Journal of Chromatography, 491 (1989) 424–431
Biomedical Applications
Elsevier Science Publishers B.V , Amsterdam — Printed in The Netherlands

CHROMBIO. 4762

Note

Peptide and protein hydrolysis by microwave irradiation

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(First received February 9th, 1989; revised manuscript received March 8th, 1989)

The recent advances in instrumentation for amino acid analysis and high-performance liquid chromatography (HPLC) have shortened the net analysis time for amino acid analysis to less than an hour for each individual sample. Therefore the rate-determining and most essential step of a successful amino acid analysis now lies in the skillful preparation of protein hydrolysates. The commonly used conventional protocol of Hirs et al. [1] utilizes 6 *M* hydrochloric acid and 110–120°C for more than 24 h and has been the method of choice for the past thirty years. Although the recent development of HPLC amino acid analysis [2,3] coupled with gas phase hydrolysis of protein samples [4] has been widely used, the reproducible and complete amino acid analysis data for most proteins are not easily obtained, except in some cases of short peptides.

We have introduced a rapid method of microwave heating for the facile preparation of protein and peptide hydrolysates [5,6]. This paper reports refinements of the method, and establishes microwave irradiation as a rapid and novel means of protein hydrolysis with special regard to its general application to amino acid analysis. It provides a radical method of protein and peptide hydrolysis via commercial microwave ovens and specially designed Teflon–Pyrex tubes with the potential of on-line automation of protein hydrolysis and analysis.

0378-4347/89/$03.50

EXPERIMENTAL

Custom-made Teflon–Pyrex reusable hydrolysis tubes (150 mm × 4 mm I.D.) were purchased locally (The Continuity Enterprise, Taipei, Taiwan). Each tube can contain up to 1.0 ml of hydrolysis solvent. In practice, ca. 0.1 ml of solvent was added for the preparation of each protein hydrolysate. The basic designs of the tubes are based on inert-gas flushing for the removal of oxygen inside the tubes, with the specially designed Teflon cap and plunger to ensure leak-free operation under microwave irradiation [7]. The Reacti-Therm dry block heating system (Pierce, Rockford, IL, U.S.A.) was used for the conventional hydrolysis procedure for 24 h at 110°C.

Constant-boiling 6 *M* hydrochloric acid, 4 *M* methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole and standard amino acid mixture in 1-ml ampoules were obtained from Pierce. Individual amino acids in crystal form were from Merck (Darmstadt, F.R.G.). Native chicken egg white lysozyme was from Sigma (St. Louis, MO, U.S.A.).

The samples (0.1–0.5 mg) were analysed for the amino acid composition by microwave irradiation or the conventional procedure [1]. The proteins or peptides were dissolved in 0.1 ml of constant-boiling 6 *M* hydrochloric acid contained in the Teflon–Pyrex tubes, which were then directly flushed with purified nitrogen for 1 min and sealed by screwing down the Teflon plunger [6,7]. The tubes were then put in the microwave oven (Model MW3500XM, Whirlpool, Benton Harbor, MI, U.S.A.) at the pre-set power (80% input power or 0.96 kW) for different time periods or on a dry heating block set at 110°C for 24 h. At the end of heating, the hydrolysed protein solutions were pipetted into acid-cleaned vials and evaporated to dryness in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.) with refrigerated condensation trap.

Amino acid compositions were determined with the Beckman high-performance amino acid analyser (Model 6300) with dual-channel data system using a single ion-exchange column. The special procedure and buffer system for the analysis of hydrolysates from methanesulphonic acid were essentially as described previously [7]. Reversed-phase HPLC was carried out on a Hitachi liquid chromatograph with a Model L-6200 pump and a variable-wavelength UV monitor. The column (300 mm × 4.0 mm I.D., SynChropak RP-C18, 6.5 μm beads) was used to analyse the hydrolysis products from microwave irradiation for completeness of protein hydrolysis. The amino acid composition data from different heating procedures were compared with the literature values for lysozyme, based on its amino acid sequence.

