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Note

Determination of amino acids on Merrifield resin by microwave hydrolysis

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An accurate and convenient method for the amino acid analysis of proteins and peptides is becoming increasingly important with the recent developments in molecular biology and biotechnology. The classical method of Hirs *et al.*[1] utilizing 6 *M* hydrochloric acid at 110–120°C coupled with the ion-exchange separation of the hydrolysates of proteins using the method developed by Moore *et al.*[2] is still the most commonly used and unambiguous methodology for the amino acid analysis of most proteins and peptides. Amino acid analysis is of special importance for researchers carrying out routine peptide synthesis. The cleavage of single amino acids from Merrifield solid-phase resin and the determination of the first amino acid coupled to the chloromethylated resin are prime requirements for the determination of the exact amounts of blocked amino acids needed for later steps of solid-phase peptide synthesis. Westall and co-workers[3,4] employed a mixed-acid solvent [propionic acid–12 *M* hydrochloric acid (1:1)] at 130°C for 2 h for the hydrolysis of peptide fragments generated in solid-phase peptide synthesis.

In this paper we describe a novel and faster alternative to the conventional hydrolysis protocol using a rapid microwave oven-based technique[5] that yields a quantitative and reproducible recovery of amino acids from solid-phase resin in as little as 6–8 min. The technique has important implications for the total automation of the amino acid analysis of peptides and proteins starting from the pre-analysis sample preparation.

EXPERIMENTAL

Equipment

Custom-made thick-walled PTFE vials (6 mm thick) were fabricated locally according to the design shown in Fig. 1. Previous experience with Pyrex glass tubes commonly used for the hydrolysis of proteins with 6 *M* hydrochloric acid indicated that the high pressure and temperature readily induced in the sealed hydrolysis tubes by the microwaves easily caused explosion of the reaction tubes inside the microwave oven.

Each PTFE vial can contain up to 2 ml of solution and is equipped with a silicone-rubber septum and a cap made of the same Teflon material. For safety

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Fig. 1. PTFE vials and container shown inside a commercial microwave oven. The thicknesses of the two PTFE vials (right) and the container (left) are 6 and 5 mm, respectively. The container is 7.5 cm high with I.D. 7.5 cm and the vials are 5.5 cm high and with O.D. 3.0 and I.D. 1.8 cm. At the top of the small vial, a sealing septum of silicone-rubber was attached (not shown) for insertion of a needle to flush the vial with inert gas (*e.g.*, nitrogen) before the microwave heating. The container can contain 1–5 vials at one time. It is preferably placed at the same location to ensure constant and reproducible heating.

precautions, a large Teflon container with a thinner wall (5 mm thick, Fig. 1) was used to hold all hydrolysis vials during the microwave heating process. The microwave oven used was a commercially available cooking apparatus (compact Model MW3500XM, Whirlpool, MI, U.S.A.) without any modification. The total power of the microwaves was 1.2 kW. There is no temperature indicator on this model, hence the exact temperatures for different settings need to be measured separately using several compounds with known melting points[5].

Reagents and amino acid-attached resin samples

Propionic acid and concentrated hydrochloric acid (30%, Suprapur) for the preparation of the mixed-acid solvent were obtained from Merck (Darmstadt, F.R.G.). Propionic acid–12 *M* hydrochloric acid (1:1, v/v) in 1-ml vials can also be obtained from Pierce (Rockford, IL, U.S.A.). N-*tert.*-Butoxycarbonyl (N-t-Boc) amino acid resin esters for peptide synthesis by the Merrifield method were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals and buffers used for the amino acid analyser were of the highest grade commercially available.

Procedures

Hydrolyses of N-t-Boc amino acid resins were carried out in thick-walled Teflon vials as described above. Samples (5–7 mg) were dissolved in 0.2–0.8 ml of propionic acid–12 *M* hydrochloric acid mixed-acid solvent. The vials were covered with tight silicone-rubber septa and flushed with nitrogen through two small-sized needles (one for gas inlet and the other outlet). They were then screw-fastened with the Teflon caps and placed in a large Teflon container. The container was placed at the centre of the

bottom plate in the microwave oven (Fig. 1). We set the "microwave power" on the command pads to 70% of full power (*i.e.*, 0.84 kW) and varied the time from 1 to 7 min.

