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Enhancement of Chemical Reactions by Microwave Irradiation

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Microwaves are low electromagnetic energy which induce molecular perturbation by stimulation of ionic diffusion and by enhancement of dipole rotation without causing rearrangement of molecular structures. Microwave energy irradiated on the samples in various liquid media is lost or absorbed to the samples by the following 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 irradiation. In contrast to conventional heating, the salient feature of "dipole rotation" constitutes one efficient form of "molecular agitation" or "molecular stirring" which can be explored for many aspects in chemical reactions. We will discuss some of the useful application of "molecular agitation" by means of microwave irradiation such as: 1. rapid hydrolysis of proteins and peptides, 2. selective hydrolysis of aspartyl peptide bond, 3. the racemization of amino acids, 4. rapid hydrolysis of sugars, 5. continuous-flow process of microwave reactions, and 6. the enhancement of coupling reactions in peptide synthesis.

INTRODUCTION

Microwaves are electromagnetic radiation of wavelength between 0.1 cm and 100 cm, i.e. microwave energy lies in the range of the radio and infrared region of the electromagnetic spectrum. The first microwave oven for commercial use was introduced in the early 1950s and has gradually become a necessity for effecient cooking. Recently there have been growing interests in utilizing microwave heating for the rapid thermal digestion prior to elemental and chemical analysis of inorganic and biological samples.[1-4] The rapid heating by the microwave, capable of saving a considerable amount of dissolution time, 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.[5-7] Microwave irradiation has also been applied to several organic reactions in a continuous-flow process by which preparative-scale samples (> 20 g) may be synthesized with ease using a kitchen microwave oven.[8] The present report is intended to give an overview of some of the chemical and biochemical applications of microwave irradiation.

RESULTS AND DISCUSSION

Protein and Peptide Hydrolysis by Microwave Irradiation

Table 1 shows the effect on hydrolysis time by microwave irradiation for the recoveries of standard amino acids. It is clearly evident that a 2 to 8 min period of microwave irradiation of amino acids in 6 M HCl is not destructive to most amino acids except some minor degradation in the labile amino acids such as serine, threonine, methionine, tyrosine and histidine, being irrelevant because these 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, lysozyme and various toxin and protein components isolated from snake venom in 6 M HCl. Table 2 shows the comparison of amino acid data of phospholipase A_2 isoenzymes from Thailand cobra (Naja naja siamensis) by a 5 min-period microwave irradiation as compared to that obtained by the conventional 110°C hydrolysis. It is evident that results obtained by short-time microwave heating are, in general, similar to those obtained by conventional heating at 110°C for 24 h. Of interest is the finding that by

Dedicated to Professor Yau-Tang Lin (林耀堂) on the occasion of his eightieth birthday.

Table 1. Effect of Microwave Irradiation on Stability of Standard 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	1.00	1.00	1.00	1.00
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	0.01
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 an asterisk indicate some destruction after irradiation for 8 min.

Table 2. Amino Acid Analyses of Phospholipase A_2 in 6 M HCl by Microwave Irradiation and Conventional Heating Protocol

Amino acids	Microwave Irrad.	110°C/24 h
1/2Cys	13.1(14)	12.8(4)
Asx	22.2(22)	21.4(22)
Thr	4.7(5)	4.3(5)
Ser	4.8(5)	4.2(5)
Glx	7.6(8)	7.8(8)
Pro	3.8(4)	3.8(4)
Gly	8.8(9)	8.9(9)
Ala	11	11
Val	3.6(4)	3.9(4)
Met	0.8(1)	0.6(1)
Ile	3.7(4)	3.6(4)
Leu	4.9(5)	4.7(5)
Tyr	8.6(9)	8.5(9)
Phe	3.9(4)	3.6(4)
His	0.7(1)	0.9(1)
Lys	4.8(5)	4.9(5)
Arg	5.3(5)	5.5(5)
Trp	-	-

Data are experssed as the number of residues per molecule of protein using alanine as the reference. Values represent the mean of triplicate determinations. The hydrolysis condition is microwave irradiation for 5 min using custom-made Teflon-Pyrex hydrolysis tubes flushed with N2. The values in the parentheses are the theoretical residue numbers of amino acid compositions based on the protein sequence.

raising the hydrolysis temperature and shortening the exposure time, higher recoveries of some labile amino acids such as serine and threonine were observed. These higher recoveries 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 accuracy of the analysis of these amino acids are commonly observed in the amino acid analysis of HCl-hydrolyzed proteins.

