Journal of the Chinese Chemical Society, 1989, *36*, 435–442

RAPID PROTEIN HYDROLYSIS BY MICROWAVE IRRADIATION USING HEAT-RESISTANT TEFLON-PYREX TUBES

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A rapid peptide-bond hydrolysis by means of microwave irradiation is introduced for the facile preparation of protein hydrolysates used for amino acid analysis. The optimal hydrolysis condition has been determined using several enzymes with known amino acid compositions. The effects of hydrolysis time on the recovery of various labile and hydrophobic amino acids are also exemplified in the microwave heating of standard amino acids. The method has been applied to the complete amino acid analysis with a single nonvolatile solvent of methanesulfonic acid with good recovery of tryptophan and half-cystine. It provides a radical expedition of protein and peptide hydrolysis via commercial microwave ovens and specially-designed Teflon-Pyrex tubes, circumventing the tedious procedures using vacuum-sealed pyrex tubes heating at 110°C for more than 24 h. This novel type of microwave chemistry associated with rapid peptide-bond cleavage is of great potential in the automation of the complete process of amino acid analysis starting from the preparation of protein hydrolysates.

INTRODUCTION

During the last few years we have frequently conducted the conventional anaerobic hydrolysis of purified proteins[1-5] using 6 M HCl and 110°C/24 h according to the commonly-used protocol of Hirs *et al*[6]. Time-course study of hydrolysis for various time periods is always needed to ensure a complete hydrolysis of peptide bonds next to some stable hydrophobic amino acids and to account for losses of some labile amino acids such as cystine, tyrosine, serine and threonine residues in the protein samples[7,8]. Therefore an alternative utilizing the elevated temperature induced by microwave irradiation and simple inert-gas flushing method for the removal of oxygen from hydrolysis tubes has been evaluated with regard to the completeness of the peptide-bond hydrolysis and the accuracy and reliability of amino acid composition data from such simpler and rapid hydrolysis protocol[9-11]. The upshot of this report is to refine and establish microwave irradiation as a rapid and novel means of protein hydrolysis with special regard to its application to amino acid analysis.

EXPERIMENTAL

Custom-made Teflon-Pyrex reusable hydrolysis tubes (4 mm I.D. x 150 mm) were ordered from the local glass-plastic shop (The Continuity Enterprise, Taipei, Taiwan). Each tube can hold up to 1.0 mL of hydrolysis solvent. In practice, less than 0.5 mL of hydrolysis solvent is added to each individual sample for the preparation of protein hydrolysates. The basic designs of the tubes are based on the inert-gas flushing for the removal of oxygen inside the tubes. The Reacti-Therm dry block heating system (Pierce, Rockford, IL. U.S.A.) was used for the conventional protocol of 110°C/24 h hydrolysis for the comparison of our new protocol and the conventional heating.

Constant boiling 6 M HCl and standard amino acid mixture in 1 mL ampules were obtained from Pierce. Individual amino acids in crystal forms were from Merck (Darmstadt, W. Germany). Native chicken egg white lysozyme, and oxidized ribonuclease were from Sigma (St. Louis, MO., U.S.A.).

The samples (0.1–0.5 mg) were analyzed for the amino acid compositions by microwave irradia-

Dedicated to Professor K.T. Wang on the occasion of his 60th birthday.

tion or the conventional 110°C/24 h protocol (1). The proteins were dissolved in 0.1 mL of constant boiling 6 M HCl contained in Teflon-Pyrex reusable hydrolysis tubes, and the tubes with samples were directly flushed with purified nitrogen for 1 min and sealed by screwing down the Teflon plug[11,14]. The tubes were then put in a microwave oven (Model MW3500XM, Whirlpool Corp., Benton Harbor, MI. U.S.A.) at the pre-set power (80% input power or 0.96 kilowatt) for different time periods or on a dry heating block set at 110°C for 24 h. At the end of heating, the hydrolyzed protein solutions were pipetted into acid-cleaned vials and evaporated to dryness using 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 Analyzer (Model 6300) using a single-column system based on ion-exchange chromatography. Reversed-Phase HPLC was carried out on a Hitachi liquid chromatograph with a model L-6200 pump and a variable UV monitor. The column (4.0 x 300 mm, Syn-Chropak RP-C_{18}, 6.5 μm bead) was used to analyze the hydrolysis products from microwave irradiation and the solvent systems were as described in the figure legends. The amino acid composition data from different heating procedures were compared with the literature values of amino acid contents reported for these enzymes based on their sequences.