RESULTS AND DISCUSSION

Sample preparation of protein hydrolysates plays an important role in the successful analysis of amino acid compositions for most peptides and proteins

[1]. The standard protocol for the preparation of protein hydrolysates involves the evacuation of Pyrex tubes in vacuo to ensure anaerobic conditions before protein hydrolysis at 110°C for more than 24 h. Although the conventional method is widely used and quite satisfactory for the purpose of obtaining the amino acid contents of peptides and proteins in general, it is always a prime requisite for a skilled researcher to do a time-course study of hydrolysis in order to have a more accurate estimation of some labile amino acids plus other stable amino acids such as isoleucine, valine and leucine [8,9]. Therefore an alternative procedure, involving a higher temperature and simple inert-gas flushing for the removal of oxygen from the hydrolysis tubes, has been previously evaluated with regard to the completeness of the peptide-bond hydrolysis and the accuracy and reliability of amino acid composition data [10]. Our method circumvents the tedious procedures of using vacuum-sealed Pyrex tubes by adopting the specially designed Teflon–Pyrex hydrolysis tubes. The hydrolysis time is further shortened, to a matter of minutes, by the application of microwave irradiation.

Previous experience with Pyrex tubes commonly used for 6 *M* hydrochloric acid hydrolysis of proteins has indicated that the high pressure and temperature induced in the sealed hydrolysis tubes by microwave irradiation caused tubes to explode. The design of Teflon–Pyrex tubes that can resist high temperatures and pressures under microwave oven conditions was described in the previous report [7], and thus rapid protein hydrolysis can be achieved by microwave irradiation without expensive instrumentation.

Table I shows the effect of hydrolysis time by microwave irradiation on the recoveries of standard amino acids. It is clearly evident that microwave irradiation of amino acids in 6 *M* hydrochloric acid for 2–8 min did not cause destruction of most amino acids, except for some minor degradation of labile amino acids such as serine, threonine, methionine, tyrosine and histidine, which is also commonly observed during conventional analysis. These findings formed the basis for the potential application of microwave irradiation in the routine hydrolysis of peptides and proteins before amino acid analysis.

We first applied microwave hydrolysis to native egg white lysozyme in 6 *M* hydrochloric acid. Fig. 1 shows the chromatogram of amino acid analysis of the hydrolysate after microwave irradiation for 4 min. The retention times of various amino acids are exactly the same as those obtained from the conventional 24-h hydrolysis at 110°C (data not shown). It is noteworthy that the chromatogram is generally clean, with no ambiguous peaks, which is indicative of the specific cleavage of peptide bonds by microwave irradiation. The amino acid content is also close to that obtained conventionally and from the theoretical residue numbers of amino acids in lysozyme. However, the time required for the hydrolysis of proteins has been decreased from 24 h to less than 5 min. The results of microwave hydrolysis for less than 4 min were not so

TABLE I

EFFECT OF MICROWAVE IRRADIATION ON STABILITY OF AMINO ACIDS

Data are expressed as relative molar ratios of seventeen amino acids in a standard amino acid mixture (Pierce) detected in the chromatograms of amino acid analyser before and after different times of microwave irradiation in 6 *M* hydrochloric acid, using alanine as the reference. Values marked with an asterisk indicate some degradation after irradiation for 8 min.

Amino acid	Relative molar ratio			
	0 min	2 min	4 min	8 min
½ Cys	1.18	1.17	1.16	1.14
Asx	1 04	1.05	1.04	1.04
Thr	1.07	1.06	1.04	0.95*
Ser	1.04	1.02	0.95	0.89*
Glx	1.06	1.05	1.06	1.07
Pro	1.12	1.13	1.15	1.15
Gly	1.04	1.05	1.04	1.05
Ala	1	1	1	1
Val	1.08	1.07	1.05	1.06
Met	1.00	0.98	0.96	0.93*
Ile	0.94	0.96	0.97	1.01
Leu	1.00	1.02	1.01	1.02
Tyr	1.03	1.04	0.97	0.92*
Phe	1.01	1.03	1.02	0.99
His	1.03	1.04	1.01	0.91*
Lys	1.01	0.99	1.05	0.98
Arg	0.97	0.98	0.97	0.99

satisfactory, and several unknown peaks were present in the chromatogram (data not shown).