As the exact pressure and temperature induced inside commercial microwave ovens are difficult to measure, it is advisable to make a preliminary calibration of the microwave oven using a series of compounds with known melting points in order to find the approximate temperature ranges obtained at specified settings.

At the end of rapid heating (less than 10 min), the acid-digested mixtures were centrifuged on a microcentrifuge to remove the undigested resins and the supernatants were pipetted into acid-cleaned vials and evaporated to dryness using a Speed Vac Concentrator (Savant, U.S.A.) with a refrigerated condensation trap. The amino acid compositions of the digested samples were determined with an LKB-4150 amino acid analyser based on an ion-exchange method using a single-column system.

The samples were also hydrolysed at 140°C for 3 h by using a modified protocol of Westall and co-workers[3,4] with the same mixed-acid solvent. The amino acid resins were dissolved in 0.6 ml of propionic acid–12 *M* hydrochloric acid contained in Pierce re-usable vacuum hydrolysis tubes designed for a dry heating block. The sample tubes were freeze–thawed several times in liquid nitrogen, evacuated with an oil pump and sealed by screwing down the cap. The hydrolysis were conducted in a heating block set at 140°C for 3 h. Thereafter the sample analyses with the amino acid analyser were the same as those described for the microwave method. The data from the different heating procedures were compared with the reported values of millimoles of N-t-Boc-amino acid per gram of resin given by the manufacturer (Sigma).

RESULTS AND DISCUSSION

The conventional anaerobic hydrolyses of synthesized peptides and purified proteins with 6 *M* hydrochloric acid and 110°C for 24 h or more have been widely used for more than 30 years[1,2]. Although the conventional method is in general satisfactory for the purpose of obtaining amino acid contents of peptides and proteins, it is always time consuming for a skilled researcher to obtain accurate values for some labile amino acids such as serine, threonine, tyrosine, cysteine and methionine. Therefore, we have attempted to devise a novel approach of utilizing microwave technology for the rapid hydrolysis of peptide bonds with the aim of shortening the analysis time and obtaining data as reliable as those given by the conventional method[5]. We have extended the previous microwave heating protocol for the cleavage of N-t-Boc amino acid derivatives attached to Merrifield resins.

The designs of the PTFE vials and accessories for the purpose of conducting hydrolysis are shown in Fig. 1 together with the microwave oven. It should be emphasized that the high pressure and temperature developed under the microwave oven conditions often result in explosion of the sealed Pyrex tubes even with short heating times (less than 5 min). Therefore, the use of PTFE vials is strongly recommended. It is known that the nature of microwave heating precludes the conventional means of temperature determination. The preliminary calibration of the approximate temperature inside the microwave oven using thermocouples in conjunction with a digital thermometer failed owing to strong arcing. We therefore conducted a preliminary calibration of the temperature inside the microwave oven by use of several organic compounds with known melting points. The setting of 70% power and

7 min on the control pads corresponded to the temperature range between the melting points of semicarbazide hydrochloride (178°C) and *p*-anisic acid (186°C). The temperature of our microwave hydrolysis is tentatively concluded to be about 180°C. The pressure factor inside the hydrolysis vial remains to be determined. Nevertheless, reproducible data can always be obtained by seting the microwave at a specified "power" and "time".

