

Specific Peptide-Bond Cleavage by Microwave Irradiation in Weak Acid Solution

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A rapid and selective peptide-bond cleavage in weak acid, induced by microwave irradiation, has been developed. The specific cleavage sites of peptide bonds located only at the carboxyl- and amino-terminal ends of aspartyl residues along the peptide chain. A systematic study including the time course for the cleavage of various aspartyl-containing peptides, the effect of the acidity of the reaction solution on the completeness of peptide-bond cleavage, and the relationship between the power of microwave irradiation and the reaction time of cleavage are studied.

KEY WORDS: Specific cleavage; peptide hydrolysis; microwave irradiation; rate enhancement; weak acid hydrolysis; aspartyl residues.

1. INTRODUCTION

Recently, there has been growing interest in applying microwave heating to the rapid thermal digestion prior to elemental and chemical analysis of inorganic and biological samples (Nadkarni, 1984; Fischer, 1986; Fernando *et al.*, 1986; Kingston and Jassie, 1986). The rapid heating capability of the microwave oven leads to a considerable saving in dissolution time, which may eventually replace some of the conventional flame and hot-plate heating protocols. During the last few years, we have introduced a rapid method of microwave heating for the facile preparation of protein and peptide hydrolysates prior to amino acid analysis (Chen *et al.*, 1987; Yu *et al.*, 1988; Chiou and Wang, 1989). Microwave irradiation has also been applied to several organic reactions in a continuous-flow process by which preparative-scale samples may be synthesized with ease using a kitchen microwave oven (Chen *et al.*, 1990).

Previously, partial hydrolysis of proteins in acidic or basic media by conventional heating has been

reported (Schultz *et al.*, 1962; Tsung and Frankel-Conrat, 1965; Schultz, 1967; Inglis, 1983; Marcus, 1985; Piszkiwicz *et al.*, 1970; Light, 1967). The protocols are usually laborious and time-consuming. We report here a novel application of microwave technology to the specific cleavage of peptide bonds only at the aspartyl residues (Asp and Asn). The new chemical cleavage can circumvent the inherent contamination problems associated with the microscale structural analysis of peptides and proteins using some proteolytic enzymes.

2. MATERIALS AND METHODS

2.1. Apparatus, Chemicals, and Reaction Vessels

Custom-made Teflon vials and Teflon-Pyrex reusable hydrolysis tubes (Chiou and Wang, 1990; Wang *et al.*, 1991) were purchased locally (The Continuity Enterprise, Taipei, Taiwan). Each vial can contain up to 2.0–5.0 ml of hydrolysis solvent. In practice, about 0.3 ml of solvent was added for the preparation of protein hydrolysates. The basic designs of the vials and tubes are based on the inert-gas flushing instead of vacuum-sealing procedure for the removal of oxygen inside the tubes (Chiou and Wang, 1990). The Reacti-Therm dry block heating system (Pierce

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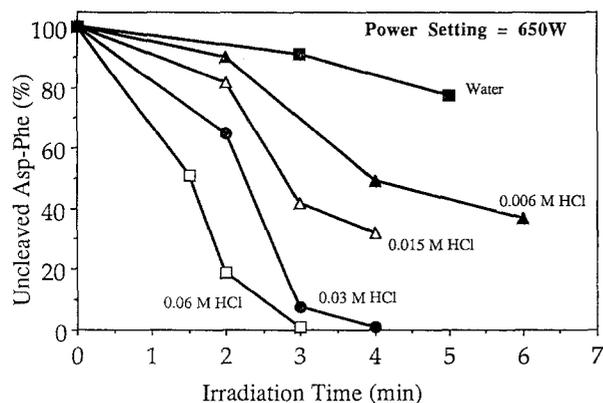


Fig. 1. The effect of solvent acidity on the hydrolysis of peptide bond in dipeptide Asp-Phe. The procedures for microwave irradiation and quantitative analysis of irradiated products are as described in Section 2.2 of **Materials and Methods**.

