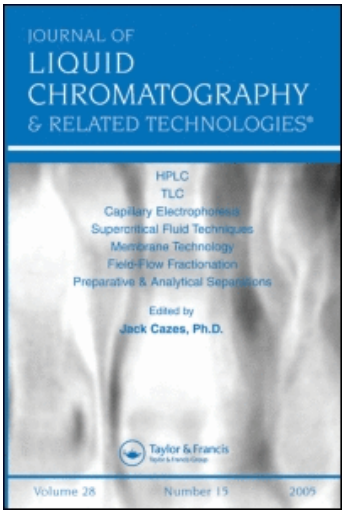

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MECHANISTIC STUDY OF MICROWAVE-ASSISTED ACID HYDROLYSIS OF PEPTIDES ON TLC PLATES

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ABSTRACT

Microwave-induced acid hydrolysis was performed with isolated peptides on TLC layers. This vapor phase method was compared to solution phase techniques. A mechanistic study of peptide bond clevage was carried out by varying exposure conditions and reaction time. Acid hydrolysis products were identified by ninhydrin visualization. Also, TLC and TLC/MS were used to elucidate hydrolysis mechanisms.

INTRODUCTION

Recently, microwave-induced acid hydrolysis of proteins and peptides has been reported in the literature. Rapid, solution-phase hydrolysis of proteins and peptides has been

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described by Chen and coworkers (1). Hydrolysis times as short as three minutes were shown to provide results equivalent to the 24 hr conventional method for the amino acid composition of insulin B chain. A mixed acid solvent system of propionic acid/12N HCl (1:1) proved comparable to constant bioling 6 N HCl. Only 4 minutes was necessary to completely hydrolyze oxidized ribonuclease A, giving better recoveries for threonine and serine than the conventional method. Numerous aerobic (2-5) and anerobic (1,6) methods have been described in the literature or presented at scientific meetings. A vapor phase microwave hydrolysis method was recently reported by Gilman and Woodward (7). In this study, vials containing vacuum dried methionyl human growth hormone were irradiated inside a Teflon digestion vessel, which was connected to a microwavable gas manifold. The digestion vessel was charged with 10 mL of 6N HCl and the dried protein was in vapor equilibrium with the acid. The heating regime was under constant digestion vessel pressure control; though a maximum power of 650 Watts was used throughout. Hydrolysis times between 8 and 12 minutes were found to be optimum.

We have been developing a column - planar chromatographic technique for amino acid composition analysis of proteins and peptides. The technique utilizes a miniature column liquid chromatographic separation of protein/peptide mixtures where the column effluent is transferred to a planar separation medium, such as a TLC plate. Subsequent to hydrolysis of the separated proteins/peptides on the plate, the amino acids are separated by

TLC and visualized by conventional chemistries. Our initial work has been devoted to small peptides and basic measurements of hydrolysis conditions. We quickly became aware of the capability and importance of obtaining mechanistic data on peptide hydrolysis with the unique multidimensional format of the chromatographic media. By exposing peptides to 1) increasing levels of microwave energy, 2) radiative thermal heating from the hot glass plate and 3) corrosive atmospheres we were able to "control" the extent of hydrolysis, thus enebling mechanistic information to be gained. To our knowledge, microwave "processing" of TLC plates has not been studied, suggesting that this may be a new application for microwave sample preparation and processing (10).

We present, here, data on the partial and total hydrolysis of model di- and tri- peptides, with emphasis on the method as a tool for studying the mechanisms of peptide acid hydrolysis. Microwave treatment in TLC may be a beneficial method for enhancing chemical reactivity and facilitating layer conditioning.

MATERIALS AND METHODS

Instrumentation

The major instrumentation consisted of a commercial microwave oven (Model JE48, General Electric, Louiville, KY) and standard

equipment for thin layer chromatography. Twin trough chambers and a scanning densitometer, Scanner II, (Camag Scientific, Wrightsville Beach, NC) were used for the TLC separations. A Biospec chemical ionization mass spectrometer (CIMS) with spot sampling probe (11) were used for mass spectral analyses.

Chemicals and Reagents

High performance liquid chromatography-grade solvents (Burdick & Jackson, Muskegon, MI, USA) were used throughout. All chromatographic plates were 5 X 20 cm non-F254 K6 TLC plates (Whatman, Clifton, NJ). All amino acids were purchased from Sigma Chem. Corp. (St. Louis, MO). Model peptides were obtained from Sigma Chem. Corp., National Biochemicals Corp. (Cleveland, OH) and International Chemical and Nuclear Corp. (City of Industry, CA). All other chemicals and reagents were ACS or reagent grade and were used without purification.