Scotchler *et al.*[6] first improved the procedure of Merrifield[7] and Gutte and Merrifield[8] by substituting one protocol of propionic acid–hydrochloric acid hydrolysis for the two-step (first with anhydrous hydrofluoric acid–anisole treatment followed by hydrolysis with constant-boiling hydrochloric acid) removal of the resin and determination of amino acid residues in the initial stage of the solid-phase peptide synthesis. We performed experiments to optimize the conditions for the amino acid analysis of attached amino acid resin esters using the microwave method. In our experience, insufficient solvation of the Merrifield resin often adversely affected the analytical data owing to some resin samples being charred by the high temperature induced inside the microwave. Fig. 2 shows the effect of solvent volume on the recovery of alanine from the resin. Clearly the minimum volume needed for the complete hydrolysis of resin samples (5–7 mg) is about 0.6 ml. Hence 0.6 ml of the mixed-acid solvent [propionic acid–12 *M* hydrochloric acid (1:1, v/v)] were used for all hydrolyses of resin samples measured accurately with an analytical balance in the range 5–7 mg. N-t-Boc-alanine resin was also used to calibrate the hydrolysis time for the complete recovery of amino acids from the Merrifield resin. The recovery curve reached a plateau at times longer than 6 min (Fig. 3). We chose 7 min for safety reasons as heating times longer than 10 min at the specified power caused leakage of the reaction mixtures from the Teflon vials, probably due to the release of isobutylene gas generated from the butoxycarbonyl blocking group by acid hydrolysis.

Table I gives the results for 18 amino acid resin esters (histidine and tryptophan resin derivatives are not available from Sigma) obtained by the microwave technique using the above mixed-acid solvent with a pre-set power of 70% of full power and a heating time of 7 min. It is evident that this fast microwave heating yielded reproducible recoveries with a standard deviation of less than 5% for most samples. It

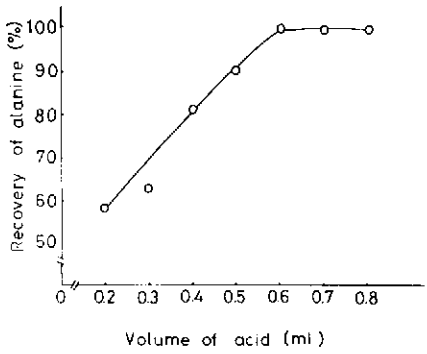

Fig. 2. Effect of solvent volume on the recovery of alanine from N-t-Boc-Ala resin for microwave hydrolysis. Resin samples of about 5–7 mg were used and different amounts of the mixed-acid solvent were added to the vials for microwave hydrolysis with a setting of 70% power for 7 min.

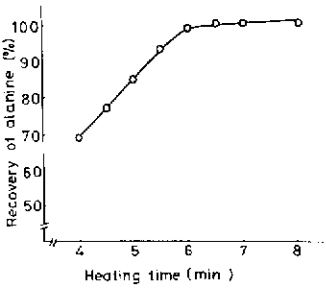

Fig. 3. Determination of heating time for the maximum recovery of alanine from N-t-Boc-Ala resin for microwave hydrolysis. Resin samples of 5–7 mg were used and 0.6 ml of the mixed-acid solvent were added to each vial for microwave hydrolysis with a setting of 70% power and different heating times.

compared favourably with those obtained by the conventional method[6] with the exception that our data for serine and threonine amino acid derivatives were much higher than those obtained by the old heating method. Using aerobic hydrolysis with propionic acid–hydrochloric acid, Westall and Hesser[4] claimed complete hydrolysis of peptides in 15 min at 150–160°C. In our experience with various peptides, application

