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Account

# The Studies of Microwave Effects on the Chemical Reactions

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Microwave heating involves direct absorption of energy by functional groups that bear ionic conductivity or a dipole rotational-effect, and this energy is then released into the surrounding solution. This absorption of energy causes the functional groups involved to have higher reactivity to other surrounding reactants than when they are simply incubated with the reactants at the same temperature. In other word the enhanced rate of the reaction can be due to the reactant stirred by the molecular dipole rotation and molecules themselves acting as a stirring bar. In contrast to conventional heating, the salient feature of "dipole rotation" constitutes one efficient form of "molecular agitation" or "molecular stirring" many aspects of which can be explore in chemical reactions. We will discuss some of the useful applications of this "molecular agitation" by means of microwave irradiation. Using this unique technology, we have developed: 1) a method to control the cleavage sites of peptide bonds, especially those bonds connected to aspartic acid residues inside the native peptides and proteins, 2) a method to increase coupling efficiency in solid-phase peptide synthesis using a common microwave oven, 3) a novel procedure that increases the rate of alcalase-catalyzed reactions using microwave irradiation in peptide-bond formation with proline as a nucleophile and selective benzoylation of a pyranoside derivative, 4) a procedure to solubilize and hydrolyze retrograded starch, 5) a novel procedure to enhance the rate of saponification in a serum sample for very long chain fatty acid analysis.

## INTRODUCTION

Microwaves are electromagnetic radiation of wavelengths between 0.1 cm and 100 cm. Microwave energy lies between the radio and infrared regions of the electromagnetic spectrum. The first microwave oven for commercial use was introduced in the early 1950s and has gradually become a necessity for fast cooking. Recently, interest has been growing in applying microwave heating to rapid thermal digestion prior to elemental and chemical analysis of inorganic and biological samples.[1-5] Microwave irradiation perturbs molecules into dipole rotation without causing rearrangement of their structures. The energy absorbed by polar substances stimulates dipole rotation of molecules. If the molecules are water, friction caused by dipole rotation of water molecules generate heat after microwave irradiation. This makes fast cooking of foodstuffs possible in the home microwave. If the polar organic molecules dissolved in non-polar solvents, in *n*-hexane for example, the irradiated molecule would act as a stirring bar and accelerates the rate of the reactions. 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.[6-8] Microwave irradiation has also been applied to several organic reactions in a continuous-flow process by which preparative-scale samples (20 g) have been synthesized with ease using a kitchen microwave oven.[9] The present report is intended to give an overview of some of the chemical and biochemical applications of microwave irradiation studied in this lab.

### Specific Cleavage of Peptide Bonds

The use of microwave irradiation in rapid hydrolysis of peptides and proteins has been documented. Microwaves reduced the time required for peptide bond hydrolysis from 24 hours to 5 minutes. Previously partial hydrolysis of proteins in acidic or basic media by conventional heating has been reported.[1-4,10-11] The protocols are usually laborious and time-consuming. We have developed 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

Dedicated to Professor Yu-Shia Cheng on the occasion of her 65th birthday.

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contamination problems associated with the microscale structural analysis of peptides and proteins using some proteolytic enzymes. To prevent the inherent hazard of explosion due to a rapid pressure buildup caused by microwave irradiation,[9] 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. 1). It was found that an input power of 572 W or 650 W is very suitable for the hydrolysis reaction, and the peptide bonds can be hydrolyzed safely within 3 min.

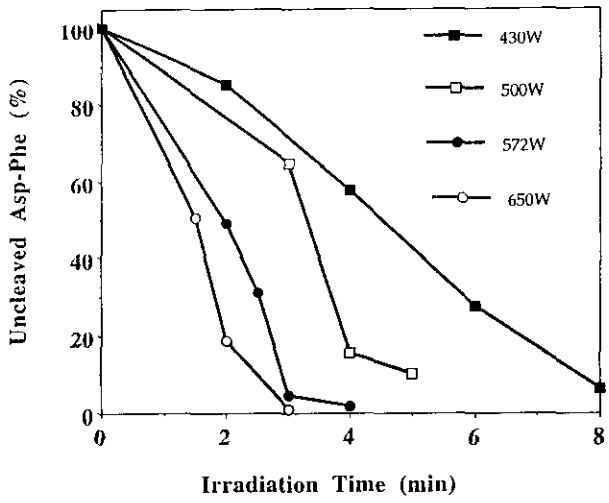



Fig. 1. The effect of solvent acidity on the hydrolysis of peptide bond between Asp and Phe.

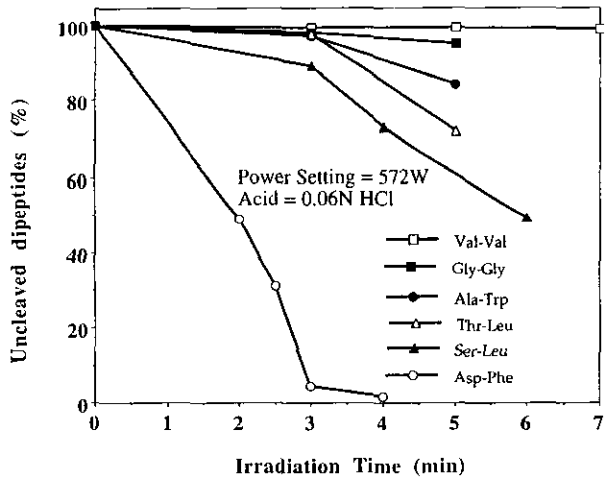



Fig. 2. The effect of input power of microwave irradiation on the hydrolysis of peptide bond between Asp and Phe.

In order to compare the relative susceptibility of the peptide bonds in various dipeptides compared to those of Asp-Phe, five dipeptides Ala-Trp, Val-Val, Gly-Gly, Thr-Leu, and Ser-Leu were reacted under identical irradiated conditions in 0.06 M HCl and 572 W input power (Fig. 2). 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 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 of 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 rapid microwave heating with mild acid and short irradiation.

A comparison of time courses between peptide-bond hydrolysis in Asp-Phe by the conventional method (145 °C, heating block) and microwave irradiation (power 572 W) shows that hydrolysis by microwave irradiation is much faster than by conventional heating as judged by the slopes in Fig. 3. 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

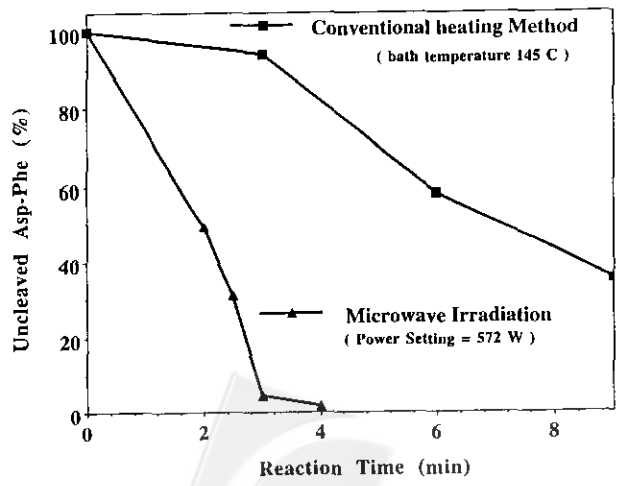



Fig. 3. The time course of various peptide bonds hydrolyzed by microwave irradiation with input power 572 W in 0.06M HCl.