Fig. 2 shows the effect of prolonged irradiation on the recovery of amino acids, with special regard to serine and threonine. It is of interest to note that doubling the irradiation time to 8 min did not worsen the results of amino acid analysis (top of Fig. 2), in contrast to the effect of doubling the conventional hydrolysis time to 48 h (bottom of Fig. 2). In the latter case, threonine and serine were decreased 40 and 80%, respectively. It is well known that the shorter the time of sample preparation and hydrolysis, the lower is the risk of amino acid degradation during hydrolysis [8]. In this respect, microwave hydrolysis offers a much greater advantage than the conventional protocol in obtaining accurate estimate of labile amino acids [5].

In the previous report [7], we have shown that the complete amino acid compositions of proteins can be obtained by using methanesulphonic acid [11] in place of hydrochloric acid for protein hydrolysis at higher temperatures. It is known that the nature of microwave heating precludes the conventional means of temperature determination. We have conducted a preliminary cali-

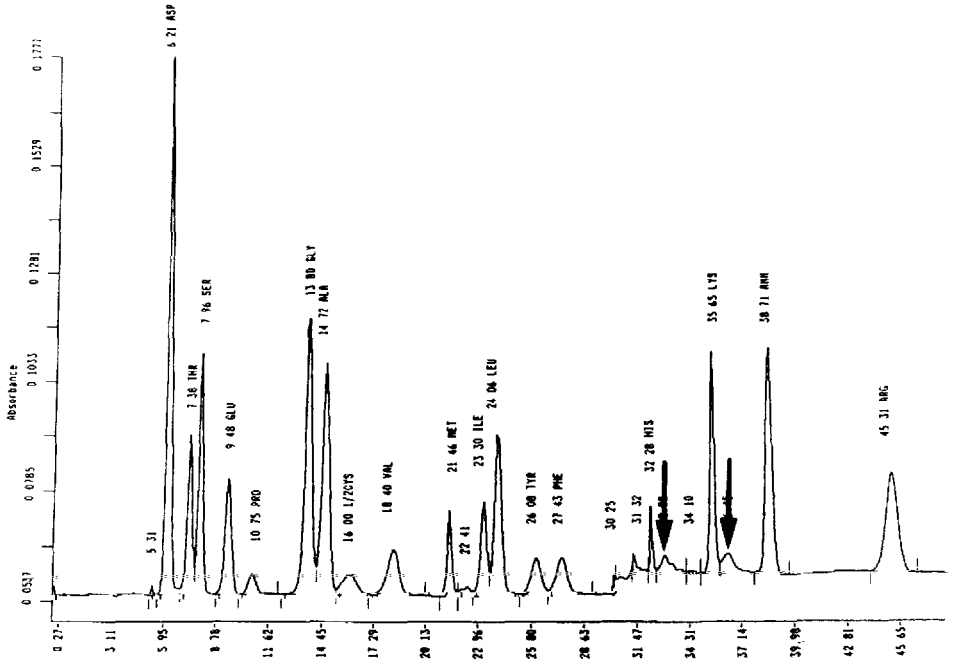

Fig. 1. Amino acid separation patterns of the 6 *M* hydrochloric acid hydrolysate of lysozyme by 4 min microwave irradiation on Beckman 6300 high-performance amino acid analyser. The buffer formulation and conditions are similar to those employed in conventional ion-exchange chromatography of amino acid analysis [1]. The names of the various amino acids, with their retention times, are indicated on the peaks. The arrows show two unknown ninhydrin-positive peaks eluted just after the histidine and lysine peaks, which also appear in the chromatogram obtained after hydrolysis at 110 °C for 24 h

bration of the temperature inside the microwave oven by use of several organic compounds with known melting points. The setting of '80%' full input power and 4 min on the control pad of the microwave oven corresponded to the temperature range between the melting points of semicarbazid hydrochloride (178 °C) and *p*-anisic acid (186 °C), similar to our previous calibration for 70% power and 7 min irradiation [6]. Therefore the temperature of our microwave hydrolysis is tentatively shown to be ca. 180 ± 5 °C by the indirect method; however, the pressure inside the hydrolysis tube remains to be determined. Nevertheless, reproducible data can always be obtained by setting the microwave at a specified 'power' and 'time'. It is to be emphasized that any commercially available microwave oven can be adapted for the purpose of rapid protein hydrolysis. It is advisable for first-time users to carry out a preliminary calibration of their own ovens, by setting a specified power output and varying the irradiation time from 2 to 8 min using standard proteins with known amino acid compositions, such as lysozyme, to find the optimal conditions for com-

