Resolution of Amino Acids. I. Resolution of Racemic Phenylalanine and γ-Phenyl-α-aminobutyric Acid by Leucine Aminopeptidase

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Several methods have been described for the resolution of racemic phenylalanine[1,2] and phenylaminobutyric acid[3], but no method involving the asymmetric hydrolysis of the amides of these two racemic amino acids by aminopeptidase has yet been shown. As Smith[4] has reported that the highly active leucine aminopeptidase was prepared easily from swine kidney and found to hydrolyze L-phenylalaninamide at a moderate rate, we tried in the present work to resolve racemic phenylalaninamide and phenylaminobutyric acid amide by making use of the enzyme.

We prepared a partially purified leucine aminopeptidase of Step 4 according to Smith[4] by which phenylaminobutyric acid amide was also found to be hydrolyzed at a higher rate than that for L-phenylalaninamide. For the resolution, the initial substrate concentration was set at 0.1 mol. for DL-phenylalaninamide and 0.05 mol. for DL-phenylaminobutyric acid amide, the course of hydrolysis was followed by measurements of evolved ammonia, and the digest was allowed to stand beyond the point at which analysis reveals the theoretical 50% hydrolysis. In the course of the digestion of phenylaminobutyric

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1) J. P. Greenstein, *Advances in Protein Chem.*, **9**, 121 (1954).

2) K. Michi and H. Tsuda, *J. Jap. Biochem. Soc.*, (*Seikagaku*) **29**, 646 (1957).

3) V. du Vigneaud and O. J. Irish, *J. Biol. Chem.*, **122**, 349 (1938).

4) 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; D. H. Spackman, E. L. Smith and D. M. Brown, *J. Biol. Chem.*, **212**, 255 (1955).

acid amide, the L-isomer which is remarkably insoluble in water was partly precipitated.

The products of the reaction were separated in two ways: namely, by differential solubility and the use of ion-exchange resin. It was found that the former way is conveniently applied for the separation of the products from DL-phenylaminobutyric acid amide and the latter way is for that of DL-phenylalaninamide.

For the resolution of phenylaminobutyric acid, only one method has been reported by du Vigneaud and Irish[3], which is based on the fractional crystallization of the brucine salt of the racemic formyl derivative. They assigned the L optical configuration to the isomer of $[\alpha]_D^{30}$ +48.8° (in 1 N hydrochloric acid) according to the Lutz-Jirgensons rule and on the basis of the animal feeding experiment. Since the enzyme employed here was completely inactive toward D-leucinamide, and since no D-leucine aminopeptidases have yet been found in animal tissues, it was assumed that the susceptible isomer was L in optical configuration. The digest contained, therefore, L-amino acid and D-amino acid amide at the end of the incubation. L-Phenylaminobutyric acid obtained here possessed $[\alpha]_D^9$ of +48.1° in 1 N hydrochloric acid, which coincides with the results of du Vigneaud and Irish[3].

Experimental

Preparation of leucine aminopeptidase. A partially purified enzyme solution (Step 4 of Smith) was prepared as described[4]. Table I shows the proteolytic coefficients (C_1) of L-amino acid amides. The C_1 of L-leucinamide is included for comparison. The rate of enzyme action on the amides was followed by measurement of the extent of ammonia liberation in Conway micro-diffusion vessels[5]. The rate of hydrolysis ($C_1 \times$ substrate concentration) of phenylalaninamide and phenylaminobutyric acid amide with the enzyme preparation slightly increased with increase in concentration of the substrates.

TABLE I

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

Substrates	C_1 at different concn. of substrates**		
	0.01 M	0.025 M	0.05 M
L-Leucinamide	5.5	3.6	2.2
DL-Phenylalaninamide	2.4	1.0	0.5
DL-Phenylaminobutyric acid amide***	8.2	3.4	2.1

* Enzyme was preincubated in 0.1 M Tris buffer of pH 8.0 with 0.008 M manganous 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.