Chemical Company, Rockford, Illinois) set at 145°C for conventional hydrolysis was also used for comparison. The microwave oven used was a commercially available cooking apparatus without any modification (Tatung microwave oven TMO-1110, Tatung Co., Taipei, Taiwan). The total power of microwaves was 650 W, with 10 power setting starting from 36 W and operated at a frequency of 2450MHz.

Constant boiling 6 M HCl in 1 ml ampules was obtained from Pierce. Dipeptides were obtained from Sigma (St. Louis, Missouri). The HPLC system consisted of two Waters Model 6000 pumps, a Waters Model 450 UV detector, and an M-660 solvent programmer. All HPLC-grade solvents were obtained from a local supplier (Alps Chem. Co., Taiwan). The oligopeptides were synthesized with the established methods and purified to homogeneity.

2.2. General Procedure for Microwave-Irradiated Peptide Hydrolysis

The peptides (about 1 mg/ml each) were dissolved in dilute hydrochloric acid, and aliquots of 0.3 ml were transferred to the Teflon vials. The vials were covered with a silicon septum and flushed with nitrogen gas for about 1 min. Afterward, the vials were placed in the center of a microwave oven and irradiated for a specified time at a desired power setting. After irradiation, the hydrolysates were treated with *o*-phthaldehyde to form fluorescent derivatives and analyzed by reversed-phase HPLC. The column (4.0 × 300 mm, SynChropak RP-C₁₈, 6.5 μm bead) was used to analyze the cleavage products from

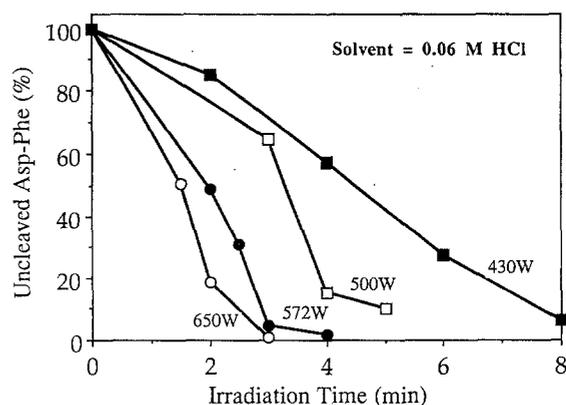


Fig. 2. The effect of input powers of microwave irradiation on the hydrolysis of peptide bond in dipeptide Asp-Phe. The procedures and conditions for microwave irradiation are as described in **Materials and Methods**.

microwave irradiation under different conditions. The peaks were monitored with a fluorescence detector.

2.3. Dipeptide Hydrolysis by Conventional Method

The same peptide solutions as described above were placed in Teflon-Pyrex tubes, flushed with nitrogen gas, and hydrolysis was carried out in a heating block set at 145°C for different time periods. The hydrolysates were analyzed by HPLC with the same fluorescent technique described above.

2.4. Solid-Phase Synthesis of Peptides

t-Boc-amino acid was attached to the resin (0.6 mmol Cl⁻/g resin) according to the method as described by Gisin (1973). The coupling yield of the amino acid attached to resin was 0.54 mmol/g resin. The subsequent additions of other *t*-Boc-amino acids to the solid support were according to the sequences shown in Table II (nonapeptide) and Table III (tetradecapeptide). The final peptides were cleaved from the resin by HF method as follows: (1) 1 g of peptide resin in 0.2 ml of anisole was poured into the reaction vessel (HF-reaction apparatus, type II, from Peptide Institute). The vessel was cooled in a dry ice-isopropanol bath for 30 min and evacuated. (2) 10 ml of HF was charged into the reaction vessel and reacted at 0°C for 45 min. (3) The HF was evaporated and residues were washed with ether (3 × 30 ml) on a sintered glass filter to remove side products. (4) The peptide was separated from resin by washing with 20 ml of