Microwave Hydrolysis Procedure

Spotted TLC plates were partially covered with plain glass plates, allowing the spotted region to be exposed to acid vapors. The plate sandwich was placed on ceramic blocks (2 cm high) with the exposed silica surface oriented downward. A ceramic reaction chamber (12 cm diameter X 5 cm deep plus a loosely-fitting cover) was used with 5 mL of 6N hydrochloric (HCl) acid for each exposure

period. A single hydrolysis experiment (heating program) consisted of three, 5 minute exposure periods, each one separated by a 1 minute cooling period. After each cooling period 5 mL of 6N HCl was added to the chamber. The total exposure time for each experiment was, therefore, 15 min. The microwave was rated at 500 watts excitation power, which we did not verify by separate measurement. Acid vapors were removed from the oven compartment with a vacuum line, inserted through the oven wall, that was connected to a 12N sodium hydroxide solution in a gas washer.

Thin Layer Chromatography

All TLC plates were washed in methanol and baked at 100 C for 30 min prior to use. After cooling to room temperature (following microwave treatment) the plates were developed in a phenol/water/aqueous 0.4% trifluoroacitic acid (72.5/25/2.5) mobile phase. Visualization was accomplished by reaction with Ninhydrin, performed according to the manufacturer's directions (12). Scanning densitometry pas performed in the absorbance mode at 480 nm.

TLC/MS Measurements

Entire spots, from unknown peptides, were transfered to the sampling probe of a chemical ionization mass spectrometer (11). Ammonia reagent gas and a source temperature of 210 C were used with positive ion scanning.

RESULTS AND DISCUSSION

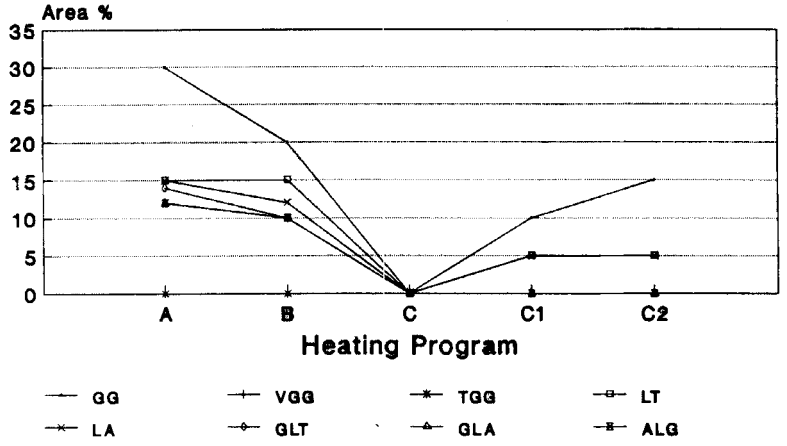
Study of Reaction Conditions

Several preliminary experiments were devoted to the study of reaction conditions. There were several points that we considered as being important to microwave-induced acid hydrolysis on TLC plates. First, we surmized that the glass could get quite hot, especially without a heat sink, resulting in radiant heating of the layer from the hot glass substrate. Second, we were sensitive to the fact that microwave absorption by the layer could be significant, hopefully so, in promoting enhanced reactivity. Third, since the reaction was primarily vapor phase, we hypothesized that total reaction time would be very important because we had no provision for controlling reactor pressure. Reaction conditions were controlled by incorporating heating and cooling periods in a multicycle heating program through varying the total microwave exposure and total reaction time.

In separate experiments (not shown) we measured the temperature of TLC plates, with and without a layer using a cement-on thermocouple. With long exposure times (>7 min) we were able to achieve temperatures greater than 300 C! To prevent thermolysis we limited individual exposure times to 6 min. The presence of a layer did not affect temperature. We also discovered that microwave excitation was pulsed, with changes in temperature setting producing only changes in duty cycle. According to the

manufacturer (13) the time base for our microwave is 22 seconds and a 2 second start time is required for every heating program. A "high" setting (100% duty cycle) corresponded to (22-2)/22 or 90.9% effective "on" time for a given program. Therefore, a programmed run time of 5 min resulted in 4.54 min as the total "on" time. The integrated power for this setting is calculated as 500 Watts X 4.54 min = 2270 Watt min. It was apparent that temperature and time were both important in facilitating the reaction. Due to the high temperatures and acidic vapors we noted extensive chemical modification of the layer. We noted about a 5 % decrease in Rf, indicating a more polar layer, compared to untreated layers. To minimize this modification we covered the chromatographic layer area with a glass plate and allowed only the region containing peptides to be exposed to acid vapors.