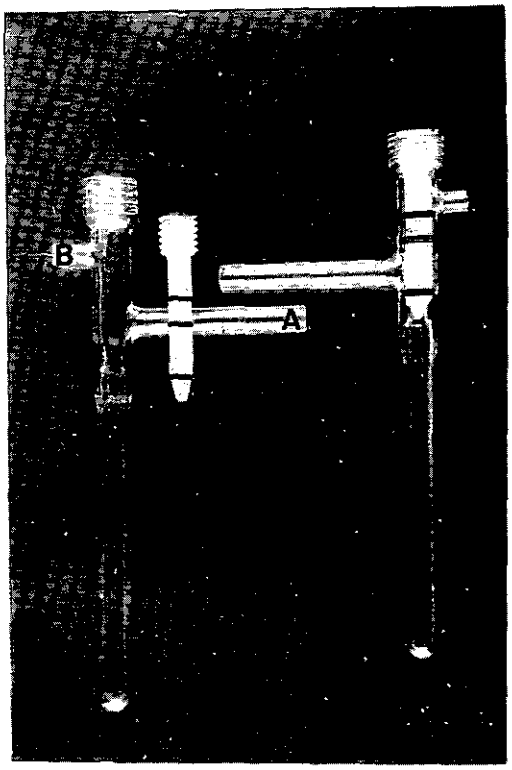

Fig. 1 The design of reusable Teflon-Pyrex tube for fast inert-gas flushing and microwave hydrolysis. The Teflon plunger contains three sealing O-rings to ensure leak-free operation during microwave irradiation. The long-arm opening A is the inlet for flushing the tube with nitrogen before heating. The short-arm opening B with a small bore is the outlet for the flushing gas. The tubes with samples were flushed with pure nitrogen gas for 1 min one at a time with gentle shaking. The outlet of the tube was closed with a finger at the end of 1 min flushing and sealed by screwing down the Teflon cap until the Teflon plunger is flushed with the Pyrex surface of the constricture and the O-ring squeezed in a circle.

RESULTS AND DISCUSSION

Fig. 1 shows the design of hydrolysis tubes for the purpose of conducting inert-gas flushing before microwave hydrolysis. Each Teflon cap contains three sealing O-rings (replaceable) to ensure complete leak-free operation during high-temperature heating. The design is based on the inert-gas flushing instead of vacuum-sealing procedure for the removal of oxygen inside the atmosphere of tubes[11,12]. Therefore one long arm of the pyrex tube with wide bore is used as the inlet of the flushing inert gas and the short arm opening with small bore as the outlet of the inert gas. It differs from the commercially available reusable tubes (Pierce) and conventional disposable Pyrex tubes which are all based on vacuum evacuation. It is well known that the less amount of pre-sample preparation and hydrolysis time re-

quired, the lower is the risk of amino acid degradation in the amino acid analysis of proteins and peptides[7]. The inert-gas (purified nitrogen) flushing has been shown to be as effective as the traditional vacuum-sealing protocol in preventing the amino acids from oxidation effected by the residual air or oxygen in the protein solutions during hydrolysis[12]. In general, the approach of adopting inert-gas flushing and short-time microwave irradiation gives the results similar or superior to that of the tedious method employing a strong oil-pump in achieving a good vacuum condition inside the hydrolysis tubes before sealing the pyrex tubes. Previous experience with Pyrex tubes commonly used for 6 M HCl hydrolysis of proteins has indicated that high pressure and temperature induced in the sealed hydrolysis tubes by the microwave irradiation easily caused explosion of the tubes. The design of custom-made Teflon plunger and cap (Fig. 1) which can resist high temperature and pressure under microwave oven set at 80% input power or 0.96 kilowatt for about 10–12 min without leaking or explosion. The main advantage of rapid protein hydrolysis has been achieved by microwave irradiation coupled with these Teflon-Pyrex tubes without expensive instrumentation.