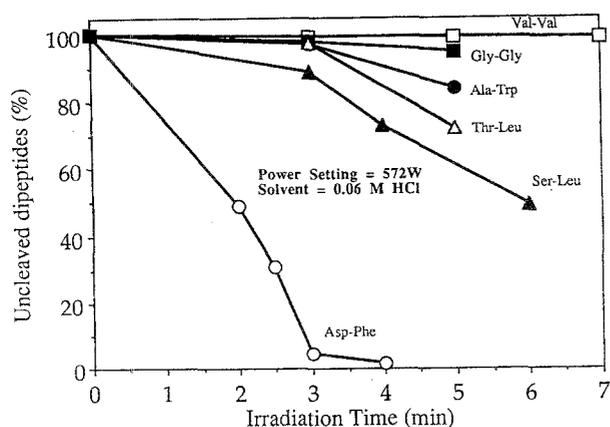


Fig. 3. Time-course study of peptide-bond cleavage in different dipeptides by microwave irradiation in 0.06 M HCl and an input power of 572 W.

0.1 M TFA three times and the combined TFA solution lyophilized to give the crude peptides.

The crude peptides thus obtained were purified by preparative HPLC. They all gave a single peak on reversed-phase HPLC, and amino acid analysis also indicated compositions consistent with the desired synthesized peptides.

3. RESULTS AND DISCUSSION

The dipeptide Asp-Phe was first used to establish the effect of solvent acidity on the peptide-bond hydrolysis. The reaction was performed at five different concentrations of hydrochloric acid (0.06 M, 0.03 M, 0.015 M, and 0.006 M) and water (pH 7.0), respectively, with an input power of 650 W, and the time course of peptide-bond hydrolysis was monitored by HPLC analysis of uncleaved dipeptide. The hydrolysis of the peptide bond was completed within 3 min in 0.06 M HCl and 4 min in 0.03 M HCl (Fig. 1). On the other hand, the hydrolysis of peptide bond in neutral aqueous solution was less than 15% after 4 min microwave irradiation. These observations emphasized the important role of specific acid catalysis in the reaction of peptide hydrolysis.

Microwave energy irradiated on the samples in various liquid media is lost or absorbed to the samples by two mechanisms: ionic conduction and dipole rotation. These two effects take place simultaneously to account for the phenomenon of rapid heating associated with many practical applications of microwave heating (Jassie and Kingston, 1988; Neas and Collins, 1988). Because the inherent hazard of explosion due

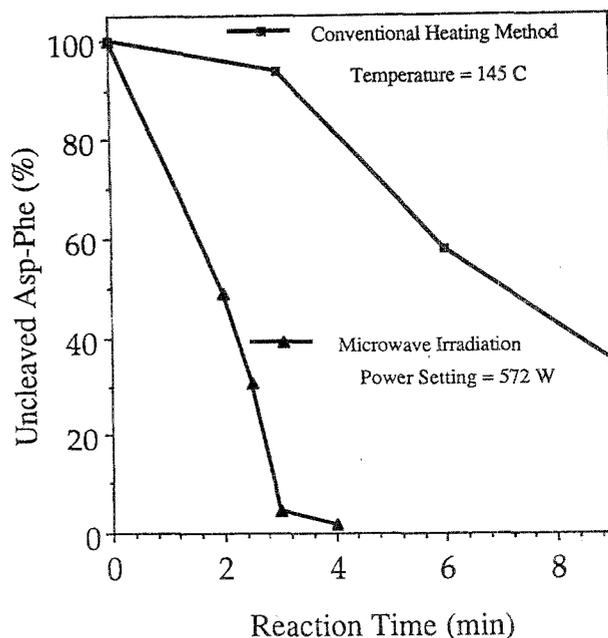


Fig. 4. Comparison of the rate of peptide-bond cleavage in Asp-Phe using microwave irradiation and conventional heating at 145°C in 0.06 M HCl solvent.