*** Crystals of L-amino acid appeared after 50~70% hydrolysis.

The effect of various manganous ion concentrations on hydrolysis of the substrates under the same conditions of the resolution was examined. In the presence of manganous ions (0.0005~0.008 M), the apparent increase of hydrolysis was observed in the case of phenylalaninamide, while little effect of the presence of manganous ions was shown with phenylaminobutyric acid amide.

Substrates. *1. DL-Phenylalaninamide hydrochloride.*—This was synthesized from DL-phenylalanine ethyl ester hydrochloride by the same procedure as that for the L form[6]; yield 95%; m. p. 234~236°C (decomp.).

Anal. Found: N, 13.80. Calcd. for $C_9H_{13}ON_2Cl$: N, 13.96%.

2. DL-γ-Phenyl-α-aminobutyric acid.—Procedure[7] Rothstein and Miller's was slightly modified, the yield being remarkably improved. After refluxing the mixture of ethyl acetamidocyanoacetate, sodium and β-phenylethyl bromide in ethanol, the precipitated salt was filtered off and a small amount of acetic acid was added as described in Rothstein and Miller's paper. The filtrate was then evaporated in vacuo to an oil which was changed to crystals by the addition of water. The crystals were collected and washed with water. A part of the crude crystals was recrystallized from ethanol-water; m. p. 116°C. This is ethyl acetamidocyanophenylbutyrate.

Anal. Found: N, 10.36. Calcd. for $C_{15}H_{18}O_3N_2$: N, 10.21%.

The main part of the crude crystals was refluxed with concentrated hydrochloric acid and turned to phenylaminobutyric acid in the usual manner; yield 66% (based on ethyl acetamidocyanoacetate); m. p. 300~302°C (decomp.). The reported melting point is 305~306°C[3].

3. DL-γ-Phenyl-α-aminobutyric acid ethyl ester hydrochloride.—DL-Phenylaminobutyric acid (81g.) was suspended in ethanol (1.5 l.), dry hydrogen chloride was introduced to saturation at room temperature, and the solution was refluxed for one hour. Removal of the solvent in vacuo and treatment of the residue with dry ether yielded crystals. This was recrystallized from ethanol-ether; yield 98 g. (88%); m. p. 135~136°C.

Anal. Found: N, 5.69. Calcd. for $C_{12}H_{18}O_2NCl$: N, 5.75%.

4. DL-γ-Phenyl-α-aminobutyric acid amide hydrochloride.—This was prepared from DL-phenylaminobutyric acid ethyl ester hydrochloride in

5) R. B. Johnston, M. J. Mycek and J. S. Fruton, ibid. **185**, 629 (1950).

6) E. L. Smith and D. H. Spackman, ibid. **212**, 271 (1955).

7) M. Rothstein and L. L. Miller, ibid. **199**, 209 (1952).

the same way as that for the L-phenylalaninamide hydrochloride[6] and recrystallized from methanol-ether; yield 91%; m. p. 214~217°C (decomp.).

Anal. Found: N, 13.21. Calcd. for $C_{10}H_{15}ON_2Cl$: N, 13.05%.

Resolution of DL-phenylalaninamide. *A. Separation method involving ion-exchange chromatography. 1. D-Phenylalaninamide hydrochloride.*—DL-Phenylalaninamide hydrochloride (45.2 g.) was dissolved in water (1.5 l.) containing manganese chloride tetrahydrate (0.224 g.). The pH was adjusted to 7.5 with 1 N aqueous ammonia (about 180 ml.), the enzyme solution containing the equivalent of 2.25 mg. of protein nitrogen was added, and the volume was made up to 2.25 l. After forty hours' incubation at 38°C, the results of ammonia determination indicated complete hydrolysis of the susceptible L-isomer, when the pH of solution was found to be 8.5. The course of a typical hydrolysis is shown in Fig. 1.

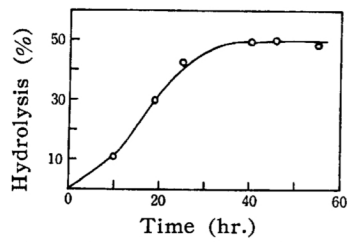

Fig. 1. Time-course of hydrolysis of DL-phenylalaninamide.