Table 1 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 HCl from 2 to 8 minutes did not cause destruction of most amino acids except for some minor degradation in the labile amino acids such as serine, threonine, methionine, tyrosine and histidine, which are also commonly observed by 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 have applied microwave hydrolysis on oxidized ribonuclease in 6 M HCl. Table 2 shows the comparison of amino acid data of oxidized ribonuclease obtained from the 5 min microwave irradiation and the conventional 110 °C/24h hydrolysis. It is clearly evident that short-time microwave heating has essentially similar result to that of heating on the dry heating block at 110°C for 24 h. Of interest is the finding that by elevating the hydrolysis temperature and shortening the time higher recoveries of some labile amino acids such as serine and threonine were observed. The recoveries of amino acids from the elevated temperature and shorter time fall within a constant range of 97–102 % of the theoretical values of each amino acid except those of Val, Ile, Thr, Ser and Tyr. The difficulties encountered in the accurate analysis of these amino acids are commonly observed in the amino acid

Table 1 Effect of Microwave Irradiation on the Stability of Amino Acids

Amino acids	Irradiation duration			
	0	2 min	4 min	8 min
1/2 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

Data are expressed as relative molar ratios of 17 amino acids in standard amino-acid mixture (Pierce) detected in the chromatograms of amino acid analyzer before and after different times of microwave irradiation in 6 M HCl using alanine as the reference. Values marked with * indicated some destruction with 8 min irradiation.

Table 2 Amino Acid Analysis of Oxidized Ribonuclease A by Different Protocols

Amino acids	Predicted	Protocols (6 M HCl)	
		110°C/24 h	Microwave 5 min
Cysteic acid	8	6.6	6.8
Asx	15	14.3	14.5
Thr	10	8.4	9.2
Ser	15	10.3	12.8
Glx	12	11.4	11.8
Pro	4	3.7	4.2
Gly	3	3.1	3.0
Ala	12	12	12
Val	9	7.6	7.5
Met sulfone	4	5.1	4.3
Ile	3	2.4	2.1
Leu	2	2.5	2.0
Tyr	6	4.6	5.3
Phe	3	2.4	2.8
His	4	3.7	3.8
Lys	10	9.4	9.7
Arg	4	3.9	4.1
Trp	–	–	–

Data are expressed as the number of residues per molecule of ribonuclease using alanine as the reference. Values represent the average of duplicate analyses.

analysis of HCl-hydrolyzed proteins.

The trend of recoveries of labile amino acids with time is in general agreement with that of the previous report[12] using a dry heating block set at a temperature of 160 °C. From the present study it is obvious that good recoveries of amino acids can still be obtained at a higher temperature (> 160°C) under microwave irradiation and the hydrolysis time has been decreased from 45 to 5 min. Since the recovereis of isoleucine and valine are usually low[9-12], a time-course study for a pre-set microwave irradiation may be needed in order to have a more accurate estimate of these two hydrophobic amino acids.