Although it has been quite popular to use gas-phase hydrolysis for the amino-acid analysis of small quantities of peptides, we found that the microwave irradiation of protein or peptide samples in the liquid phase can also be adapted for the gas-phase hydrolysis in a microwave oven. There is, however, an extra step of drying the protein-HCl solutions before applying the microwave irradiation. In some cases if the protein samples are completely dry without any residual HCl solvent, rapid microwave heating can cause charring in the protein samples even within one minute irradiation exposure time. Actually by adopting liquid-phase hydrolysis for most peptides using microwave oven, the same sensitivity of gas-phase hydrolysis can be obtained if the hydrolysates in the Teflon-Pyrex tubes can be rinsed more thoroughly to ensure all hydrolysates are pipetted into the vials before Speed-Vac drying. The lowest sample limit of lysozyme for the liquid-phase microwave hydrolysis is 1 μg or 72 picomoles.

Specific Cleavage of Peptide Bonds Next to Aspartic Acid

Also investigated, was the kinetics for the release of amino acids from various hydrophobic dipeptides to provide some basis for the optimization of irradiation time for peptide-bond cleavage. It was found that any dipeptides containing valine and isoleucine are more difficult to cleave than the dipeptides of other combinations in agreement with the previous report using conventional heating method.[9] This observation prompted a search for the weakest peptide-bond present in most proteins. The selective cleavage at aspartic acid residue of protein was discussed by Inglis[10] who described the use of dilute hydrochloric acid and formic acid heating in vacuum for 2 hours at 108°C. This investigation included using these same acid solvents in the hydrolysis of peptides by microwave irradiation. As shown in Fig. 1 various dipeptides containing aspartic acid were hydrolyzed much faster than other

dipeptides without aspartic acid. This indicated a potential of employing microwave irradiation to the isolation and characterization of the defined acid-cleaved fragments for peptide mapping and protein sequencing. Microwave irradiation of protein solutions in dilute acids should prove its general applicability in the current microsequencing work. It can also favor the use of chemical cleaving agents over proteases to avoid the contamination of proteases in the protein fragments generated by conventional proteolysis.

Rapid Racemization of Amino Acids by Microwave Irradiation

Most amino acids are synthesized by conventional chemical methods. A resolution process is needed for the separation of racemic amino acids obtained from chemical synthesis. Many methods for the racemization of one form of optically pure compound into racemic mixture have been developed in order to recycle the undesired enantiomer and increase the recovery of the useful optical isomer. The conventional methods for racemization are usually tedious, rather laborious and prone to decomposition because of long incubation in strongly acidic or basic conditions at high temperature. This experiment has shown the success of applying the microwave-based heating method for the facile racemization of amino acids (Fig. 2). All amino acids tested can be rapidly racemized within 2 min and the yields are generally greater than 90%. The new technique can overcome the drawback of conventional methods, i.e. the instability of some optically active compounds during the long process of racemization. As compared to microwave irradiation, the conventional heating takes much longer time to reach the same extent of racemization at similar temperatures.[11] In addition there is probably pressure effect besides high temperature induced by the microwave oven to account for the acceleration of racemization by microwave irradiation

Microwave Hydrolysis of Sugars

The linkages for polysaccharides are in general more labile than the peptide bonds encountered in proteins. In our preliminary experiments with sugars we found that microwave irradiation provides a more efficient means than the traditional heating at 100°C under acidic conditions (Fig. 3). Microwave hydrolysis of biological macromolecules should prove an efficient alternative if the aim of shortening the digestion time by conventional heating is needed.

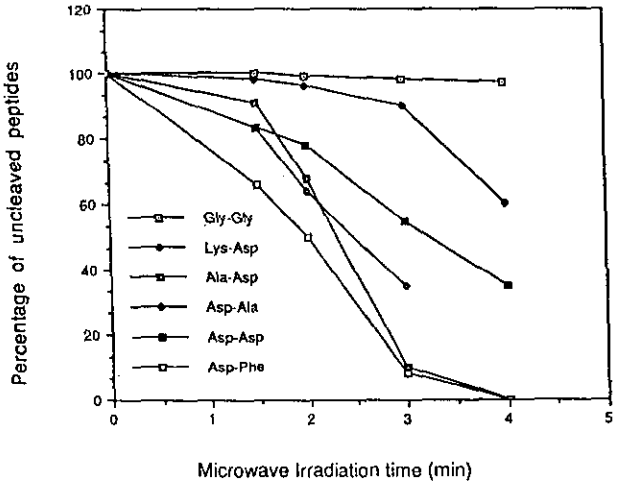

Fig. 1. Cleavage of dipeptides by mcirowave irradiation. Various dipeptides in a concentration of 0.1 mg/mL in 0.06 M HCl were irradiated (100% full power or 1.2 kilowatt) at different time intervals and measured the released amino acids by fluorescence detector. The dipeptide Gly-Gly was not cleaved under the described conditions.