TABLE I

RECOVERY OF AMINO ACIDS AFTER MICROWAVE HYDROLYSIS

*Boc-amino acid resin**	*Stated concentration (mmol/g resin)***	*Found concentration (mmol/g resin)****	*Recovery (%)*§
N-t-Boc-Ala	0.61	0.64 ± 0.03	103.0 ± 2.2
N-t-Boc-NO_2-Arg	0.17	0.10 ± 0.01	100.3 ± 3.1
N-t-Boc-Asn	0.40	0.33 ± 0.02	104.9 ± 0.9
N-t-Boc-Bzl-Asp	0.38	0.44 ± 0.02	99.4 ± 1.4
N-t-Boc-(S-Bzl)-Cys	0.29	0.33 ± 0.08	105.2 ± 1.7
N-t-Boc-Gln	0.35	0.41 ± 0.04	102.2 ± 3.2
N-t-Boc-Bzl-Glu	0.13	0.32 ± 0.06	102.1 ± 4.7
N-t-Boc-Gly	0.44	0.36 ± 0.03	102.4 ± 3.7
N-t-Boc-Ile	0.29	0.34 ± 0.05	117.2 ± 1.6
N-t-Boc-Leu	0.24	0.36 ± 0.03	97.6 ± 4.4
N-t-Boc-Met	0.27	0.50 ± 0.06	102.2 ± 5.1
N-t-Boc-Phe	0.70	0.78 ± 0.05	101.0 ± 4.6
N-t-Boc-Pro	0.38	0.44 ± 0.07	103.1 ± 2.5
N-t-Boc-(O-Bzl)-Ser	0.20	0.13 ± 0.04	343.7 ± 3.6
N-t-Boc-(O-Bzl)-Thr	0.37	0.30 ± 0.03	113.8 ± 2.7
N-t-Boc-Cbz-Lys	0.46	0.23 ± 0.02	103.8 ± 3.7
N-t-Boc-(O-Bzl)-Tyr	0.05	0.31 ± 0.04	107.0 ± 2.8
N-t-Boc-Val	0.13	0.40 ± 0.03	102.0 ± 3.6

* All N-t-Boc amino acid resin esters were obtained from Sigma, except that of alanine, which was synthesized in our laboratory. NO_2 = nitro; Bzl = benzyl; Cbz = chlorobenzyloxycarbonyl.

** Analytical data on the labels supplied by Sigma.

*** Data (mean ± S.D., $n = 3$) obtained by hydrolyses of the amino acid resin esters using propionic acid–12 *M* hydrochloric acid at 140°C for 3 h.

§ Recovery (mean ± S.D., $n = 3$) by the method of microwave heating with the mixed acid assuming the corresponding data in the previous column to represent 100%.

of the same solvent at 140°C for 3 h under anaerobic conditions is more suitable for achieving a higher yield and reproducible cleavage of peptides from the resin in the final step of solid-phase synthesis[9]. Therefore, we calculated the recovery data for the corresponding amino acid resin esters obtained by the microwave method on the basis of data obtained by hydrolysis at 140°C for 3 h. It was also shown previously[5] that microwave heating yielded much better recoveries of serine and threonine than those obtained by traditional hydrolysis at 110°C for 24 h. We believe that the more than 3-fold recovery of serine obtained here with microwave heating probably indicates a great improvement in the analysis of labile amino acids by this fast method over the previous methods.

The reason for the low concentrations of Boc-Glu, Boc-Tyr and Boc-Val in the resins reported by the manufacturer, in contrast to the much higher concentrations obtained by our analyses, is not clear at present. However, it is well known that determinations of the concentrations of amino acid resin esters often vary greatly among different manufacturers using different methods of analysis. It is noteworthy that the extra benzyl groups introduced in Glu and Tyr resin esters probably account for the low recoveries of these amino acid derivatives. The hydrophobic nature of valine in Boc-Val is also known to yield a low recovery in the regular amino acid analysis of proteins and peptides[10,11].

CONCLUSION

Microwave hydrolysis has been applied with success for the first time to the cleavage of amino acids from Merrifield solid-phase resins. The fast and accurate analysis of the first amino acid attached to the solid-phase resin is an essential step in peptide synthesis. The analysis time of the rate-determining step in the initial stage of solid-phase peptide synthesis can be shortened by a factor of 2–5 by using the mixed-acid solvent and microwave heating. Improvements in the design of commercial microwave ovens should prove valuable in the important application of amino acid analysis of peptides and proteins. Currently we are in the process of refining the microwave-heating step in order to interface it with the amino acid analyser for the on-line automation of protein hydrolysis and analysis.

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