Table 1. The Half Life Time (min) of Peptide Bond Cleaved in Various Conditions

| Tested Dipeptides | in 0.015N HCl at 110 °C* | in 0.06N HCl by microwave | in 0.06N HCl at 145 °C |
|---|---|---|---|
| Asp-Lys | 228 | 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 |

* The concentration of amino acid released was determined by HPLC analysis (RP-18) and the time required for 50% hydrolysis (min).

Table 3. The Amino Acid Analysis of Tested Synthetic Peptide Gly-Phe-Lys-Phe-Ser-Phe-Glu-Phe-Gly-Asp-Phe-Ala-Leu-Gly and Peptide Fragments Which Was Isolated by HPLC After Cleavage by Microwave Irradiation

| Amino Acid residues | amino acid found | Isolated fragment | |
|---|---|---|---|
| | | 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 | | |

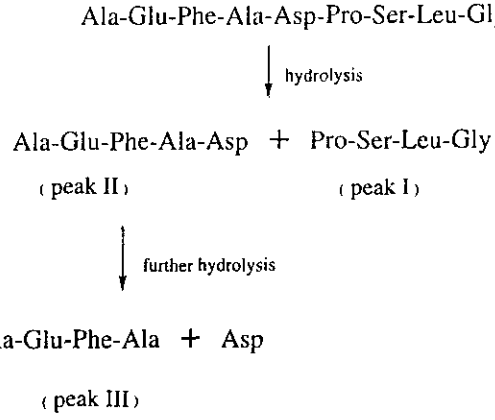
transparent. The temperature of the reaction solution was determined to be about 140-155 °C and that of the vessel about 40-50 °C after 2-3 min irradiation. We have also compared the hydrolysis times required for 8 different Asp-containing dipeptides using three different protocols (Table 1). In general the time needed for complete hydrolysis by microwave irradiation is only about 1/6 to 1/3 that of 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 and amino acid analysis (Tables 2 and 3). The composition data of the cleaved fragments, clearly indicated that the peptides were cleaved 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 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.

Table 2. The Amino Acid Analysis of Tested Synthetic Peptide Ala-Glu-Phe-Ala-Asp-Pro-Ser-Leu-Glyand Peptide Fragments Which Was Isolated by HPLC After Cleavage by Microwave Irradiation

| Amino Acid residues | amino acid found | Isolated fragment | | |
|---|---|---|---|---|
| | | 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 | | |

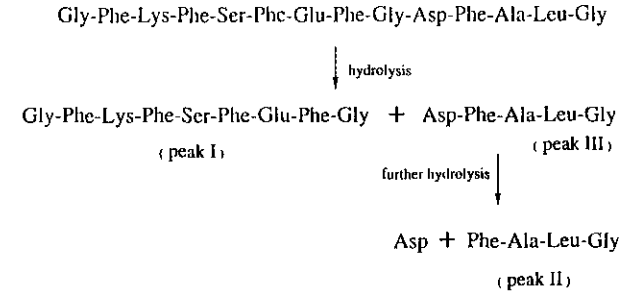
**Scheme I** Microwave irradiation cleavage synthetic peptide Ala-Glu-Phe-Ala-Asp-Pro-Ser-Leu-Gly in to two fragments peak I & peak II and the peak I was further hydrolyzed to peak III and Asp




**Application of Microwave-Assistant Specific Cleavage of Peptide Bonds to Determining a Side-Reaction Occurring in Peptide Synthesis**

Automatic peptide synthesizer is currently a convenient way to obtain peptides which have less than one hundred amino-acid residues.[12] Many biologically active peptides have been synthesized by this general method.[13,14] Recently, we found that a side reaction occurred in the synthesis of a fragment of the acyl carrier protein ACP$^{(65-74)}$. The

**Scheme II** Microwave irradiation cleavage synthetic peptide Gly-Phe-Lys-Phe-Ser-Phe-Glu-Phe-Gly-Asp-Phe-Ala-Leu-Gly into two fragments peak I & peak III and the peak III was further hydrolyzed to peak II and Asp




$ACP^{(65-74)}$ has the amino-acid sequence $^{65}$Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-$^{74}$Gly which corresponds to the sequence 65-74 of the acyl carrier protein. Two major side-products and a desired peptide (each was one-third) were found in analysis of a synthesized $ACP^{(65-74)}$ (see Fig. 4). The peptide mixtures were separated and collected by HPLC. Each isolated peptide was characterized via amino-acid composition analysis and mass spectra. The specific peptide-bond cleavage had been used to prove the sequence as follows.

The isolated peptides were hydrolysed by microwave irradiation. Each peptide (A, B, and C) was completely hydrolyzed under microwave irradiation in a mixture of 6M HCl/TFA = 4:1, and the amino-acid composition of each peptide was determined. The results were shown in Table 4. Only the amino-acid composition of peptide B agreed with the composition of the $ACP^{(65-74)}$, and the compositions of peptides A and C lacked one amino-acid as compared with the data of $ACP^{(65-74)}$. Peptide A lacked one Ile and peptide C lacked one Asx (i.e. Asp or Asn). The peptides A, B, and C were also confirmed by the molar mass of each peptide in their mass spectra. Peptide A has $MM^{(M+1)+}$: 950, peptide B has $MM^{(M+1)+}$: = 1063, and Peptide C has $MM^{(M+1)+}$: 949. Because there are two Ile and two Asx in the sequence of the $ACP^{(65-74)}$ (i.e.: $^{69}$Ile and $^{72}$Ile, and $^{70}$Asp and $^{73}$Asn), at this stage, it was still premature to determine the position of the missing amino-acid. We had to find other methods to confirm that $^{69}$Ile or $^{72}$Ile was absent in peptide A and $^{70}$Asp or $^{73}$Asn was lacking in peptide C.

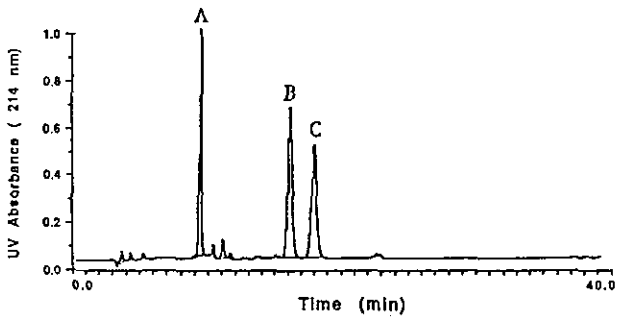



Fig. 4. HPLC profile for the mixtures of $^{65-74}$Acp. Conditions: 0-5 min linear gradient of 12%-22.2% acetonitrile in water containing 0.1% TFA and 5-35 min line gradient of 22.2%-28.8% acetonitrile in water containing 0.1% TFA.

