CHEMISTRY LETTERS, pp. 51- 56, 1974. Published by the Chemical Society of Japan

SOLID PHASE PEPTIDE SYNTHESIS USING ULTRASONIC WAVES

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The introduction of ultrasonic waves into solid phase peptide synthesis was devised. Using the technique peptide synthesis on solid support was accelerated. Bradykinin was synthesized easily in good yield and high purity, demonstrating that it is a powerful tool for solid phase peptide synthesis.

The solid phase method introduced by Merrifield[1] has brought about remarkable improvement in peptide synthesis. However, it has been reported in some preparations[2,3] that failure or truncated sequences occur. In order to circumvent these problems several modifications of solid support have been reported.[4] The present authors wish to report the introduction of ultrasonic waves to the reaction system of solid phase synthesis, which increases the diffusion of reagents into resin matrix resulting in enhancement of the reaction on resin.

In a preliminary experiment, it was found that p-nitrophenol, which was steeped in resin (polystyrene-co-2% divinylbenzene) together with dimethylformamide, was removed from resin more easily under ultrasonic wave irradiation than under the ordinary condition, as shown in Fig. 1. Namely, the amount of p-nitrophenol squeezed from resin reached the maximum before 120 sec under ultrasonic wave irradiation, whereas it did not reach the maximum after 240 sec under the usual treatment. Furthermore, the residual amount of p-nitrophenol in resin matrix after 240 sec treatment with ultrasonic waves was smaller than that with the usual treatment, as shown in Fig. 1. This finding indicates that the attainment of the equilibrium between inner and outer matrix was hastened by ultrasonic wave irradiation.

Next, the authors applied ultrasonic wave irradiation to the reaction system of solid phase peptide synthesis. Conditions for deblocking of t-butyloxycarbonyl (t-Boc) group by N HCl in acetic acid, neutralization by 10% triethylamine solution

in methylene chloride and coupling between t-Boc-amino acid and amino acyl- or peptidyl-resin by dicyclohexylcarbodiimide (DCC) were examined. From these investigations, it was found that a complete coupling of one amino acid residue could be performed according to the schedule, as shown in Table I. Following this schedule, some model peptides were synthesized on resin using ultrasonic waves and then cleaved from

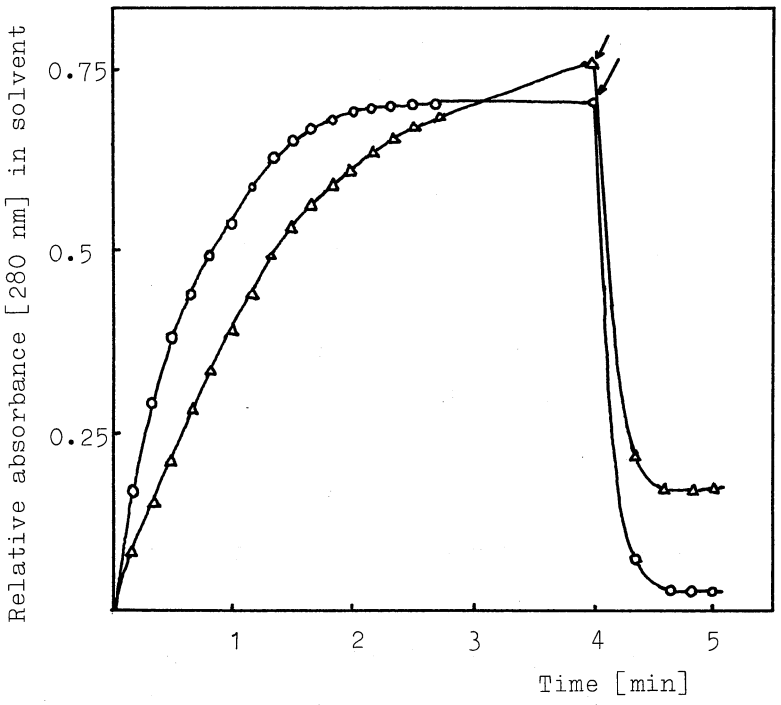

Fig. 1 Washing out of p-nitrophenol from resin. The amount of p-nitrophenol (vertical) squeezed from resin to solvent (DMF) against the lapse of time (horizontal) was measured by its UV-absorption in solvent at 280 nm. At the point of arrow solvent was removed from resin, and fresh solvent was immediately added to the residual resin.
-Δ-Δ- ordinary treatment, -O-O- ultrasonic wave treatment.

resin by treatment with anhydrous hydrogen fluoride.[5,6] The peptides thus synthesized were checked not only by paper-electrophoresis or -chromatography, but also by the recovery of amino acids in their hydrolysates performed by amino acid analysis. The results are given in Table II.

To test the applicability of this technique for longer peptides, bradykinin was selected because this peptide can be specifically assayed by its physiological activity. In this experiment, t-Boc-ω-nitro-L-arginyl-resin (2.00 g; 0.21 mmol of arginine per g of resin) was transfered into a glass column, fitted with a G-2 sintered filter and a jacket, in which water with a constant temperature was circulated.