Figs. 2A and 2B show the use of HPLC and reversed-phase column to follow the microwave hydrolysis of lysozyme in the non-volatile and non-oxidizing solvent, 4N methanesulfonic acid[13]. In the previous study[14] we have shown the applicability of this solvent in place of HCl to achieve the complete amino acid analysis of proteins. In this report we have extended the microwave irradiation to the hydrolysis of proteins with methanesulfonic acid in order to have an estimate of tryptophan which is usually destroyed during protein hydrolysis using 6 M HCl. It clearly indicates that with increasing the irradiation times, the protein peak disappears with concomitant appearance of a large amino-acid peak in the early chromatogram. The elution patterns of the hydrolysates from 4 and 8 min irradiation are similar, indicating a complete hydrolysis of lysozyme has been reached in 4 min (Fig. 2B). The results of amino acid compositions from these two hydrolysates are shown in Table 3. Surprisingly, half-cystine and tryptophan are quite close to the expected values by this simpler hydrolysis protocol as compared with that of Simpson *et al.*[13] using conventional hydrolysis and tedious carboxymethylation for the determination of cysteine. This also corroborates our previous results[12,14] on the advantages of higher temperature and shorter hydrolysis times in achieving good recoveries of some labile amino acids in the amino acid analysis.

Fig. 3 shows the chromatogram of the HCl-hydrolysate of lysozyme from 4 min microwave irradiation in the amino acid analysis determined on the Beckman High-Performance Amino Acid Analyzer. The retention times for various amino acids are exactly the same as those obtained from the conventional 110°C/24h hydrolysis (data not shown). It is noteworthy that the chromatogram is generally clean with no other ambiguous peaks present in the analysis, which is indicative of the specific cleavage of peptide bond under microwave irradiation. The analyzed amino acid content is also close to those obtained from conventional hydrolysis protocol and the theoretical residue numbers of amino acids in lysozyme. However the time required for the hydrolysis of proteins has been decreased