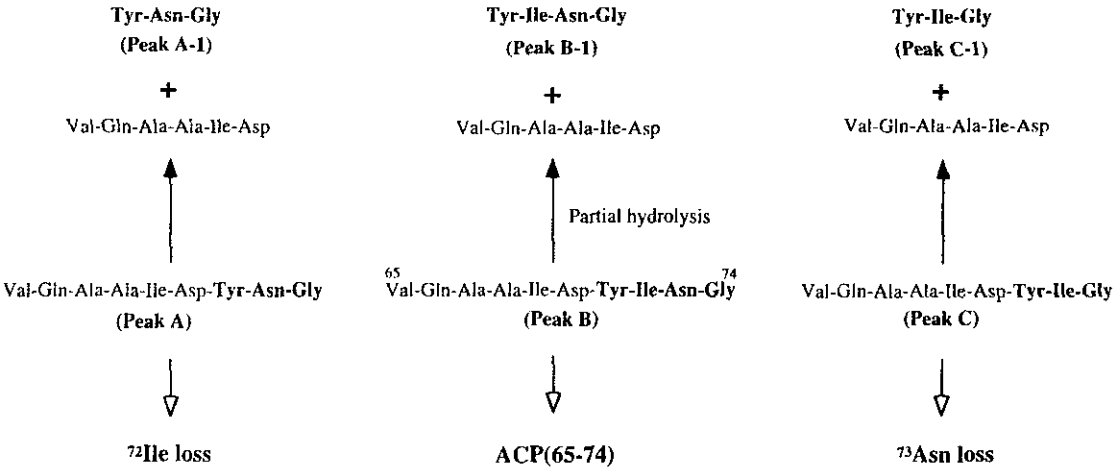
Table 4. Amino Acid Composition of the Three Major Peaks of Synthesized ACP (65-74)

| | Asp | Glu | Gly | Ala | Val | Ile | Tyr |
|---|---|---|---|---|---|---|---|
| Theoretical: | 2 | 1 | 1 | 2 | 1 | 2 | 1 |
| (found:) | | | | | | | |
| Peak-A | 1.94 | 1.04 | 1.07 | 2.15 | 1.00 | 0.93 | 0.96 |
| Peak-B | 1.95 | 1.02 | 1.06 | 2.12 | 1.00 | 1.78 | 0.91 |
| Peak-C | 0.98 | 1.02 | 1.00 | 2.03 | 1.00 | 1.78 | 0.93 |

The selective peptide-bond hydrolysis method allowed the peptide-bond to be specifically cleaved only at the C-terminal or N-terminal of the aspartyl residue. Because the $ACP^{(65-74)}$ had only one aspartyl residue, we could selectively cleave the peptides A and B into two fragments and further analyze each isolated fragment to determine its sequence. In the case of peptide C, if the missing amino-acid were Asp, this method would not be applicable. Then other methods may be necessary for further proof of the sequence of peptide C. The peptides A, B and C were selectively cleaved by microwave irradiation in 0.06M HCl and the hydrolysate of each reaction was analysed by HPLC. The results are shown in Figs. 5-A, B, and C. Each peptide was cleaved into three peaks, designed A-1, A-2, A-3 for peptide A, B-1, B-2, B-3 for peptide B, and C-1, C-2, C-3 for peptide C.

Because peptide C could be cleaved into two small fragments, this preliminary test showed that the missing amino-acid in peptide C is Asn. Those peaks were isolated by preparative HPLC. We identified the amino-acid compositions of the hydrolysate of each isolated peak with the amino-acid analyzer. Table 5 shows the results. The hydrolysate of the isolated peak A-1 has the amino-acid composition (Asp:Gly:Tyr = 0.97:1.00:0.89) and only the peptide which contained the C-terminal three amino-acid residues had this composition. Thus it was proved to be the fragment Tyr-Asn-Gly. Obviously the missing Ile was $^{72}$Ile and not $^{69}$Ile of peptide A. By using the same method, the peak C-1 proved to be the fragment Tyr-Ile-Gly generated by partial

**Scheme III**



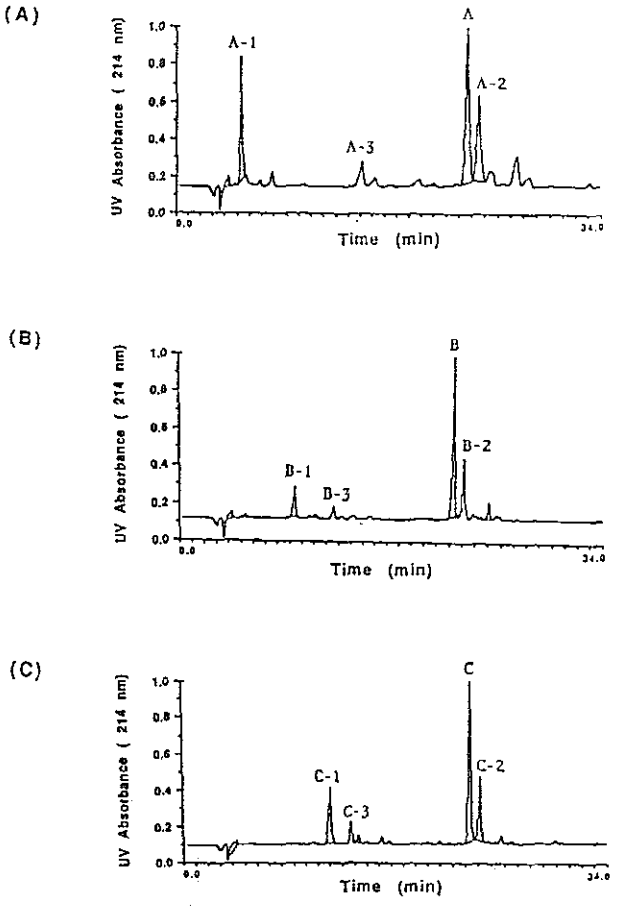




Fig. 5. HPLC profile for the hydrolysate of partial hydrolysis of peptides. (A) Peptide A, conditions: RP-18 column, 25 min linear gradient of 5%-22.5% acetonitrile in water containing 0.1% TFA. (B) Peptide B, conditions: RP-18 column, 30 min linear gradient of 5%-36.5% acetonitrile in water containing 0.1% TFA. (C) Peptide C, conditions: the same as (B).

hydrolysis of peak C; this result indicated that the lacking amino-acid residue in peptide C was $^{73}$Asn (see Scheme III).

According to the above results, the side reaction occurred when the second or third amino-acid residue was coupled by the automatic peptide synthesizer. A side reaction of this type also happened in the manual method of solid-phase peptide synthesis. We thought that this kind of side reaction was caused by impurities from the air or moisture or water in the solvent. The polar compound that decomposed from the reagent for peptide synthesis might also cause a side reaction.

## Enhanced Coupling Efficiency in Solid-Phase Peptide Synthesis by Microwave Irradiation

We have developed a novel microwave technology to enhance coupling efficiency in solid-phase peptide synthesis. A significant improvement of the coupling efficiency (a rate increase of at least 2-4 fold), especially in side-chain

Table 5. Amino Acid Composition of A-1, A-2, B-1, B-2, C-1 and C-2

| | Asp | Glu | Gly | Ala | Val | Ile | Tyr |
|---|---|---|---|---|---|---|---|
| Theoretical: | 1 | 0 | 1 | 0 | 0 | 1 | 1 |
| Found | | | | | | | |
| Peak A-1 | 0.97 | 0 | 1.00 | 0 | 0 | 0 | 0.89 |
| Peak B-1 | 0.95 | 0 | 1.00 | 0 | 0 | 0.80 | 0.85 |
| Peak C-1 | 0 | 0 | 1.25 | 0 | 0 | 1.00 | 0.96 |
| Theoretical: | 2 | 1 | 1 | 2 | 1 | 2 | 1 |
| Found | | | | | | | |
| Peak A-2 | 1.89 | 1.08 | 0.83 | 2.02 | 1.00 | 0.89 | 0.93 |
| Peak B-2 | 1.97 | 1.06 | 0.76 | 2.19 | 1.00 | 1.85 | 0.95 |
| Peak C-2 | 0.95 | 0.98 | 0.95 | 1.98 | 1.00 | 1.70 | 0.90 |

hindered amino acids, was obtained in the study.

