Anal. Found: N, 7.24. Calcd. for $C_{22}H_{26}O_5N_2$: N, 7.03%.

*α-Carbobenzoxy-δ-benzoyl-*DL*-ornithine amide.* — This compound was prepared from the above compound (6.0 g.) by the same procedure as that for benzoyllysine amide hydrochloride and recrystallized from methanol-ether; yield, 4.7 g. (85%); m. p. 205~206°C.

Anal. Found: N, 11.52. Calcd. for $C_{20}H_{23}O_4N_3$: N, 11.38%.

*δ-Benzoyl-*DL*-ornithine amide hydrochloride.*—This compound was prepared from benzoylornithine ethyl ester hydrochloride (150 g.) in the same way as that for benzoyllysine amide hydrochloride and recrystallized from methanol-ether; yield, 87 g. (64%); m. p. 195~197°C.

Anal. Found: C, 53.24; H, 6.68; N, 15.24. Calcd. for $C_{12}H_{18}O_2N_3Cl$: C, 53.04; H, 6.68; N, 15.46%.

This compound was also prepared as follows. α-Carbobenzoxy-δ-benzoylornithine amide (3.7 g.) dissolved in 0.3 N methanolic hydrochloric acid (36 ml.) was treated with dry hydrogen in the presence of palladium black in the usual manner[8]. The filtrate from the catalyst was evaporated in vacuo, and the residual syrup was crystallized easily; yield, 2.5 g. (92%); m. p. 196~197°C.

Anal. Found: N, 15.41. Calcd. for $C_{12}H_{18}O_2N_3Cl$: N, 15.46%.

*δ-Carbobenzoxy-*DL*-ornithine.*—This compound was prepared from ornithine hydrochloride (101 g.) by the same procedure as that for the L-isomer[4,9]; yield, 121 g. (76%); m. p. 250~253°C (decomp.).

Anal. Found: N, 10.71. Calcd. for $C_{13}H_{18}O_4N_2$: N, 10.52%.

*δ-Carbobenzoxy-*DL*-ornithine amide hydrochloride.*— The above compound (106 g.) was treated with 1.5 N methanolic hydrochloric acid (4.4 l.) as described in the case of the L-isomer[10], and the hygroscopic crystalline carbobenzoxyornithine methyl ester hydrochloride obtained was amidated with methanolic ammonia in the usual manner. The product was recrystallized from methanol-acetone; yield, 86 g. (71%); m. p. 153~154°C.

TABLE II. R_f VALUES OF REFERENCE COMPOUNDS

Compounds	R_f*
ε-Benzoyllysine (DL, L and D form)	0.58
ε-Benzoyl-DL-lysine ethyl ester	0.82
ε-Benzoyllysine amide (DL and D form)	0.65
Lysine (L and D form)	0.14
δ-Benzoylornithine (DL, L and D form)	0.55
δ-Benzoyl-DL-ornithine ethyl ester	0.80
δ-Benzoylornithine amide (DL and D form)	0.60
Ornithine (L and D form)	0.11
δ-Cabobenzoxyornithine (DL and L form)	0.61
δ-Carbobenzoxyornithine amide (DL and D form)	0.68

* The solvent system of *n*-butanol:acetic acid: pyridine: water (4:1:1:12, by volume) and Toyo filter paper No. 50 were used.

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[4] A. C. Kurtz, ibid., **180**, 1253 (1950).

[5] J. C. Eck and C. S. Marvel, "Organic Syntheses", Col. Vol. 2, edited by A. H. Blatt, John Wiley & Sons, Inc., New York (1948), p. 374; E. E. Howe and E. W. Pietrusza, *J. Am. Chem. Soc.*, **71**, 2581 (1949).

* When the paper chromatography of the crystals shows an additional spot of ε-benzoyllysine except benzoyllysine ester, treatment by dry hydrogen chloride of ethanol solution of the crystals should be repeated again.

[6] E. Fischer and G. Zemplen, *Ber.*, **42**, 1022, 2989 (1909).

[7] H. E. Carter, R. L. Frank and H. W. Johnston, "Organic Syntheses", Col. Vol. 3, edited by E. C. Horning, John Wiley & Sons, Inc., New York (1955) p. 167.,

[8] N. Izumiya and T. Yamashita, *J. Biochem.*, **46**, 19 (1959).

[9] R. L. M. Synge, *Biochem. J.*, **42**, 99 (1948).

[10] I. Schumann and R. A. Boissonas, *Helv. Chim. Acta*, **35**, 2237 (1952).

Anal. Found: C, 51.53; H, 6.68; N, 13.59. Calcd. for $C_{13}H_{20}O_3N_3Cl$: C, 51.74; H, 6.68; N, 13.93%.