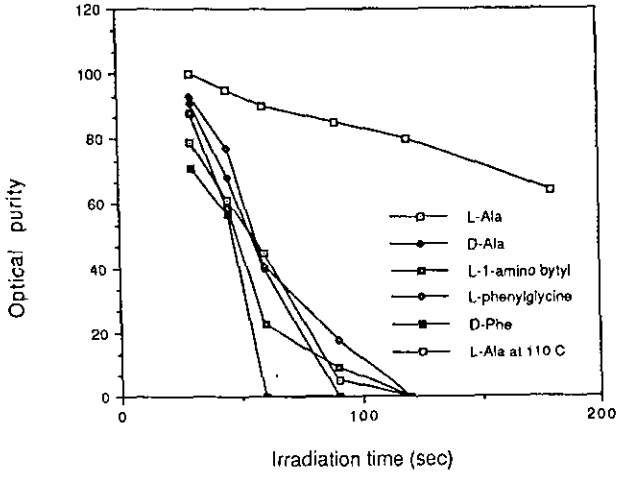

Fig. 2. Racemization of optically active amino acids in 0.1% acetic acid catalyzed by benzaldehyde. The microwave power was set at 40% full power (0.48 kilowatt). L-Alanine was used as the control and heated at 110°C. % Racemization for each tested amino acid was measured by polarimeter and chiral HPLC analysis. L-Phe: L-phenylalanine; L-Aba: L-1-aminobutyric acid.

Organic Synthesis by Continuous-flow Microwave Irradiation

The inherent hazard of violent explosion due to the high pressure and temperature developed in a closed vessel under microwave irradiation has limited the scale at which these rapid microwave reactions can be carried out (< 1 g). Reported here is a continuous-flow reaction process by which preparative-scale samples (>20 g) may be synthesized safely. Five different types of organic reactions were studied: (1) the esterification of *p*-hydroxybenzoic acid with 1-butanol and methanol, (2) the racemization of optically pure amino acids in acetic acid, (3) the acid hydrolysis of sucrose to glucose and fructose, (4) the S_N2 reaction of phenoxide with benzyl chloride, and (5) the cyclization of butane-1,4-diol and of diethylene glycol.

Fig. 4 shows the reaction diagram of the continuous-flow system. The sample in the reservoir is pumped into a reaction coil in the microwave oven for irradiation, and then out to a product collector. A microwave oven with ten adjustable power settings operating at a frequency of 2450 MHz and having a full-power level of 650 W was used for these experiments. A microwave-transparent Teflon tube was used as the sample-carrying system and continuous-flow container for reactants and products. The total volume of the Tefion coil is about 10 mL. The reactions were carried out under the continuous-flow process and also in a sealed Teflon vessel as previously reported.[5-7,12] The products of these microwave heated reactions were then analyzed by HPLC or GC methods and the reaction efficiencies for both processes were calculated and compared.

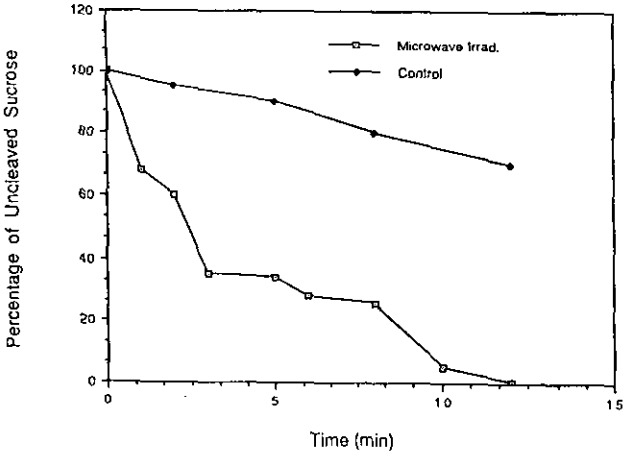

Fig. 3. Hydrolysis of sucrose in 0.01 M HCl by microwave irradiation. Reagent grade sucrose in 0.1 g/mL 0.01 M HCl was irradiated at 50% microwave full power (0.6 kilowatt) for different periods and the released glucose was measured on a carbohydrate HPLC column by an RI detector. Control sucrose solution was heated in the same solution at 100°C.