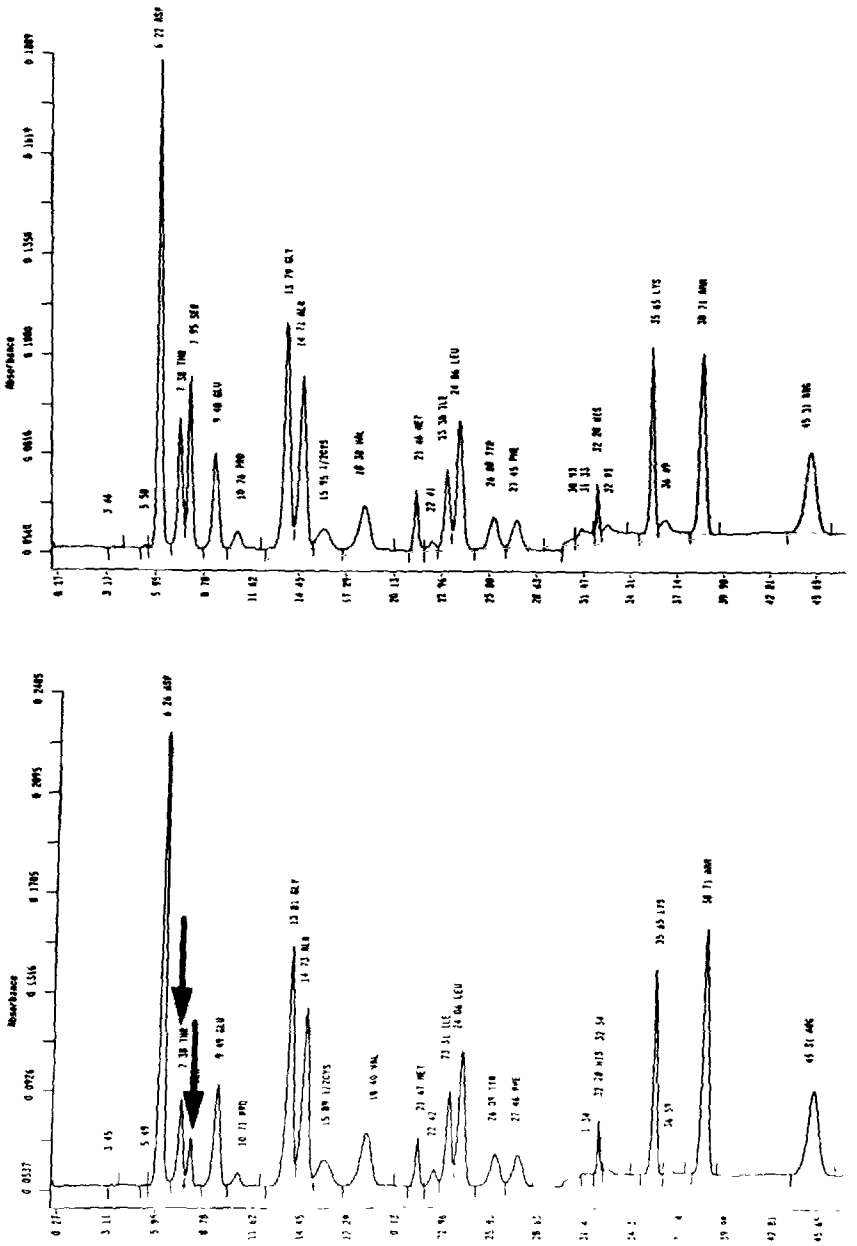

Fig. 2. Comparison of amino acid separation patterns of the 6 *M* hydrochloric acid hydrolysates of lysozyme on Beckman 6300 high-performance amino acid analyser. (Top) Amino acid analysis of lysozyme irradiated with microwaves for 8 min. (Bottom) Amino acid analysis of lysozyme hydrolysed at 110 °C for 48 h on a dry heating block. The arrows indicate the threonine and serine peaks, which are much smaller following conventional hydrolysis. Other peaks are relatively unchanged.