R_f Values of Compounds.—The R_f values of the reference compounds are given in Table II.

Resolution of ε-Benzoyl-DL-lysine Amide. — *ε-Benzoyl-L-lysine.* — ε-Benzoyl-DL-lysine amide hydrochloride (86.4 g.) was dissolved in water (2 l.) containing manganese chloride tetrahydrate (0.60 g.). The pH was adjusted to about 7.5 with 1 N aqueous ammonia (150 ml.), the enzyme solution containing the equivalent of 16.4 mg. of protein nitrogen was added, and the volume was made up to 6.0 l. After about ten hour incubation at 38°C, the results of ammonia determination indicated complete hydrolysis of the L-amide, when the pH of solution was found to be near 8.5. In the course of the digestion, benzoyl-L-lysine was partly precipitated. The course of hydrolysis is shown in Fig. 1.

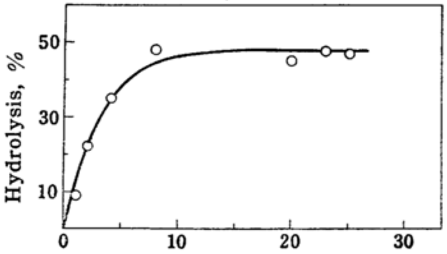

Fig. 1. Time-course of hydrolysis of ε-benzoyl-DL-lysine amide.

It was incubated for another fifteen hours, and its paper chromatography in this stage showed two spots with several solvent systems. The incubation mixture was evaporated in vacuo to a small volume, and the crystals were collected and washed with water (combined filtrate and washings saved for the D-amide). This was recrystallized from hot water; yield, 33.1 g. (88%); m. p. 244~246°C (decomp.); $[\alpha]^{15}_{D}+22.0^\circ$ (c=2, in 5 N hydrochloric acid) (lit.[11] $[\alpha]^{25}_{D}+22.3^\circ$).

Anal. Found: N, 11.10. Calcd. for $C_{13}H_{18}O_3N_2$: N, 11.29%.

L-Lysine hydrochloride. — The above compound (10 g.) was refluxed with 6 N hydrochloric acid (150 ml.) for ten hours, and benzoic acid produced was removed in the usual manner[12]. The solution was evaporated in vacuo, and the residual syrup was dissolved in water. The solution, adjusted to pH 6.4~6.6 by the addition of triethylamine, was evaporated to dryness, and the residue was recrystallized from water-ethanol; yield, 6.7 g. (92%); $[\alpha]^{15}_{D}+20.5^\circ$ (c=2, in 5 N hydrochloric acid) (lit.[13] $[\alpha]_{D}+20.7^\circ$).

Anal. Found: N, 15.50. Calcd. for $C_6H_{15}O_2N_2Cl$: N, 15.34%.

ε-Benzoyl-D-lysine amide hydrochloride. — The filtrate and washing** described already were passed through a column of Amberlite IRA-400 (5 cm.×50 cm.) in the alkaline phase, a strongly basic anion-exchange resin, and water (9 l.) was successively added to the top of the column. The fractions were combined and evaporated to dryness in vacuo. The evaporation was repeated several times with addition of methanol, the residue was dissolved in 1 N methanolic hydrochloric acid (150 ml.), and the solution was concentrated to a small volume, acetone being added. The crystals collected were recrystallized from hot methanol-acetone; yield, 35.5 g. (83%); m. p. 220~221°C (decomp.); $[\alpha]^{15}_{D}-16.8^\circ$ (c=2, in water).

Anal. Found: C, 54.61; H, 7.16; N, 14.58. Calcd. for $C_{13}H_{20}O_2N_3Cl$: C, 54.64; H, 7.05; N, 14.71%.

ε-Benzoyl-D-lysine.—The above compound (14.3 g.) was refluxed with 2 N hydrochloric acid (210 ml.) for one hour, the solution was evaporated in vacuo, and the residue was dissolved in water, triethylamine being added to neutralization. The crystals collected were recrystallized from hot water; yield, 8.5 g. (68%); m. p. 246~247°C (decomp.); $[\alpha]^{15}_{D}-21.8^\circ$ (c=2, in 5 N hydrochloric acid) (lit.[11] $[\alpha]^{30}_{D}-22.1^\circ$).

Anal. Found: N, 10.97. Calcd. for $C_{13}H_{18}O_3N_2$: N, 11.19%.