The solution that remained clear was passed through a column of Amberlite IRA-400* in the alkaline phase (5 cm.×40 cm.) and water (8 l.) was added to the top of the column. Detection of the amide and ammonia in the fractions was accomplished by means of the Nessler's reagent or the ninhydrin spot test on paper. The fractions were combined and evaporated to dryness in vacuo. The evaporation was repeated several times with addition of ethanol, and the remaining oil was crystallized after being kept in a desiccator for two to three days. The crystals were dissolved in 0.5 N methanolic hydrochloric acid (225 ml.)**. The solution was evaporated to small volume and ether was added. The resulting crystals were collected and recrystallized from methanol-ether; yield 18.6 g. (82%); m. p. 235~237°C (decomp.); $[\alpha]_D^{12}$ −20.4° (c 2, in water).

Anal. Found: C, 53.65; H, 6.55; N, 13.81. Calcd. for $C_9H_{13}ON_2Cl$: C, 53.87; H, 6.53; N, 13.96%.

The reported values for L-phenylalaninamide hydrochloride are $[\alpha]_D^{25}$ +20.7° and m. p. 246°C[6].

2. L-Phenylalanine.—Elution of the L-amino acid from the column was accomplished with 2 N hydrochloric acid (10 l.). Detection of the fractions was accomplished by means of the paper chromatography. The fractions were evaporated to dryness in vacuo three times to remove the excess of hydrochloric acid. The residue was dissolved in water and neutralized with triethylamine. The crystals were recrystallized from hot water-ethanol; yield 17.3 g. (93%); m. p. 270~273°C (decomp.); $[\alpha]_D^{12}$ −34.1° (c 2, in water).

Anal. Found: C, 65.61; H, 6.59; N, 8.26. Calcd. for $C_9H_{11}O_2N$: C, 65.44; H, 6.71; N, 8.48%.

The reported values are $[\alpha]_D^{25}$ −33.35° and m. p. 273~275°C[8].

3. D-Phenylalanine.—D-Phenylalaninamide hydrochloride (4.0 g.) was refluxed with 2 N hydrochloric acid (60 ml.) for five hours and D-phenylalanine was obtained in the usual manner; yield 3.0 g. (91%); m. p. 271~274°C (decomp.); $[\alpha]_D^{9}$ +33.8° (c 2, in water).

Anal. Found: N, 8.41. Calcd. for $C_9H_{11}O_2H$: N, 8.48%.

B. Separation method involving fractional crystallization. 1. L-Phenylalanine.—DL-Phenylalaninamide was resolved as described above. The incubation mixture was evaporated to a small volume and ethanol was added. The resulting crystals were collected and recrystallized from hot water-ethanol; yield 51~55%; $[\alpha]_D^{12}$ −35.1° (c 2, in water).

Anal. Found: N, 8.31. Calcd. for $C_9H_{11}O_2N$: N, 8.48%.

2. D-Phenylalaninamide hydrochloride.—The filtrate and washings from L-amino acid were evaporated to dryness in vacuo and the residue was treated as described later in the preparation of D-phenylaminobutyric acid amide hydrochloride; yield 77~81%; m. p. 234~237°C (decomp.); $[\alpha]_D^{12}$ −20.1° (c 2, in water).

Anal. Found: N, 14.21. Calcd. for $C_9H_{13}ON_2Cl$: N, 13.96%.