Table I. The schedule of peptide synthesis with ultrasonic waves

Stage	Reagent	Sonication time (min)	Number of treatment
1	AcOH	1	1
2	N HCl/AcOH	3	2
3	DMF	1	1
4	CH_2Cl_2	1	2
5	10% Et_3N/CH_2Cl_2	1	2
6	CH_2Cl_2	1	3
7	t-Boc-AA-OH/CH_2Cl_2	0.5	2
8	DCC/CH_2Cl_2	2.5	
9	CH_2Cl_2	1	1
10	MeOH	1	1
11	CH_2Cl_2	1	1

Fig. 2 The apparatus used for solid phase synthesis

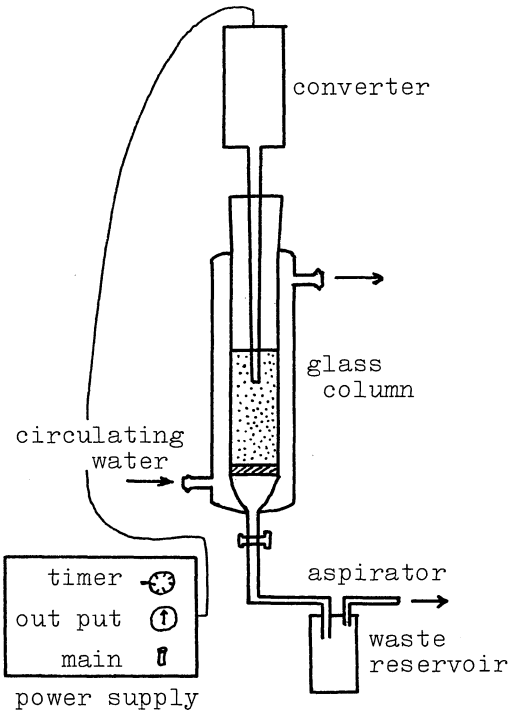

Table II. Amino acid composition and recovery of the hydrolysates of synthesized peptides

Peptide	Amino acid composition of the hydrolysates			Recovery of the hydrolysates (%)*)
(1) (2) (3)**)	(1)	(2)	(3)**)	
Asp-Leu-Gly	0.99	0.96	1.00	88.3
Lys-Lys-Ile	1.94		1.00	101.7
Gly-Pro-Pro	1.00	1.98		101.4
Gly-Val-Ile	1.00	0.82	0.82	98.1

*) Recovery calculated on values of amino acid analysis

**) Number in parenthesis means position of amino acid in peptide.

Reagents were introduced manually into a glass column and removed by suction with aspirator. Tip of ultrasonic vibrator (Ultrasonic vibrator Model UR-200P of Tomy Seiko Co., Ltd. Tokyo, Japan) were inserted into the mixture of resin and reagent, in which ultrasonic waves of 20 kc were generated, as illustrated in Fig. 2. Deblocking of t-Boc group was performed by repeating three minutes treatment with N HCl/AcOH.

The succeeding t-Boc-amino acid and DCC were added repeatedly in amount of five equivalents of the first amino acid bound to resin. Coupling solvent except for t-Boc-ω-nitro-L-arginine was methylene chloride, and for this amino acid dimethylformamide was used. Deblocking, neutralization, coupling and washing were carried out according to the schedule shown in Table I. The total time for the coupling of eight amino acids (Phe, Pro, Ser(Bzl), Phe, Gly, Pro, Pro, and Arg(NO_2)) was within 5 hours. Peptidyl-resin (2.30 g) was obtained. Cleavage of the peptide from resin (919.4 mg) was performed by treatment with anhydrous hydrogen fluoride (15 ml) at 0°C for 60 min in the presence of anisole (1.5 ml).[5] Extraction of the free peptide from resin was performed by three minutes treatment with ultrasonic waves in N acetic acid. The extract was passed through a column of IR-45 (acetate form). The eluates were lyophilized and dried over P_2O_5 in vacuo; 177 mg. The second extraction under the same condition gave only 12 mg of the free peptide. The theoretical yield of bradykinin triacetate pentahydrate is 212 mg based on the original t-Boc-ω-nitro-L-arginyl-resin. The peptide thus obtained gave the same mobility as an authentic sample[7] by paperelectrophoresis using an acetic acid-pyridine buffer solution at pH 4.8 (stained with ninhydrin and Sakaguchi reagent). Amino acid analysis of the hydrolysate gave the values as shown in Table III. The average recovery of the hydrolysate was about 95%. Bioassays of the product on guinea-pig ileum muscle exhibited 95% of the specific activity compared with the standard bradykinin.[7]

Table III. Amino acid analyses of bradykinin synthesized by ultrasonic wave method*)

Residue	Crude peptide	U-III**)	Theory for bradykinin
Arginine	1.94	1.95	2
Serine	0.95	0.95	1
Proline	3.02	3.09	3
Glycine	1.00	1.00	1
Phenylalanine	2.04	2.03	2

*) Peptides were hydrolyzed in 6N hydrochloric acid for 24 hr at 105°C and analyzed in a Shibata amino acid analyzer. All values shown are uncorrected.