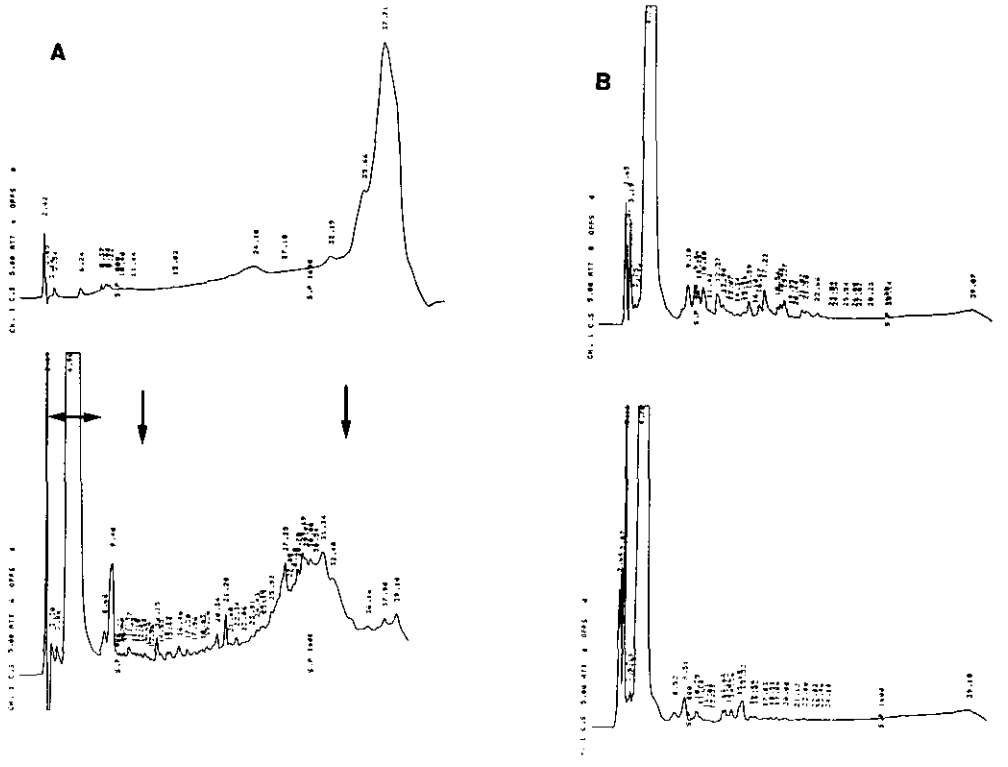

Fig. 2 Time-course study of microwave hydrolysis of lysozyme in methanesulfonic acid with different periods of microwave irradiation. The hydrolysates of lysozyme were partially neutralized with 8 M sodium hydroxide until pH-indicator papers turned to the color range of about pH 2. Five μl each of the hydrolysates was directly injected to a Hitachi liquid chromatograph with a variable UV detector set at 222 nm. The chromatographic conditions were (1) one min with solvent A of 0.1 % trifluoroacetic acid (TFA) in distilled water, (2) a linear gradient of 0 to 35% of solvent B of 0.1 % TFA in acetonitrile for 40 min and (3) return of the system to the initial conditions of 0 % solvent B in 9 min. The flow rate was set at 1 mL/min for the complete cycle. (**A**): Top-chromatogram of the control lysozyme solution in methanesulfonic acid without microwave irradiation. Bottom- elution pattern for lysozyme hydrolysate with 1-min irradiation. The double-arrow (3.0–8 min) and two single arrows (11.5 min and 32.6 min) indicated the elution positions for amino acids, dipeptide (leu-val) and insulin B chain, respectively. (**B**): Top-elution pattern for lysozyme hydrolysate with 4-min irradiation. Bottom-elution pattern for lysozyme hydrolysate with 8-min irradiation.

360-fold from 24 h to within 5 min. The results for the microwave hydrolysis shorter than 4 min were less than satisfactory and several unknown peaks were present in the chromatogram (data not shown).

Fig. 4 showed the effect of long-time irradiation on the recovery of amino acids with special regard to serine and threonine. It is of great interest to note that increasing the irradiation time 2-fold to 8 min did not deteriorate the result of amino acid analysis (Top of Fig. 4) in contrast to that obtained by 110°C/48 h hydrolysis (Bottom of Fig. 4). In the later case the threonine and serine had been decreased 40 and 80 %, respectively. In this aspect, microwave hydrolysis offers a much greater advantage than the conventional protocol in obtaining accurate estimate of labile amino acids[9,11].

It is known that the nature of microwave heating precludes the conventional means of tempera-

Table 3 Amino Acid Analysis of Lysozyme Using Microwave Hydrolysis for Different Periods

Amino acids	H-4 min	H-8 min	M-4 min	M-8 min	110°C/24 h
1/2Cys	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)

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 HCl and that for M samples is 4 M methanesulfonic acid. All analyses using microwave irradiation are carried out in Teflon-Pyrex tubes. The last column of 110°C/24 h is the data obtained by the conventional protocol using methanesulfonic acid and dry heating block[14]. The values in the parentheses are the theoretical residue numbers of amino acids predicted from the protein sequence.

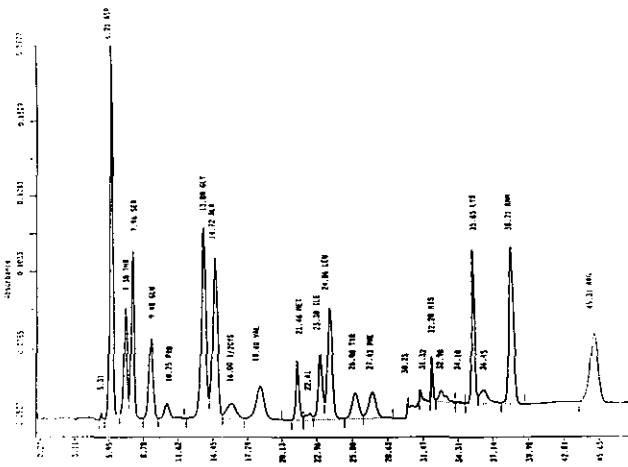

Fig. 3 Amino acid separation patterns of the 6 M HCl-hydrolysate of lysozyme by 4-min microwave irradiation on a Beckman 6300 High-Performance Amino Acid Analyzer. The buffer formulation and conditions are similar to those employed in conventional ion-exchange chromatography of amino acid analysis[6]. The eluted peaks of various amino acids with their retention times are indicated at the top of each amino acid. The unknown ninhydrin-positive peaks would appear in the chromatogram if the microwave irradiation of proteins was carried out in less than 4 min, indicating the incomplete peptide-bond hydrolysis and the presence of short peptides in the hydrolysates.