Fig. 6 shows the reaction apparatus and vessels.[15] The custom-made solid phase reaction vessel was placed in the middle of the microwave oven, and a teflon tube from the side-arm of the reaction vessel was connected to a nitrogen source to introduce a stream of nitrogen. The Fmoc-protected amino acids with two different coupling methods, symmetric anhydride and preformed *N*-hydroxybenzo-triazole active ester (HOBt), were used in the synthesis.[16-20]

In a preliminary test, two symmetric anhydride derivatives of Fmoc-Ile and Fmoc-Val were coupled with Gly-HMP-resin,[21-24] respectively. The reaction was conducted via microwave irradiation for 2-6 minutes and stopped by filtering off the reaction solution via the side-arm. Fig. 7 shows the time course for the dipeptide formation. The coupling reaction of these two refractory and sterically hindered β-branched amino acid derivatives to form protected dipeptide on resin usually takes a longer time than do most other amino acid derivatives.[25-26] The reaction was slow when the coupling was conducted without microwave irradiation. In the case of microwave irradiation using the lowest power (10% of full power), the reaction rate increased at least 2-3 fold under comparable conditions, and the coupling reaction of both dipeptides could be completed in 6 minutes whereas without irradiation both reactions only reached a level of 60% and of 79%, respectively. Under these reaction conditions, the temperature of the reaction solution was near 55 °C.

Significantly improved coupling efficiency with microwave irradiation was observed in all the tested amino acid derivatives.[27] In a further study, two peptide fragments, Fmoc-Val-Ile-OH and Fmoc-Ala-Val-Ile-OH were coupled with Gly-HMP-resin. Surprisingly, all the coupling reactions were completed within 2 minutes. The coupling yield of each step was determined by quantitative ninhydrin assay[28] and by calculating the relative concentration of the product and the unreacted peptide using HPLC analysis.[29]

For a representive test, three peptides, $^{65\text{-}74}$fragment of acyl carrier protein ($^{65\text{-}74}$Acp, for sequence see Scheme IV), Gly-Val-Gly-Phe-Val-Ile-Gly, and Gly-Phe-Gly-Val-Ala-Val-Ile-Gly were synthesized using pre-formed active ester in DMF, and each coupling step included irradiation for 4 minutes. The $^{65\text{-}74}$Acp was synthesized by stepwise coupling of amino acid derivatives, and the other two peptides were synthesized by fragment coupling. After the elongation finished, the weight increased by the peptide coupled to the resin was measured and calculated to have an average coupling yield of 99.65%. The coupling efficiency (by ninhydrine assay) in each step is shown in Scheme IV. With microwave irradiation, peptide bond formation was completed within 4 minutes. Compared with the reaction without microwave irradiation at room temperature for 30 minutes, the latter was relatively slow, especially in the case of fragment coupling. Fig. 8 shows the Hplc analysis results of crude $^{65\text{-}74}$Acp, pure $^{65\text{-}74}$Acp (obtained by collecting the desired peak in crude Hplc analysis), and a crude $^{65\text{-}74}$Acp syn-

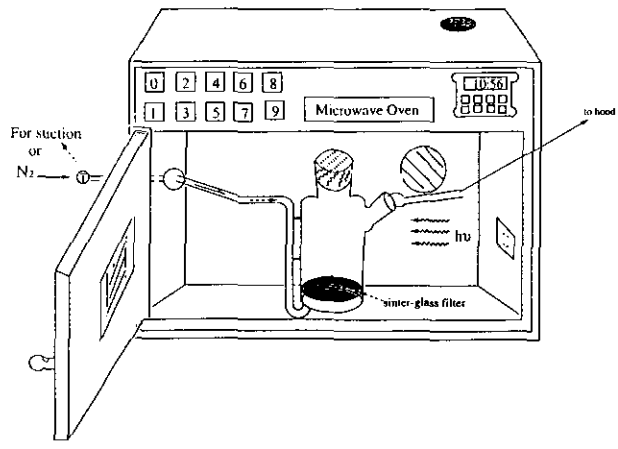



Fig. 6. The microwave oven and the custom-made solid phase reaction vessel. The vessel was left in the middle of the microwave oven, and a teflon tube from the side-arm of the reaction vessel was connected to a nitrogen source. During microwave irradiation, a stream of nitrogen was blown into the reaction vessel, and the bubbles of nitrogen gas served as a stirrer. After irradiation was stopped, the reaction solution was filtered off via the side-arm by suction.

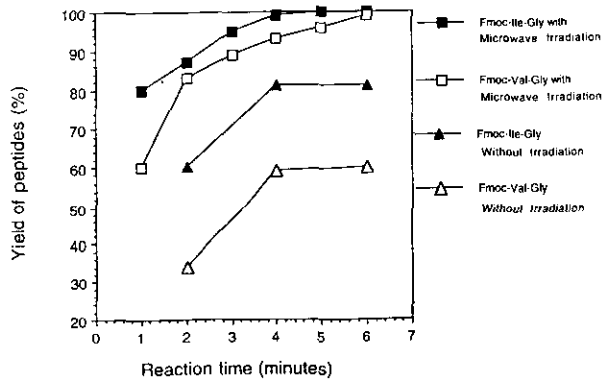



Fig. 7. Time courses for the solid phase peptide synthesis of Fmoc-peptides with and without microwave irradiation in DMF solution. (Fmoc-Ile-Gly-resin with microwave irradiation; Fmoc-Ile-Gly-resin without microwave irradiation; Fmoc-Val-Gly-resin with microwave irradiation; Fmoc-Val-Gly-resin without microwave irradiation.)

**Scheme IV**

$^{65\text{-}74}$Acp: H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-Resin

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| with microwave irradiation: | 99.9 | 100 | 100 | 99.8 | 99.5 | 99.9 | 99.9 | 99.7 | 99.8 |
| without microwave irradiation: | 80.4 | 83.1 | 87.9 | 94.8 | 93.1 | 76.7 | 70.5 | 79.9 | 83.4 |
| ABI 431-A Synthesizer: | 99.4 | 99.9 | 100 | 99.8 | 98.9 | 99.9 | 98.9 | 99.7 | 99.5 |

peptide-1: H-Gly-Phe-Gly-Val-Ala-Val-Ile-Gly-Resin

| | | | |
|---|---|---|---|
| with microwave irradiation: | 100 | 99.9 | 99.8 |
| without microwave irradiation: | 76.4 | 71.2 | 46.8 |

peptide-2: H-Gly-Val-Gly-Phe-Val-Ile-Gly-Resin

| | | | |
|---|---|---|---|
| with microwave irradiation: | 99.8 | 99.7 | 100 |
| without microwave irradiation: | 58.5 | 73.7 | 47.9 |

* Based on a quantitative ninhydrine test in which the average coupling yield in Acp$^{65\text{-}74}$ is 99.14%, for the other two peptides the positions of the peptide bond formation are denoted by an array, and the yields of each coupling step are shown on the array.

thesized by an auto-synthesizer method (ABI-431A). The peak of crude $^{65\text{-}74}$Acp synthesized by the microwave irradiation method had a yield of 79.0%(8-a), whereas the peak of $^{65\text{-}74}$Acp from the auto-synthesizer had a yield of 69.4%(8-c). Fig. 9 shows the hplc analysis of the two crude products that were made by fragment coupling of Fmoc-Val-Ile-OH and Fmoc-Ala-Val-Ile-OH. The purity of each major peak was > 85%. The product was characterized by amino acid analysis and FAB-MS. No detectable racemization was observed. This result coincided with another recently reported, in which a pre-formed active ester of Fmoc-Val or of Fmoc-Me-Leu coupled with an MBHA-resin or with an Me-Leu-BHA-resin in DCM/DMF (1:1) solution, respectively. No racemization (< 0.1%) has been found in hplc.[30] We reasoned that the reaction-step that can easily cause racemization is during DCC activated to form peptide bond in the presence of tertiary-amine[31] and not in the formation of a symmetric anhydride or an active ester in the absence of amino-component.