Resolution of DL-γ-phenyl-α-aminobutyric acid amide. *1. L-γ-Phenyl-α-aminobutyric acid.*—To DL-phenylaminobutyric acid amide hydrochloride (64.5 g.) dissolved in water, pH being brought to 7.5 with aqueous ammonia, was added the enzyme equivalent to 0.9 mg. of nitrogen. The mixture was made up to 6 l. with water and incubated at 38°C. After about thirty hours beautiful crystals appeared, and after another twenty hours the mixture was cooled for the complete precipitation. The crystals were collected and washed thoroughly with cold water; yield 15.6 g. The filtrate and washings were combined, the additional enzyme equivalent to 0.6 mg. of nitrogen was introduced, and the volume was adjusted to 9 l. After another twenty hours' incubation at 38°C, the results of ammonia determinations indicated complete hydrolysis, no crystals appearing this time. The incubation was continued for another fifteen hours. The course of hydrolysis is shown in Fig. 2. The solution was evaporated to about 150 ml., and the

* In another run, Amberlite IRC-50 in the acid phase, a weak cation-exchange resin, was applied to the separation, but the eluate of the digest contained total amount of L-amino acid with parts of D-amide and ammonia even though a large amount of resin was used.

** The paper chromatography of this solution showed only one spot by means of the ninhydrin test with several solvent systems.

8) S. Sakurai, *J. Biochem.*, **43**, 851 (1956).

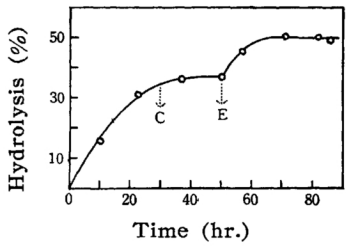

Fig. 2. Time-course of hydrolysis of DL-phenylaminobutyric acid amide. At the point of C, crystals appeared. At the point of E, additional enzyme was added.

resulting crystals were collected; yield 10.5 g. (combined filtrate and washings saved for the D-amide). The combined crystals (26.1 g.) were recrystallized from hot dilute hydrochloric acid-aqueous ammonia; yield 24.5 g. (91%); m. p. 310~313°C (decomp.); $[\alpha]_D^9$ +48.1° (c 1, in 1 N hydrochloric acid).

Anal. Found: C, 66.84; H, 7.44; N, 7.72. Calcd. for $C_{10}H_{13}O_2N$: C, 67.02; H, 7.31; N, 7.82%.

The reported value is $[\alpha]_D^{30}$ +48.8°[8].

2. D-γ-Phenyl-α-aminobutyric acid amide hydrochloride.—The combined filtrate and washings from L-amino acid were evaporated to dryness in vacuo and 5 N sodium hydroxide (75 ml.) was added thereto under cooling. The solution was extracted three times with 3 l. (total volume) of chlorofom. Chloroform solution was dried over sodium sulfate and evaporated to dryness in vacuo. The oily residue was dissolved in 0.5 N methanolic hydrochloric acid (300 ml.), solution was evaporated to small volume, and ether was added. The resulting crystals were collected and recrystallized from methanol-ether; yield 27.2 g. (84%); m. p. 253~254°C (decomp.); $[\alpha]_D^9$ −23.7° (c 2, in water).

Anal. Found: C, 56.12; H, 7.21; N, 12.96. Calcd. for $C_{10}H_{15}ON_2Cl$: C, 55.94; H, 7.04; N, 13.05%.

3. D-γ-Phenyl-α-aminobutyric acid. — This was obtained from D-amide hydrochloride by the same procedure as that for D-phenylalanine; yield 96%; m. p. 308~311°C (decomp.); $[\alpha]_D^9$ −48.7° (c 1, in 1 N hydrochloric acid).

Anal. Found: N, 7.86. Calcd. for $C_{10}H_{13}O_2N$: N, 7.82%.

The reported values are $[\alpha]_D^{30}$ −47.0° and m. p. 323~325°C[3].

Summary

DL-Phenylalaninamide and DL-phenylaminobutyric acid amide were resolved to L-amino acids and D-amino acid amides by the partially purified leucine aminopeptidase preparation.

The products in the digests were separated conveniently by the use of ion-exchange resin, Amberlite IRA-400, in the case of DL-phenylalaninamide, while by the differential solubility in the case of DL-phenylaminobutyric acid amide.

The parts of D-amino acid amide hydrochlorides obtained were changed to D-amino acids by acid hydrolysis.

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