Table 3 shows the results for each reaction tested by these microwave processes. The concentrations of various reactants were kept the same under both sets of reaction conditions. Reaction products were identified by their melting and boiling points, and their NMR and IR spectra. In the continuous flow reaction, the reaction time is actually equal to the irradiation time of the sample in the reaction coil of the microwave oven. The retention time of the sample in the coil can be controlled by adjusting the flow rate of the pump. As shown in Table 3, the conversion of *p*-hydroxybenzoic acid and 1-butanol to butyl *p*-hydroxybenzoate increased with the irradiation time in the microwave oven. This is true for all five reactions studied, and the percentage conversion can be controlled by adjusting the irradiation time. The pressure inside the reaction coil during the reaction process is higher than atmospheric pressure owing to the positive pressure from the pump plus the induced pressure increment from irradiation. Most of the reactions tested in the continuous- flow system generally have a higher conversion efficiency compared with those conducted in a closed-vessel system within the same time period of irradiation. All reactions were run safely without explosion or charring except the cyclization of diethylene glycol to dioxane, the solution of the final products turned, black after the reaction. In the esterification of *p*-hydroxybenzoic acid with methanol, the yield is low for the continuous-flow process due to the evaporation of methanol in the Teflon coil which resulted in solution spillage. The substitution of xylene-methanol (1:1) for methanol as the cosolvent improves the conversion by 25%. It is also of interest to note that the hydrolysis of sucrose using a strong acid cation-exchange resin as catalyst gives a better result than that using mineral acid.

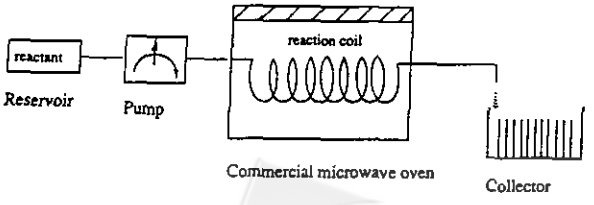

Fig. 4. Schematic diagram of continuous-flow process using microwave irradiation.

Table 3. *Comparison of Microwave Reactions under Closed Vessel and Continuous-flow Conditions*

Compounds Synthesized	Procedures Followed	Reaction[a] Time(min)	Power[b] Setting	Reaction Quantity(mmol)	Conversion (%)	Efficiency (%)
Esterification of *p*-hydroxybenzoic acid with butanol and methanol						
HO-C_6H_4-$COOC_4H_9$	closed vessel	5.0	2	1	89	-
HO-C_6H_4-$COOC_4H_9$	continuous flow	1.0	3	100	12	13
HO-C_6H_4-$COOC_4H_9$	continuous flow	2.0	2	100	29	32
HO-C_6H_4-$COOC_4H_9$	continuous flow	3.0	2	100	53	59
HO-C_6H_4-$COOC_4H_9$	continuous flow	4.0	2	100	67	64
HO-C_6H_4-$COOC_4H_9$	continuous flow	5.0	4	100	81	90
HO-C_6H_4-$COOC_4H_9$	continuous flow	6.0	3	200	89	100
HO-C_6H_4-$COOCH_3$	closed vessel	3.0	4	1	82	-
HO-C_6H_4-$COOCH_3$	continuous flow	3.0	5	100	46	56
HO-C_6H_4-$COOCH_3$	xylene/CH_3OH[d]	4.0	3	240	58	70
Racemization of L-amino acids in acetic acid[e]						
D,L-Isoleucine	closed vessel	2.0	2	1	100	-
D,L-Isoleucine	continuous flow	1.0	3	50	60	60
D,L-Isoleucine	continuous flow	2.0	3	300	100	100
D,L-Phenylalanine	closed vessel	2.0	1	1	100	-
D,L-Phenylalanine	continuous flow	0.5	2	50	7	7
D,L-Phenylalanine	continuous flow	1.0	3	50	29	29
D,L-Phenylalanine	continuous flow	1.5	3	50	89	89
D,L-Phenylalanine	continuous flow	2.0	3	200	100	100
Hydrolysis of sucrose to glucose and fructose						
glucose + fructose	closed vessel	10.0	2	1	95	-
glucose + fructose	continuous flow	10.0	2	200	93	95
glucose + fructose	continuous flow[f]	10.0	2	200	95	100
Cyclization of 1,4-butandiol and diethylene glycol						
tetrahydrofuran	closed vessel	4.0	3	1	95	-
tetrahydrofuran	continuous flow	4.0	4	200	95	100
dioxane[g]	closed vessel	4.5	6	1	37	-
dioxane[g]	continuous flow	6.0	7	180	35	97
dioxane[g]	continuous flow	8.0	6	180	43	>100
S_N2 reaction of phenoxide with benzyl chloride						
C_6H_5-O-$CH_2C_6H_5$	closed vessel	10.0	8	1	49	-
C_6H_5-O-$CH_2C_6H_5$	continuous flow	10.0	8	100	49	100

[a] In the continuous-flow processes the reaction time is equal to the irradiation time.