Using microwave irradiation to hydrolyze peptides and proteins will result in substantially less racemization.[32] We have studied microwave-accelerated coupling reactions in different solvents and at different reaction temperatures by control of energy input in microwave power.[33] It has been found that no significant racemization side reactions have occurred in the dipeptide products from the coupling reaction between different amino acid derivatives. Increased coupling efficiency in solid-phase peptide synthesis using elevated temperature has been shown,[34] but the use of microwave irradiation has not been investigated before. In conclusion, we have presented here a new reaction protocol by using a common microwave oven in solid phase peptide synthesis. The procedure not only reduced the needed reaction time of 2-3 hours reacting at room temperature, or of 30 minutes by reacting at 60 °C, to less than 6 minutes via the

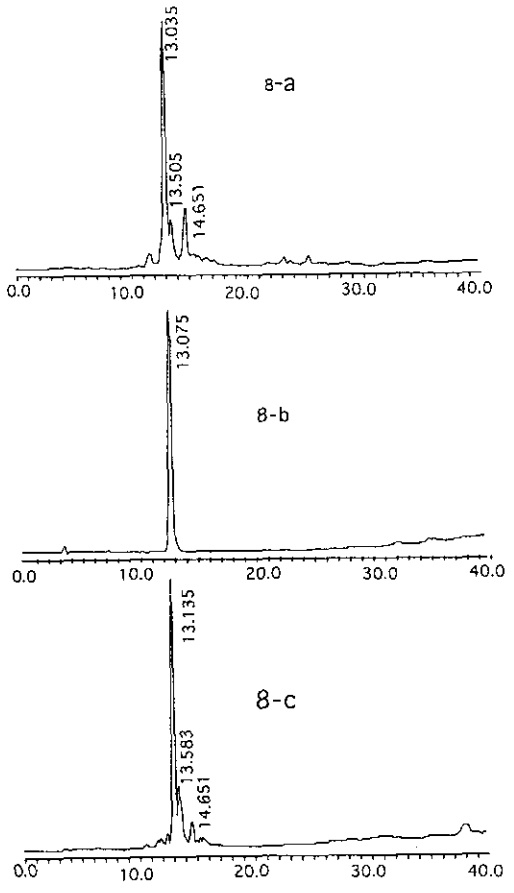



Fig. 8. Hplc analysis of three $^{65\text{-}74}$Acp's. 3-a is a crude $^{65\text{-}74}$Acp synthesized using microwave irradiation. 3-b is a pure $^{65\text{-}74}$Acp purified by Hplc method from 3-a. 3-c is a crude $^{65\text{-}74}$Acp synthesized by an auto-synthesizer method. The hplc was performed on a Gilson instrument, which consisted of a Model 302 pump, a Model 305 pump, a Model 115 uv detecter, and a Model 815b Dynamic Mixer. The signal of analysis was collected and plotted by Rainin Dynamax, which runs on a Macintosh LC. The conditions for the analysis were: eluent A, 0.1% TFA in water/acetonitrile (9:1), eluent B, 0.1% TFA in water/acetonitrile (1:9), Gradient: B%, 0%→100%, 40 min., uv: 214 nm, Flow rate: 1 mL/min., Column: RP-18, 4.6 × 250 mm.

microwave irradiation method, but also accomplished the complete coupling of difficult sequence peptides. The reaction apparatus is simple and can potentially be designed for an auto-synthesizer. Under microwave irradiation conditions, the peptide fragments have higher reactivity than do the amino acid derivatives. That is very useful for the synthesis of big peptides. Using a dipeptide or a tripeptide instead of amino acid derivatives in the same synthesis steps will make a peptide with longer amino acid residues. Also in synthesis of the same peptide, the fragment coupling will only need one-half or one-third the coupling steps, especially for a sterectically hindered amino acid. Developement of a convenient method for preparation of a peptide fragment would be very useful for the synthesis of big peptides.[35-38]

**Enhancement of the Rate of Enzyme-catalysis by Microwave Irradiation**

The use of microwave irradiation to accelarate enzyme-catalysis has not been studied before. We have developed a novel application of microwave technology to enhance the efficiency of alcalase-catalysis. A significant improvement in the efficiency of peptide bond formation (a rate increase of at least 2 to 10 fold) has been observed. Moreover, the regioselective benzoylation of sugars was also observed.

The stability of alcalase in 2-methyl-2-propanol (2.5 AU/5 mL) with or without microwave irradiation was measured according to previously described procedures.[40] Fig. 10 shows the time course of alcalase inactivation under the reaction conditions. The temperature of the solution rose from 25 °C to 50 °C after the solution was irradiated for 30 minutes.[41] The alcalase maintains 50% of the original activity with microwave irradiation, and maintains 75% of the ac-

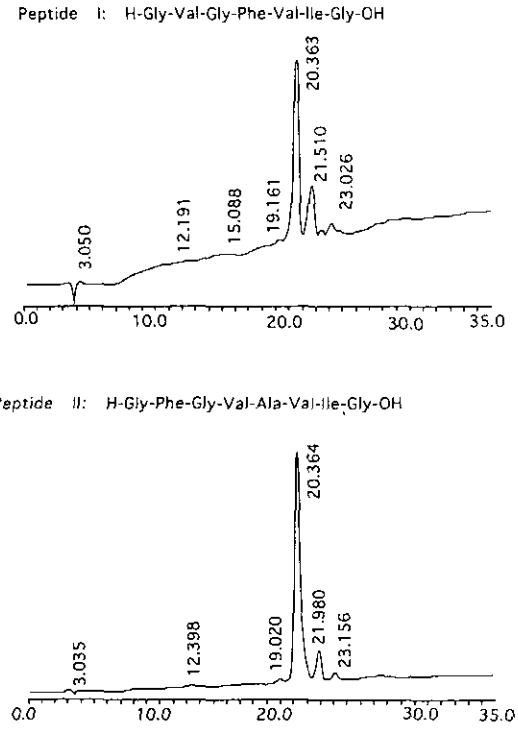



Fig 9. Hplc analysis of peptides H-Gly-Phe-Gly-Val-Ala-Val-Ile-Gly-OH (4-a) and H-Gly-Val-Gly-Phe-Val-Ile-Val-Ile-Gly-OH (4-b), using the same Hplc. The conditions for analysis of both samples were: eluent A, 0.1% TFA in water/acetonitrile (95:5), eluent B, 0.1% TFA in water/acetonitrile (25:75), Gradient: B%, 0%→100%, 0→35 min, uv: 214 nm, flow rate: 1 mL/min, Column: RP-18, 4.6 × 250 mm.