TABLE II

AMINO ACID ANALYSIS OF LYSOZYME USING MICROWAVE HYDROLYSIS FOR DIFFERENT PERIODS

Data are expressed as the number of residues per molecule of protein using alanine as the reference. Values represent the mean of triplicate determinations. The hydrolysis solvent for H samples is 6 *M* hydrochloric acid and that for M samples is 4 *M* methanesulphonic acid. All analyses using microwave irradiation are carried out in Teflon-Pyrex tubes. The last column (110 °C, 24 h) lists the data obtained conventionally using methanesulphonic acid and dry heating block [7]. The values in parentheses are the theoretical residue numbers of amino acids predicted from the protein sequence.

Amino acid	Number of residues per molecule of protein				
	H		M		110 °C, 24 h
	4 min	8 min	4 min	8 min	
½ Cys	6.1	5.2	7.2	6.9	6.6 (8)
Asx	20.5	20.1	21.4	21.9	22.2 (21)
Thr	6.2	5.9	6.3	6.0	6.2 (7)
Ser	8.3	7.5	8.6	7.8	8.1 (10)
Glx	4.6	5.1	5.0	5.4	5.2 (5)
Pro	2.3	2.7	2.5	2.6	2.4 (2)
Gly	11.7	11.9	12.4	11.8	12.5 (12)
Ala	12	12	12	12	12
Val	5.2	5.7	5.4	5.9	5.3 (6)
Met	1.9	2.2	1.8	1.7	1.8 (2)
Ile	5.2	5.7	4.7	5.2	5.0 (6)
Leu	7.4	7.6	7.8	7.7	8.3 (8)
Tyr	2.8	2.7	3.2	2.6	3.5 (3)
Phe	2.9	2.7	3.1	3.2	2.8 (3)
His	1.4	1.1	0.9	0.6	0.8 (1)
Lys	6.3	5.9	6.2	5.7	5.3 (6)
Arg	10.5	10.2	11.7	10.8	11.6 (11)
Trp	–	–	5.5	5.2	5.8 (6)

plete peptide-bond hydrolysis. In general, the heating time should never exceed 10 min in microwave ovens using Teflon vials or tubes, since they may deform at temperatures greater than 200 °C under microwave irradiation.

We have also used reversed-phase HPLC to follow protein hydrolysis in the non-volatile and non-oxidizing solvent, 4 *M* methanesulphonic acid, by microwave irradiation. The elution patterns of the hydrolysates following irradiation for 4 and 8 min are similar, indicating that lysozyme is completely hydrolysed after 4 min (data not shown). The results of amino acid compositions from these two hydrolysates are shown in Table II. Surprisingly, half-cystine and tryptophan are quite close to the expected values by this simpler hydrolysis protocol, compared with those of Simpson et al. [11] who used conventional

hydrolysis and tedious carboxymethylation for the determination of cysteine. This also corroborates our previous results [7,10] on the applicability of a higher temperature and a shorter hydrolysis time in achieving good recoveries of some labile amino acids in amino acid analysis.

The protocol described is easily adapted in most biochemical laboratories since microwave ovens are commonly used to melt agarose or polyacrylamide for the preparation of DNA or protein electrophoresis. Therefore the present method can be routinely used for liquid phase hydrolysis, which is much preferred by most protein chemists, and the expensive and specialized evacuation system of gas phase hydrolysis may be avoided. Currently we are refining our microwave-heating step in order to interface it with the amino acid analysis and HPLC for the on-line automation of protein hydrolysis and analysis. Improvements in the design of commercial microwave ovens specifically for use in biochemical research will prove valuable in the near future.

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