ture determination. We have conducted a preliminary calibration 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 microwave oven corresponded to the temperature range between the melting points of semicarbazid hydrochloride (178°C) and *p*-anisic acid (186°C). Therefore the temperature of our microwave hydrolysis is tentatively shown to be about 180±5°C by the indirect method and the pressure factor 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 adopted for the purpose of rapid protein hydrolysis. It is advisable for first-time users to have a preliminary calibration of their own microwave ovens set at a specified power input and vary irradiation time from 2 to 8 min using standard proteins with known amino acid compositions to find the optimal conditions for complete peptide-bond hydrolysis. In general 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.

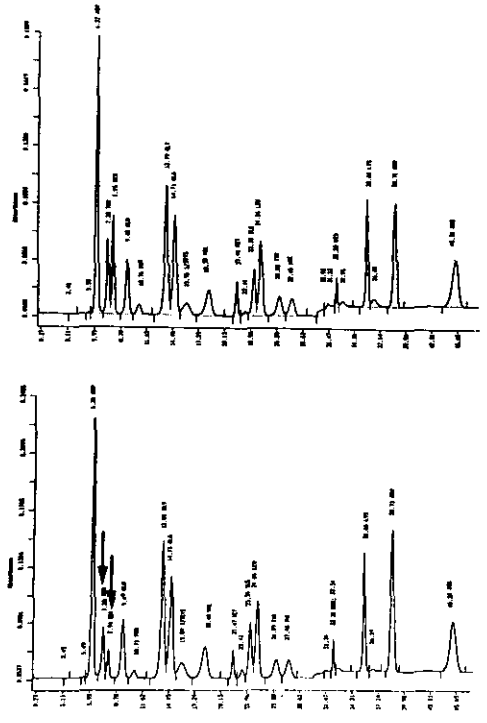

Fig. 4 Comparison of long-time microwave irradiation and conventional 48-h hydrolysis of lysozyme in 6 M HCl on a Beckman 6300 High-Performance Amino Acid Analyzer. (Top) Amino acid analysis of lysozyme irradiated with microwave for 8 min. (Bottom) Amino acid analysis of lysozyme hydrolyzed at 110°C for 48 h on a dry heating block. The arrows indicate the greatly-decreased threonine and serine peaks by the conventional heating protocol as compared to that of long-time microwave irradiation. Note that other amino acids are relatively unchanged by both procedures.

CONCLUSION

A systematic evaluation of protein hydrolysis in HCl or methanesulfonic acid using microwave oven and Teflon-Pyrex tubes has been carried out in order to facilitate easier access to the important data of amino acid compositions. This protocol is easily adapted in most biochemical laboratories since microwave ovens are so commonly used in melting 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 more preferred by most protein chemists and the expensive and specialized evacuation system of gas-phase hydrolysis is avoided. Currently we are refining our microwave-heating step in order to interface it with the amino acid analyzer and HPLC for the on-line automation of protein hydrolysis and analysis. Improvements in the design of commercial microwave ovens to the special use of biochemical research will prove valuable in the near future.

ACKNOWLEDGMENT

This paper is dedicated to Professor K.-T. Wang in celebration of his 60th birthday. I am grateful for his collaborative work during the course of the development of microwave irradiation as a novel means of peptide and protein hydrolysis.

Received April 18, 1989.

Key Word Index– Amino acid analysis; microwave irradiation; rapid protein hydrolysis; Teflon-Pyrex tubes; methanesulfonic acid; hydrochloric acid.

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