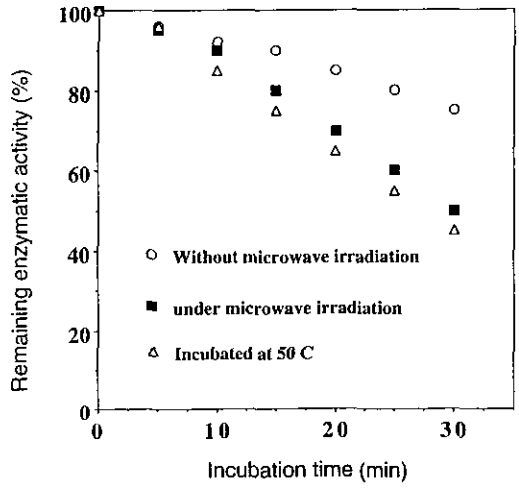



Fig. 10. The stability of alcalase in various reaction conditions was determined by measuring the *trans*-esterification of Moz-Leu-OBzl to Moz-Leu-OEt in anhydrous ethanol. The stability of the enzyme in 2-methyl-2-propanol was displayed as the activity of the enzyme remaining after incubation. In each test, alcalase solution (100 μL), from the incubation mixture of alcalase and 2-methyl-2-propanol) was added to Moz-Leu-OBzl (0.1 mmol, 46 mg) dissolved in absolute ethanol (2 mL). The resulting solution was stirred at 25 °C for 30 minutes and quenched by the addition of HCl (0.10 N, 1.9 mL) for a final volume of 4 mL. The solution was centrifuged for 5 minutes at 3000 rpm, and aliquots (20 μL) of the supernatant were analyzed by HPLC using a RP-18 column, a UV detector at 254 nm, and 15% (v/v) acetonitrile as the eluent. The peak area corresponding to Moz-Leu-OEt was determined, and the reaction rates of *trans*-esterification were measured by fitting the area to a calibration curve for Moz-Leu-OEt.

tivity when incubated at 25 °C without microwave irradiation. The alcalase has 44% of the original activity when it is incubated at 50 °C without irradiation. This result suggests that the inactivation of the alcalase with microwave irradiation is primarily due to thermo-inactivation.

The alcalase-catalyzed reaction was performed under microwave irradiation for 20 minutes in 2-methyl-2-propanol (3 mL) using Pro-$NH_2$ (0.3 mmol) as a nucleophile and Cbz-Ala-Phe-OMe (0.1 mmol) as an acyl donor. The yield of Cbz-Ala-Phe-Pro-$NH_2$ is 85% after a simple isolation procedure.[42] This reaction has been reported to have a yield of 59% by reacting at 25 °C for 4 days. To further characterize the effect of microwave irradiation on this alcalase-catalyzed reaction, high-pressure liquid chromatography (HPLC) was used to follow the product of the reactions carried out with or without microwave irradiation. Fig. 11 shows the time course of the increasing concentration of Cbz-Ala-Phe-Pro-$NH_2$ as the reactions proceed. The temperature of the reaction solution after microwave irradiation for 5, 10, 15, and 20 minutes was 35, 38, 42, and 45 °C, respectively. When the reaction was carried out with microwave irradiation, the peak corresponding to the acyl donor gradually decreased and disappeared after the reaction solution had been irradiated for 20 minutes and the product appeared to have reached its maximum concentration (91%). Without microwave irradiation, only a small quantity (7.5%) of product formed when the reaction was carried out at 25 °C for 20 minutes. When reacted at 45 °C, the product appeared rapidly for the first 30 minutes, reaching approximately 44% of total at 20 minutes and 55% of total at 30 minutes, and slowing after the first 30 minutes. The reaction continued for 5 hours with approximately 78% yield. At this point, the activity of alcalase had nearly ceased. This result indicates that microwave irradiation can enhance the rate of alcalase-catalyzed peptide bond formation between Cbz-Ala-Phe and Pro-$NH_2$ by approximately 2 to 10 times.

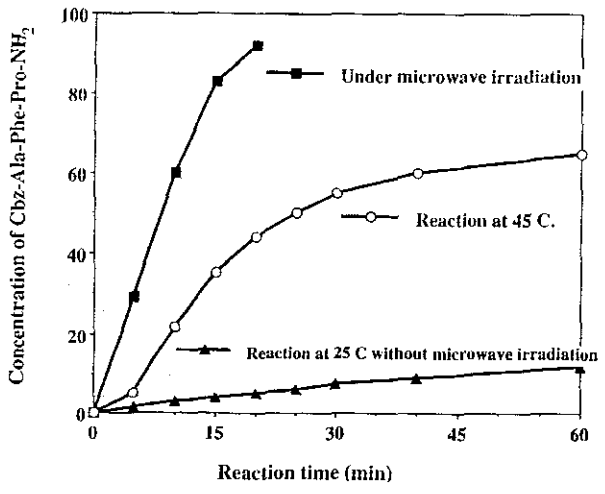
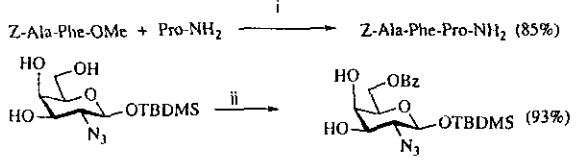



Fig. 11. Time course of the alcalase-catalyzed synthesis of Moz-Ala-Phe-Pro-$NH_2$ in 2-methyl-2-propanol under each reaction condition. The concentration of the product at each reaction interval was determined using HPLC and calculated using a calibration curve. The HPLC apparatus consisted of a Hitachi 6200 intelligent-HPLC pump and a Hitachi 4200 UV Detector. The data was collected on a Macintosh LCII using Rainin Chrompic software. Column: RP-18, 4.6 × 150 mm. Eluent: 68% $CH_3CN$ in 0.1% TFA. Flow rate: 1 mL/min. UV wavelength: 254 nm.

Two preparative scale alcalase-catalyzed reactions (2.8 and 10 mmol of substrate in 10 and 100 mL of 2-methyl-2-propanol) were carried out at 45 °C. Scheme V shows the reactions of the peptide bond formation using proline as a nucleophile and of selective benzoylation at the 6-position of *tert*-butyldimethylsilyl 2-azido-2-deoxy-β-D-galactopyranoside (BADG) using vinyl benzoate as an acyl donor.[43] The products were isolated in yields of 85% and 93%, respectively, using flash column chromatography.

**Scheme V**




i. alcalase/2-methyl-2-propanol; microwave irradiation 20 min.
ii. vinyl benzoate; alcalase/2-methyl-2-propanol; microwave irradiation 20 min.

This is the first report of the selective benzoylation of sugars by a protease. The high yield and regioselectivity of benzoylation at the 6-position of D-glucal by the microwave-enhanced alcalase-catalyzed reaction is prior to that of the Lipase-AY (Amano, Japan) catalyze benzoylation at the 6-position of D-glucal (only a 70% yield in a 6-hour reaction period in contaminating with 5 to 10 vol-% regio-isomer, and benzoylation at the 6-position of D-galactal with only a 67% yield in an 8-hour reaction period.[44] Similarly, our experiment also precedes the chemical method for the synthesis of the same products (a yield of only 78%).[45,46]

### The Effects of Microwave Irradiation on the Acid-Catalyzed Hydrolysis of Starch

The present study describes the solubilization and hydrolysis of retrograded starch by microwave irradiation. Using a household microwave oven to irradiate a suspension of starch (10%) in 0.5 M hydrochloric acid, the starch was

completely hydrolyzed in 5 min without the formation of any colored byproducts. Hydrolysis was not complete even after 24 h, when a similar starch suspension was heated at 100 °C; moreover, a deep-brown-colored byproduct appeared after heating for more than 1 h. The retrograded starch was isolated, resuspended in water or hydrochloric acid, and subjected to microwave irradiation. When resuspended in water and irradiated, the formerly insoluble retrograded starch dissolved completely. When resuspended in 0.5 M hydrochloric acid and irradiated, the retrograded starch was completely hydrolyzed, and required only a 5-min reaction time.