**) U-III is shown in Fig. 3.

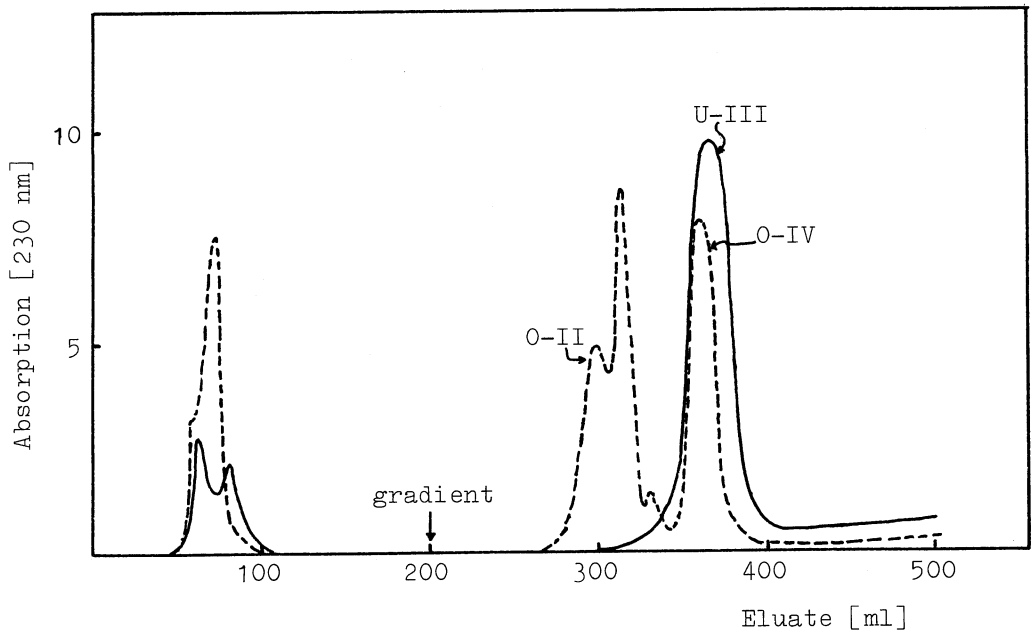

Fig. 3 Purification of synthetic bradykinin by column chromatography: Material was eluted from a column (15 mm (φ) x 360 mm (l)) of carboxymethyl-cellulose with 0.01M ammonium acetate (300 ml)/ 0.3M ammonium acetate (300 ml) gradient at pH 6.8 after washing with water (200 ml). The effluent was detected by its UV-absorption at 230 nm. ——— ultrasonic wave method, ----- ordinary method.

Table IV. Amino acid analyses of some fractions by the ordinary solid phase synthesis*)

Residue	Crude material	O-II**)	O-IV**)
Arginine	1.76	1.06	2.43
Serine	1.02	0.96	0.97
Proline	2.61	2.98	3.27
Glycine	1.00	1.00	1.00
Phenylalanine	2.26	2.14	2.22

*) Samples were hydrolyzed in 6N hydrochloric acid for 24 hr at 105°C and analyzed in a Shibata amino acid analyzer. All values shown are uncorrected.

**) Fractions O-II and O-IV are shown in Fig. 3.

This material (100 mg) was subjected to chromatography on carboxymethyl-cellulose,[8] as shown in Fig. 3. The main fractions in Fig. 3 were combined and lyophilized; 77 mg. The amino acid ratios after hydrolysis under the same condition, as described above, as shown in Table III. The bioassays of the purified material showed the same specific activity as that of the standard bradykinin.

In order to prove the merit of this technique above the ordinary method, bradykinin was synthesized by the ordinary method, using the same schedule as shown in Table I. In this case, t-Boc-ω-nitro-L-arginyl-resin (1.99 g; 0.21 mmol of arginine per g of resin) was introduced into the same glass column, as described above. Stirring was performed by a mechanical rod in place of ultrasonic waves. Other manipulations were performed by the same way, as described for ultrasonic wave irradiation method. The crude peptide, cleaved from peptidyl-resin (905 mg), was 156.7 mg. This material (155 mg) was applied to a carboxymethyl-cellulose column, as shown in Fig. 3 and gave 51.3 mg of pure nonapeptide, corresponding to the yield of about one-third that of the ultrasonic wave method. Amino acid analyses of the hydrolysates of each fraction obtained in Fig. 3 are given in Table IV.

These results presented in this report showed clearly that the use of ultrasonic waves in the solid phase synthesis improved the peptide synthesis by diffusing reagents into resin matrix easily and therefore accelerating the rate of reaction.

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(Received November 12, 1973)