to a rapid pressure buildup caused by microwave irradiation was well known (Chen *et al.*, 1990; Chiou and Wang, 1990), a systematic study of suitable reaction conditions to find an appropriate input power for the complete hydrolysis of peptide bonds without overheating is highly desirable. Four different input powers were chosen and the reactions were carried out in 0.06 M hydrochloric acid to find the optimal condition for complete hydrolysis of the peptide bond in Asp-Phe (Fig. 2). It was found that an input power of 572 W or 650 W is very suitable for the hydrolysis

Table I. Reaction Half-Life Times of Peptide-Bond Cleavage in Various Conditions

| Tested dipeptides | 0.015 M HCl at 110°C ^a | 0.06 M HCl by microwave | 0.06 M HCl at 145°C |
|-------------------|-----------------------------------|-------------------------|---------------------|
| Asp-Lys | 228 ^b | 2.6 | 8.5 |
| Lys-Asp | 165 | 4.3 | 8.0 |
| Asp-Phe | 130 | 2.0 | 5.2 |
| Asp-Asp | | 3.0 | 7.5 |
| Asp-Glu | | 2.5 | 8.0 |
| Asp-Ala | | 2.3 | 3.0 |
| Ala-Asp | 108 | 2.2 | 9.8 |
| Gly-Asp | | 1.6 | 12.0 |

^a The concentrations of amino acids released were determined by HPLC analysis (RP-C₁₈ column).

^b Time required for 50% hydrolysis (min).

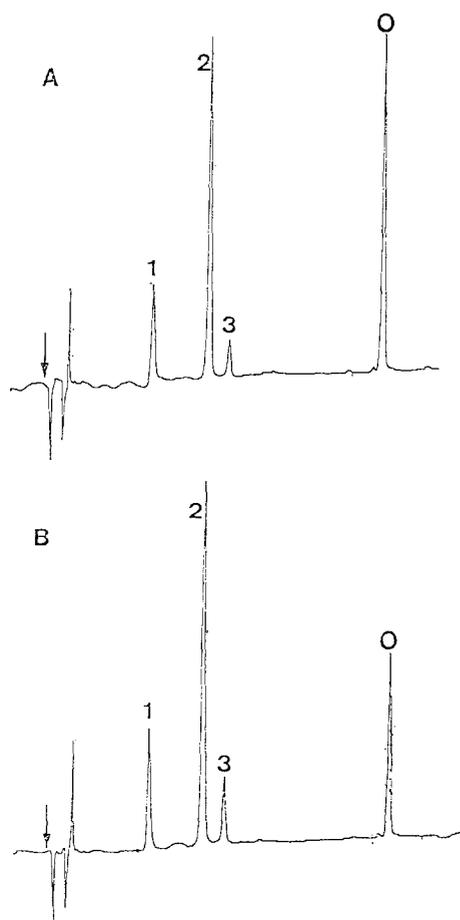


Fig. 5. HPLC analysis of peptide fragments of the synthetic peptide Ala-Glu-Phe-Ala-Asp-Pro-Ser-Leu-Gly by microwave irradiation in 0.06 M HCl under conditions of different input power and irradiation time. (A) 143 W, 3 min; (B) 72 W, 6 min. Conditions for reversed-phase HPLC: RP-C₁₈ column; A 30 min linear gradient of 11–23% acetonitrile in water containing 0.1% TFA; and Peaks monitored by UV absorbance at 214 nm. The peaks 1, 2, and 3 were identified by amino acid analysis as shown in Table II. Peak O and arrow indicate the uncleaved peptide and starting point for sample injection, respectively.

reaction and the peptide bonds can be hydrolyzed within 3 min safely.