D-Lysine hydrochloride.—Benzoyl-D-lysine amide hydrochloride (8.6 g.) was refluxed with 6 N hydrochloric acid for ten hours, benzoic acid was removed, and the solution was evaporated to dryness in vacuo. The evaporation was repeated several times with addition of water and of some triethylamine until free of ammonia, this procedure replacing ammonium chloride with triethylamine hydrochloride which is soluble in ethanol. The product remained was recrystallized from water-ethanol; yield, 4.7 g. (86%); $[\alpha]^{15}_{D}-20.3^\circ$ (c=2, in 5 N hydrochloric acid) (lit.[14] $[\alpha]_{D}-20.7\sim-21.0^\circ$).

Anal. Found: N, 15.71. Calcd. for $C_6H_{15}O_2N_2Cl$: N, 15.34%.

Resolution of δ-Benzoyl-DL-ornithine Amide.—*δ-Benzoyl-L-ornithine.* — To δ-benzoyl-DL-ornithine amide hydrochloride (54.4 g.) and manganese chloride tetrahydrate (0.40 g.) dissolved in water, pH being brought to 7.5, was added the enzyme

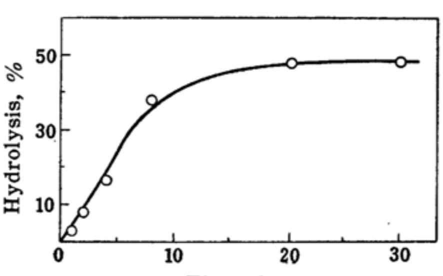

Fig. 2. Time-course of hydrolysis of δ-benzoyl-DL-ornithine amide.

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[11] N. Izumiya and S. Ota, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **72**, 445 (1951).

[12] N. Izumiya and K. Kitagawa, ibid., **79**, 65 (1958).

[13] J. P. Greenstein, S. M. Birnbaum and M. C. Otey, *J. Biol. Chem.*, **204**, 307 (1953).

** A certain amount of the combined filtrate and washings were evaporated, aqueous sodium hydroxide was added, and the solution was extracted with chloroform as that for D-phenylalanine amide[1], but about 20% of the D-amide could be extracted.

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

containing 12.5 mg. of protein nitrogen. The mixture was made up to 4.0 l. and incubated at 38°C, the course of hydrolysis being shown in Fig. 2. The solution was then evaporated to a small volume, and the resulting crystals were collected and washed with water; yield, 12.8 g. The combined filtrate and washings were passed through a column of Amberlite IRA-400 (4.5 cm.×40 cm.), and the column was washed with water (6 l.) (combined fractions saved for the D-amide). Elution of the additional L-amino acid from the column was accomplished with 2 N hydrochloric acid (7 l.) The eluate was evaporated, the residue was dissolved in water, and the solution was neutralized with triethylamine; yield, 9.7 g. The combined crystals (22.5 g.) were recrystallized from hot water; yield, 20.3 g. (86%); m.p. 243~245°C (decomp.); $[\alpha]^{15}_{\mathrm{D}}+26.0°$ ($c=2$, in 5 N hydrochloric acid) (lit.[15] $[\alpha]^{10}_{\mathrm{D}}+19.8°$).

Anal. Found: N, 11.61. Calcd. for $C_{12}H_{16}O_3N_2$: N, 11.86%.

L-Ornithine hydrochloride.—This was prepared from the above compound (11.8 g.) as that for L-lysine hydrochloride; yield, 7.6 g. (90%); $[\alpha]^{20}_{\mathrm{D}}+22.0°$ ($c=2$, in 5 N hydrochloric acid) (lit.[13] $[\alpha]_{\mathrm{D}}+22.1°$).

Anal. Found: N, 16.57. Calcd. for $C_5H_{13}O_2N_2Cl$: N, 16.60%.

δ-Benzoyl-D-ornithine amide hydrochloride.—The combined fractions described already were treated in the same way as that for benzoyl-D-lysine amide hydrochloride; yield, 23.4 g. (86%); m. p. 208~210°C; $[\alpha]^{15}_{\mathrm{D}}-16.5°$ ($c=2$, in water).

Anal. Found: C, 52.41; H, 6.77; N, 15.38. Calcd. for $C_{12}H_{18}O_2N_3Cl$: C, 53.04; H, 6.68; N, 15.46%.

δ-Benzoyl-D-ornithine.—This was prepared from the above compound (13.6 g.) as that for benzoyl-D-lysine; yield, 5.3 g. (45%); m. p. 242~245°C (decomp.); $[\alpha]^{15}_{\mathrm{D}}-25.8°$ ($c=2$, in 5 N hydrochloric acid).

Anal. Found: N, 11.73. Calcd. for $C_{12}H_{16}O_3N_2$: N, 11.86%.