Starch (10%) suspended in dilute hydrochloric acid (0.5 M) was hydrolyzed using either microwave irradiation (a household microwave, 2.45-GHz microwaves, 10-microsecond pulses, 20% to 40% full power, temperature setting at 95 °C)[47] or a traditional heating block (100 °C). The hydrolysis of starch to glucose was monitored using HPLC and gel filtration columns. For reactions under heated conditions, the starch suspension (4.0 mL) was placed in a sealed test tube, purged with nitrogen, and heated at 100 °C. After 5, 10, 20, or 60 min, an aliquot (10 μL) of reaction suspension was collected, and the extent of hydrolysis was measured using HPLC. For reactions with microwave irradiation, the starch suspension (1.0 mL) was placed in reaction vessels, irradiated for 3.0, 4.0, 4.5, 5.0, 5.5, or 6.0 min. Each timed suspension was analyzed using HPLC.

Fig. 13 shows the results of the reaction. Three samples: dextran (MW 40 000 daltons), dextran (MW 8800 daltons), and glucose, were used as references (Fig. 12-A). Fig. 12-B shows the results of HPLC analysis of the starch suspensions incubated at 100 °C for 5, 10, 20, or 60 min. Following 60 min, the soluble starch was completely converted to glucose and the retrograded starch remained suspended in the solution. This suspension was centrifuged at 7000 rpm for 10 min at 25 °C and decanted to isolate the retrograded starch.[48] The four types of starch used (rice starch, corn starch, and wheat starch from Sigma, St. Louis, MO., and tapioca starch from Thailand) contained 5.1%, 6.6%, 9.8%, and 2.5% retrograded starch, respectively.

Fig. 12-C shows the gel filtration HPLC analysis of the starch suspensions subjected to microwave irradiation for 3, 4, or 5 min at 30% full power. After irradiation for 5 min, the solution was clear, and no retrograded starch remained in suspension. This result led to the hypothesis that the β-structure of the retrograded starch was deformed by microwave irradiation, making it soluble in the solution, and therefore, subject to hydrolysis. To test this hypothesis, the isolated retrograded starch from the thermo-hydrolysis experiments (25 mg/mL) was resuspended either in water or in dilute hydrochloric acid (0.5 M) and irradiated in the same manner as above for 5 min. The retrograded starch became soluble in water and was completely hydrolyzed in hydrochloric acid, resulting in a clear solution as measured by HPLC in the same manner as above.

When the thermo-hydrolysis was prolonged at high temperature, the reaction solution became colored, and the absorbance of the solution increased significantly at wavelengths between 400 and 500 nm. Fig. 13 shows the absor-

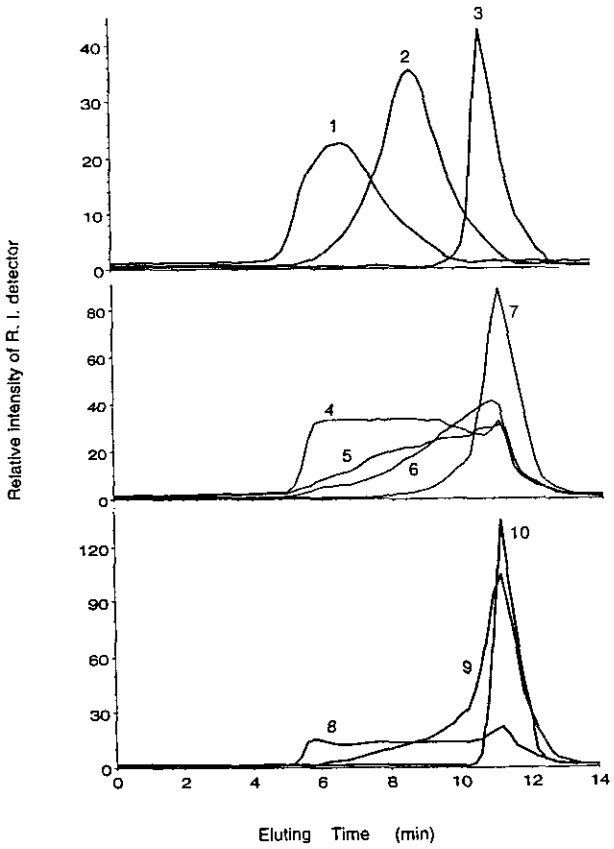



Fig. 12. Analysis of hydrolysate of starch solution after microwave irradiation or thermo-hydrolysis in acidic suspension. The HPLC system consisted of a Hitachi 6200 pump and a Hitachi Reflex Index Detector, and the data were collected on a Macintosh LCII using Rainin Chrompic' software. The conditions for analysis were: column, TSK G2000SW, 7.5 × 300 mm; eluent, phosphate buffer 0.05 M, pH 5.0; flow rate, 1 mL/min; RI, 4 RIU/FS. The curve 1, is the HPLC profile of dextran (MW 40 000 daltons); curve 2, is dextran (MW 8800 daltons); curve 3, is glucose; curves 4, 5, 6, and 7 represent starch suspensions incubated at 100 °C for 5, 10, 20, or 60 min, respectively; curves 8, 9, and 10, represent the starch suspensions subjected to microwave irradiation at 40% full power for 3, 4, or 5 min, respectively.

bance of the starch solution at wavelengths between 395 and approximately 500 nm. When the solution was digested for 5 min either via microwave irradiation or incubation, the absorbance of the starch solution was near zero. In the samples heated at 100 °C, the absorbance increased as the incubation time increased. After incubation for 7 days the solution was dark-brown, and the retrograded starch remained suspended in the solution. Using standard methods, the retrograded starch in soluble starch does not hydrolyze, and can be problematic by sticking onto the filter in large scale separation experiments.

The results show that with microwave irradiation, the suspension of starch (10%) in dilute hydrochloric acid (0.5 M) is completely hydrolyzed within 5 min without the formation of colored byproducts. In contrast, when the standard heating block is used to hydrolyze the starch, the retrograded starch remains as suspension in solution.

**Enhanced Rate of Saponification of Fatty Acid for Determination of Very Long Chain Fatty Acids in Serum Samples**

The phenotypic variant of adrenoleukodystrophy (ALD) due to the X chromosome-linked genetic mutant results in the accumulation of very long chain fatty acids (C26) in serum.[49] A rapid and precise method for determining very long chain fatty acid levels in serum would be very useful for diagnosising adrenoleukodystrophy. One of the reliable procedures currently used in this analysis contains two steps: saponification of the serum sample to liberate the free fatty acids and making derivatives of fatty acids with 2-bromo-2′-acetonaphthone for HPLC analysis. Scheme VI shows the procedures for preparing the free fatty acids.[50] Incubation of the sample in alkali KOH/ethanol solution at elevated temperatures (56-59 °C, 2 hours) to accelerate the rate of saponification is necessary.