In order to compare the relative susceptibility of the peptide bonds in various dipeptides as compared to that of Asp-Phe, five dipeptides Ala-Trp, Val-Val, Gly-Gly, Thr-Leu, and Ser-Leu were reacted under the identical irradiated conditions in 0.06 M HCl and 572 W input power (Fig. 3). The peptide bond of Asp-Phe was completely hydrolyzed within 4 min, whereas all other non-Asp containing dipeptides remained at least 75% intact. The optimized condition of inducing higher than 95% peptide cleavage in Asp-Phe while keeping more than 90% peptide bonds



Fig. 6. HPLC analysis of peptide fragments of the synthetic peptide Gly-Phe-Lys-Phe-Ser-Phe-Glu-Phe-Gly-Asp-Phe-Ala-Leu-Gly by microwave irradiation in 0.06 M HCl under conditions of different input power and irradiation time. (A) 215 W, 3 min; (B) 286 W, 4.5 min. Conditions for HPLC are the same as Fig. 5. The peaks 1 and 2 were identified by amino acid analysis as shown in Table III. Peak O and arrow indicate the uncleaved peptide and starting point for sample injection, respectively.

intact in other dipeptides was found to be that of the milder condition for 3 min irradiation. It is noteworthy that under this reaction condition the peptide bond and Trp residue in Ala-Trp dipeptide remained more or less intact in contrast to the complete peptide hydrolysis of Asp-Phe employing 3 min microwave irradiation. It is well known that Trp residues in most proteins are totally destroyed under the regular 6 M

HCl hydrolysis. This observation may be applied to the sequencing study of Trp-containing peptides to prevent the Trp from decomposition by using the rapid microwave heating under mild acid and short irradiation.

Figure 4 shows the comparison of time courses between peptide-bond hydrolysis in Asp-Phe by conventional method (145°C, heating block) and microwave irradiation (power 572 W). It is evident that the hydrolysis by microwave irradiation is much faster than that by conventional heating method as judged by the slopes shown in Fig. 4. The drastic difference in the reaction profiles may be explained by a more rapid energy transfer in microwave irradiation than conventional heating. In the microwave-irradiated reactions, all microwave energy is adsorbed by the solution since Teflon vessels used are almost microwave transparent. The temperature of the reaction solution was determined to be about 140–155°C and that of the vessel about 40–50°C after 2–3 min irradiation. We have also compared the hydrolysis times required for 8 different Asp-containing dipeptides using three different protocols (Table I). In general, the time needed for complete hydrolysis by microwave irradiation is only about 1/6 to 1/3 that of the conventional heating at 145°C.

Two Asp-containing peptides Ala-Glu-Phe-Ala-Asp-Pro-Ser-Leu-Gly and Gly-Phe-Lys-Phe-Ser-Phe-Glu-Phe-Gly-Asp-Phe-Ala-Leu-Gly were synthesized by the standard solid-phase method and purified to homogeneity as judged by both TLC and HPLC. The time-course studies of the cleavage of these peptides were followed by HPLC (Figs. 5 and 6) and amino acid analysis (Tables II and III). From the composition data of the cleaved fragments, it was clearly indicated that the peptides were cleaved

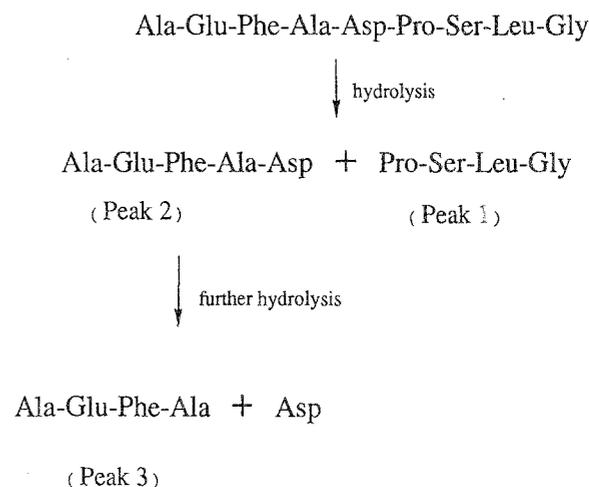
Table II. Amino Acid Analysis of Tested Synthetic Peptide Ala-Glu-Phe-Ala-Asp-Pro-Ser-Leu-Gly and Its Peptide Fragments Isolated by HPLC After Cleavage by Microwave Irradiation in 0.06 M Hydrochloric Acid