[b] According to the operation manual the power setting of each number: is 1: 36w; 2: 72w; 3: 143w; 4: 215w; 5: 286w; 6: 358w; 7: 430w; 8: 500w; 9: 572w; 10: 650w.

[c] Calculated on the basis of the % conversion obtained by closed-vessel system.

[d] Xylene-methanol (1:1) was used as the reaction cosolvent to improve the yield of conversion.

[e] Change in optical purity was measured by polarimeter and HPLC using a Diacel chiral column.

[f] Using acid cation-exchange resin (Doulite C25D, ROHM & HAAS) as catalyst.

[g] Some side products were present in both reactions, % conversion measured by GC.

Peptide Synthesis by Microwave Irradiation

Recently we have introduced microwave irradiation for the rate enhancement of coupling reactions in peptide synthesis. The coupling rates of some refractory β-branched amino acids in the conventional coupling reactions can be improved 4-8 fold by microwave irradiation in 2-5 minutes (Fig. 5). We have studied the microwave-accelerated coupling reactions in different solvents and at different reaction temperatures by control of energy input in microwave power. It was found that no racemization side reactions occurred to significant degree in the dipeptide products from coupling reactions between different amino-

acid derivatives by microwave heating. This new heating protocol has been applied to the coupling reactions between various N-protected Boc-amino acids and immobilized amino-acid Merrifield resin in the routine solid-phase peptide synthesis with high efficiency. By comparison of the rate enhancement in the coupling reactions with solvents of different microwave absorbability, it is concluded that the high efficiency achieved in the coupling reaction by microwave irradiation is effected by both a temperature elevation and the perturbation in molecular dipole moments induced by microwaves. The current detailed theory to account for these effects are poorly defined, especially in some cases where temperature elevation induced by microwaves does not appear to quantitatively delineate the observed rate enhancement. Via these findings it is proposed that this latter effect is the result of the "molecular agitation or stirring" caused by microwave irradiation to account for the nonthermal microwave effect.

CONCLUSION

In conclusion, a systematic evaluation of some chemical and biological processes using microwave irradiation has been carried out in order to circumvent the limitation of conventional heating protocols. This novel application of microwave technology is easily adapted in most analytical and biochemical laboratories since a microwave oven is now a common apparatus for rapid heating purpose. Currently our efforts are directing to the refinement of microwave-heating step as a new avenue in exploring the potentials of applying this rapid and convenient heating method to various important reactions of biochemical analysis and organic synthesis.

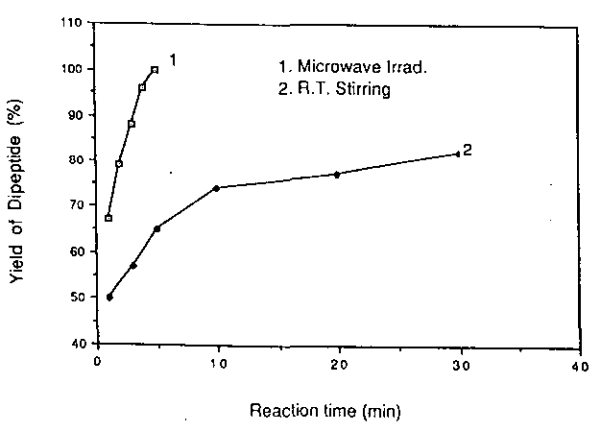

Fig. 5. Comparison of coupling reactions of Moz-Val and Val-OMe in DMF by microwave irradiation and mechanical stirring at ambient temperature. To each reaction 2 mmole Moz-Val was dissolved in 15 mL DMF and reacted with dicyclohexylcarbodiimide (2 mmol) for an hour before adding 1 mmol Val-OMe to start the coupling reactions by microwave heating or mechanical stirring. The dipeptides under different conditions were isolated and yields were calculated on the basis of the initial weight of Val-OMe.

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Key Words

Microwave irradiation; Rate enhancement; Selective hydrolysis; Preparative scale reactions; Peptide synthesis.

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