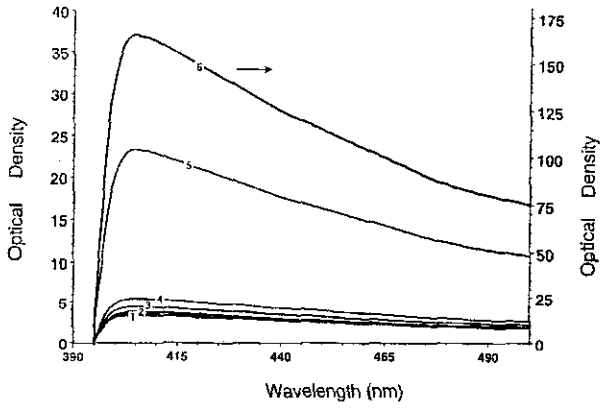



Fig. 13. Measurement of the absorbance (395 to 500 nm) of the starch hydrolysate in 0.5 M hydrochloric acid after microwave irradiation for 5 min (curve 2) or after incubation at 100 °C for 5 min (curve 1), 30 min (curve 3), 1 h (curve 4), 8 h (curve 5), and 24 h (curve 6). The scale of the vertical column for curve 6 is plotted on the right, and all others are on the left. The spectra were measured by diluting the hydrolysates with water (0.1 to 3.0 mL), centrifuging to remove the precipitates, and directly measuring the absorbance of the supernatants at wavelengths ranging from 395 to 500 nm.

**Scheme VI** Procedure for saponification of serum sample

sample in saturate KOH/ethanol 1:3 (1 mL)

↓

heating: water bath (56~59°C) or under microwave irradiation

↓

Acidify to pH < 2.0 (6N HCl)

↓

Extraction with n-Hexane (10 mL)

↓

Evaporating the Organic layer to obtain free fatty acids

We have found that microwave irradiation can enhance the rate of saponification in this procedure. TLC was used to follow the reaction of saponification.[51] As shown in Fig. 14, using the same reaction solution, the lipid (Rf 0.66) completely converted to the free fatty acid (Rf 0.33) within 5 min using 10% of full power of microwave irradiation. While incubated at 56-59 °C, the time required to complete the reaction was 80 min. Microwave irradiation enhanced the rate of saponification about 15 times over conventional heating. The double bond is more active than the single bond toward many chemical reactants. The fatty acids may contain mixtures of double bonds in the molecules. We found that the current microwave irradiation conditions will not cause side-product formation. We compared the 2-bromo-2′-acetonaphthone derivatives of the fatty acids obtained from both procedures and analyzed the derivatives by the established HPLC method.[52] Both derivatives were identical in the HPLC profiles.

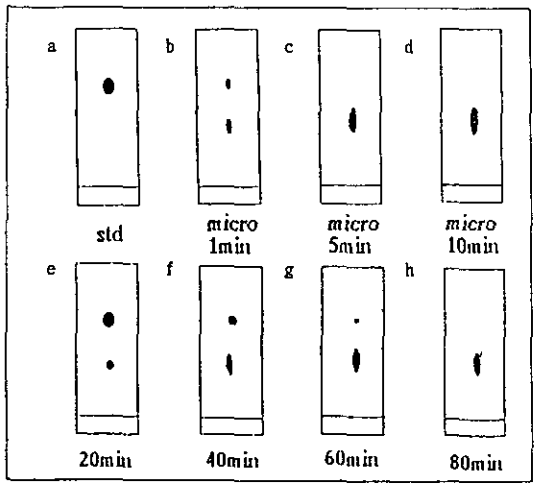



Fig. 14. TLC of lipid after saponification to liberate free fatty acids under either microwave irradiation (upper line) or incubation at 56-59 °C (lower line). The developing solvent system is *n*-hex:ether:HoAc (85:15:0.1 v/v) and the spots were visilized by incubation in presence of $I_2$ vapor. The Rf value of lipid is ~0.66 and of free fatty acid is ~0.33.

## CONCLUSIONS

Microwave heating of laboratory-scale organic reaction mixtures is moving into both the undergraduate laboratory and industrial production. Microwave irradiation is a convenient and fast way to accelerate chemical reactions, due to its "MOLECULAR STIRRING" mechanism and can be applied to many reactions.

The microwave-assisted specific cleavage of peptide bonds has proved to be very useful to obtain the defined acid-cleaved peptide fragments of proteins and circumvents the contamination problems inherently associated with microscale purification and sequence analysis of fragments generated from proteolytic enzymes.

Under microwave irradiation conditions, the peptide fragments have higher reactivity than do the amino acid derivatives to form the peptide bond, which is very useful for the synthesis of big peptides. This method is useful in the coupling of peptides in solution and in solid-phase for the synthesis of large peptides.

This is the first time that enzymatic reactions can be accelerated by microwave irradiation. Microwave irradiation appears to significantly increase the rate of alcalase-catalyzed reactions and, moreover, increase the regioselective benzoylation of sugars with high yield utilizing a protease. The microwave technology procedure described in the present report may be applicable to thermo-stable enzyme-catalysis and aid in the development of new synthetic reactions. It is necessary to design a microwave instrument fit for these purposes.

Microwave irradiation not only increases the rate of energy transfer to accelerate a reaction, it also alters the structure (or conformation) of the reactant to facilitate the reaction.

## ACKNOWLEDGMENTS

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**Key Words**

Microwave irradiation; Molecular agitation; Rate enhancement; Enzymatic catalysis; Specific cleavage; Peptide bond; Saponification; Hydrolysis.

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41. In this study, approximately 10% of full power (i.e., 72 W) was used. For the preparative scale reaction, a Synthewave 402 microwave oven (Prolabo, France) was used, and the temperature of the reaction solution was maintained at 45 °C.
42. Cbz-Ala-Phe-OMe (3.34 g, 10 mmol), Pro-$NH_2$ (3.90 g, 30 mmol) in 2-methyl-2-propanol (100 mL) and alcalase 2.5 L (2 mL, with anhydrous 2-methyl-2-propanol) was stirred at 25 °C. After all the Cbz-Ala-Phe-OMe was reacted (after about 20 hours, monitored by HPLC), the mixture was evaporated, and the residue was dissolved in ethyl acetate (200 mL). The resulting solution was washed three times with 5% citric acid (25 mL), three times with water (25 mL), three times with 5% sodium

bicarbonate (25 mL), dried over anhydrous sodium sulfate, and evaporated to produce crude Cbz-Ala-Phe-Pro-$NH_2$, which was further purified via silica gel flash column chromatography eluted with MeOH:$CH_2Cl_2$ (4:1, v/v) to yield pure Cbz-Ala-Phe-Pro-$NH_2$ (3.62 g, 85% yield).

43. A mixture of BADG (441 mg, 1.4 mmol), vinyl benzoate (415 mg, 2.8 mmol) in 2-methyl-2-propanol (11 mL), and pretreated alcalase (14 mL) was reacted under Synthewave irradiation for 20 minutes. The enzyme was filtered away, the residue was washed with EtOAc, and the resulting solution was evaporated in reduced pressure. The product was separated via flash column chromatography using a column packed with silica gel, and eluted with EtOAc-Hexane (1:1). The 6-OBz product (382 mg, 93% isolated yield) was re-crystallized from diethyl ether/hexane.
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47. In this study, 20% to 60% full power was used.
48. The resulting suspension containing retrograded starch was centrifuged (7000 rpm) for 10 min at 25 °C, and the supernatant was decanted off. The precipitate was resuspended in water and centrifuged. This procedure was repeated three times and the final precipitate was lyophilized to yield the retrograded starch.
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