| Amino acid residues | Uncleaved synthetic peptide | Isolated fragments | | |
|---------------------|-----------------------------|--------------------|--------|--------|
| | | Peak 1 | Peak 2 | Peak 3 |
| Ala (2) | 2.00 | | 2.00 | 2.00 |
| Glu (1) | 1.07 | | 1.02 | 1.00 |
| Phe (1) | 0.99 | | 1.00 | 0.98 |
| Asp (1) | 1.04 | | 1.00 | |
| Pro (1) | 1.04 | 0.98 | | |
| Ser (1) | 0.73 | 0.75 | | |
| Leu (1) | 1.05 | 1.04 | | |
| Gly (1) | 1.07 | 1.00 | | |

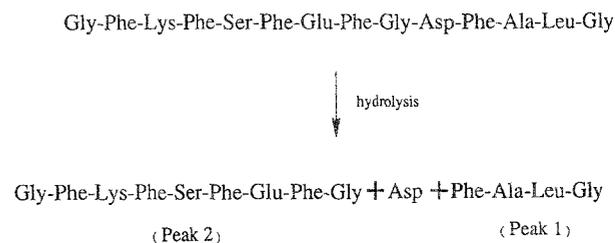
Table III. Amino Acid Analysis of Synthetic Peptide Gly-Phe-Lys-Phe-Ser-Phe-Glu-Phe-Gly-Asp-Phe-Ala-Leu-Gly and Its Peptide Fragments Isolated by HPLC After Cleavage by Microwave Irradiation in 0.06 M HCl

| Amino acid residues | Uncleaved synthetic peptide | Isolated fragments | |
|---------------------|-----------------------------|--------------------|--------|
| | | Peak 1 | Peak 2 |
| Leu (1) | 1.01 | 0.96 | |
| Ala (1) | 0.95 | 0.91 | |
| Gly (3) | 3.00 | 1.00 | 2.00 |
| Phe (5) | 4.86 | 0.96 | 3.89 |
| Lys (1) | 1.01 | | 0.99 |
| Ser (1) | 0.75 | | 0.71 |
| Glu (1) | 1.07 | | 1.04 |
| Asp (1) | 1.30 | | |

specifically at both ends of aspartic acid along the peptides. In Schemes I and II, we show the cleavage sites and the sequences of the resulting fragments. It is noteworthy that the peptide bonds of Asp-Pro and



Scheme I. Proposed hydrolytic pathway of the synthetic peptide Ala-Glu-Phe-Ala-Asp-Pro-Ser-Leu-Gly by microwave irradiation in 0.06 M HCl; three fragments (Peaks 1, 2, and 3) were detected by HPLC.



Scheme II. Proposed hydrolytic pathway of the synthetic peptide Gly-Phe-Lys-Phe-Ser-Phe-Glu-Phe-Gly-Asp-Phe-Ala-Leu-Gly by microwave irradiation in 0.06 M HCl; only two fragments (Peaks 1 and 2) were detected by HPLC.

Ala-Asp are more labile than all other peptide-bond linkages in the first peptide and those of Gly-Asp and Asp-Phe in the second peptide are weaker than other peptide bonds.

In conclusion, a method using microwave irradiation and weak acid have been developed to control the cleavage sites of peptide bonds, especially those bonds connected to aspartic acid residues inside the native peptides and proteins. The method should prove very useful to obtain the defined acid-cleaved peptide fragments of proteins and circumvent the contamination problems inherently associated with microscale purification and sequence analysis of fragments generated from proteolytic enzymes.

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