D-Ornithine hydrochloride.—This was prepared from benzoyl-D-ornithine amide hydrochloride (8.2 g.) as that for D-lysine hydrochloride; yield, 4.5 g. (89%); $[\alpha]^{20}_{\mathrm{D}}-21.7°$ ($c=2$, in 5 N hydrochloric acid) (lit.[14] $[\alpha]_{\mathrm{D}}-20.1°$).

Anal. Found: N, 16.33. Calcd. for $C_5H_{13}O_2N_2Cl$: N, 16.60%.

Resolution of δ-Carbobenzoxy-DL-ornithine Amide.— *δ-Carbobenzoxy-L-ornithine.* — δ-Carbobenzoxy-DL-ornithine amide hydrochloride (60.2 g.) was incubated with the enzyme containing 17.4 mg. of protein nitrogen in the similar manner as that for benzoyl-DL-ornithine amide, the course of hydrolysis being shown in Fig. 3. During incubation, the precipitate was deposited gradually. After about sixty hours, the mixture was kept in a refrigerator for two days, and the crystals were collected and washed with cold water (combined filtrate and washings saved for the D-amide). This was recrystallized from dilute hydrochloric acid-aqueous ammonia; yield, 24.5 g. (92%); m. p. 246~248°C (decomp.); $[\alpha]^{18}_{\mathrm{D}}+18.1°$ [$c=2$, in aqueous acetone (1 : 1, by volme) containing two equivalents of of hydrogen chloride] (lit.[9] $[\alpha]^{16}_{\mathrm{D}}+17°$); $[\alpha]^{18}_{\mathrm{D}}+18.9°$ ($c=2$, in 5 N hydrochloric acid).

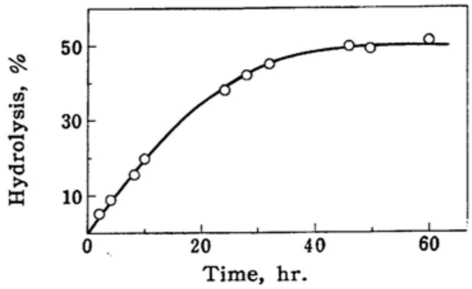

Fig. 3. Time-course of hydrolysis of δ-carbobenzoxy-DL-ornithine amide.

Anal. Found: N, 10.55. Calcd. for $C_{13}H_{18}O_4N_2$: N, 10.52%.

L-Ornithine hydrochloride.—The above compound (5.3 g.) was refluxed with 6 N hydrochloric acid (80 ml.) for four hours, the solution was evaporated to dryness in vacuo, and the residue was treated as that for L-lysine hydrochloride; yield, 2.8 g. (83%); $[\alpha]^{20}_{\mathrm{D}}+21.9°$ ($c=2$, in 5 N hydrochloric acid) (lit.[13] $[\alpha]_{\mathrm{D}}+22.1°$).

Anal. Found: C, 35.89; H, 7.87; N, 16.00. Calcd. for $C_5H_{13}O_2N_2Cl$: C, 35.58; H, 7.77; N, 16.60%.

δ-Carbobenzoxy-D-ornithine amide hydrochloride.— The combined filtrate and washings described already were treated in the same way as that for benzoyl-D-lysine amide hydrochloride. The product obtained was recrystallized from hot methanol-ether; yield, 25.7 g. (85%); m. p. 175~176°C; $[\alpha]^{18}_{\mathrm{D}}-8.7°$ ($c=2$, in water).

Anal. Found: C, 51.32; H, 6.71; N, 14.04. Calcd. for $C_{13}H_{20}O_3N_3Cl$: C, 51.68; H, 6.68; N, 13.92%.

D-Ornithine hydrochloride. — The above compound (6.0 g.) was treated as that for L-ornithine hydrochloride in this section and D-lysine hydrochloride; yield, 2.9 g. (86%); $[\alpha]^{20}_{\mathrm{D}}-22.2°$ ($c=2$, in 5 N hydrochloric acid) (lit.[14] $[\alpha]_{\mathrm{D}}-20.1°$).

Anal. Found: C, 35.91; H, 7.76; N, 15.97. Calcd. for $C_5H_{13}O_2N_2Cl$: C, 35.58; H, 7.77; N, 16.60%.

Summary

ε-Benzoyl-DL-lysine amide, δ-benzoyl-DL-ornithine amide and δ-carbobenzoxy-DL-orithine amide were resolved to ω-acyl-L-amino acids and ω-acyl-D-amino acid amides by the partially purified leucine aminopeptidase preparation. ω-Acyl-L-amino acids and ω-acyl-D-amino acid amides were hydrolyzed to L- and D-amino acid hydrochlorides, respectively. The parts of ω-benzoyl-D-amino acid amides were changed to ω-benzoyl-D-amino acids by acid hydrolysis.

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[15] N. Izumiya, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **72**, 150 (1951).