Three heating programs were developed to investigate the importance of total microwave exposure. They were constructed as follows: Program A had three, 3 min heating periods with two, 1 min intervening cooling cycles; Program B had three, 4 min heating periods with two, 1 min intermediate cooling cycles; and Program C had three, 5 min heating cycles with two, 1 min cooling periods. From the above calculation, we determined the total, integrated microwave power for each of the periods to be 4091, 5454 and 6818 Watt min, respectively. After hydrolysis, TLC and Ninhydrin visualization densitomograms were recorded. The area % of each unhydrolyzed peptide was calculated. Figure 1 shows relative contributions of unhydrolyzed peptides for these three heating



	GG	VGG	TGG	LT	LA	GLT	GLA	ALG
A	30	0	0	15	15	14	12	12
B	20	0	0	15	12	10	10	10
C	0	0	0	0	0	0	0	0
C1	10	0	0	5	5	0	0	0
C2	15	0	0	5	5	0	0	0

Figure 1. Effects of Microwave Heating Conditions on Peptide Hydrolysis

programs. Notice that VGG and TGG are both absent after just 4091 Watt min, while more than 5454 Watt min was required for all other peptides. The total "on" time for program A was 13.6 min, a bit long compared to the optimum reported for a similar vapor phase method (7). We did not determine the relative contribution from dipeptides for the 5 tripeptides, during this study.

Significance of the relative amounts of "heating" and "cooling" time was determined by increasing the percentage of cooling time through modifications to program C (above) as follows: Program C1: 15 min heating/4 min cooling and Program C2: 15 min heating/6 min cooling. The heating to cooling time ratio varied from 7.5 to 3.75 to 2.5, respectively. As shown in Figure 1, increasing the percentage of heating time had no effect among the tripeptides. The dipeptides, especially GG, were affected. This may indicate that the tripeptides each lost one amino acid residue, very easily. Doubling the ratio of heating to cooling time produced the most dramatic reduction in unhydrolyzed peptide contribution. These results can be explained by the possibility that a higher average temperature was attained in Program C, compared to Programs C1 and C2. Exceeding a "threshold" temperature appears to be consistent with acid hydrolysis of proteinaceous materials. A more extensive study of microwave heating of TLC plates is documented in a separate paper (14).

Tripeptide Hydrolysis

Model tripeptides were hydrolyzed in separate experiments in order to study their peptide bond clevage mechanisms. "Partial" hydrolysis was achieved with heating Program A. "Complete" hydrolysis was performed with heating Program C. Figure 2 shows a compositional map of the partial hydrolysis of the model peptides. Spot identity was based on either Rf matching with authentic compounds or, in the case of VG, TG and GL, mass spectral

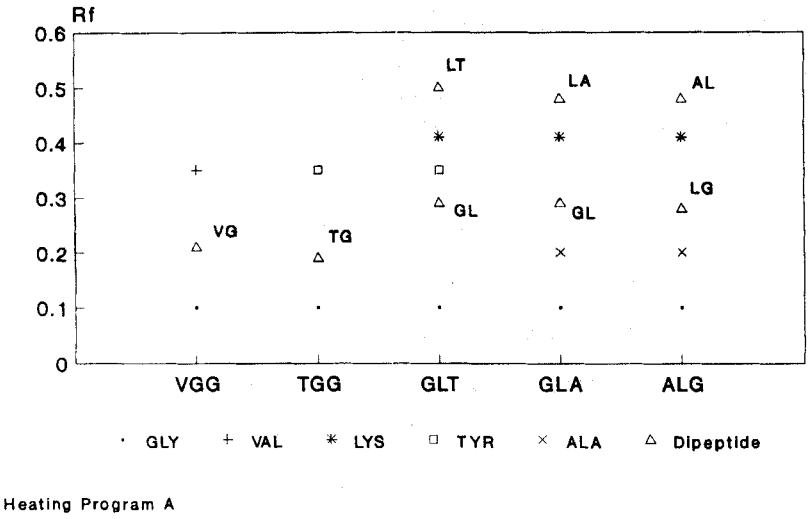


Figure 2. Microwave Hydrolysis Profiles of Model Tripeptides Under Partial Hydrolysis Conditions

confirmation. Only positions are indicated for the various components due to time constraints and problems with the densitometer. From the results of Figure 2, we conclude the following mechanisms:

VGG --> G + VG VG m/e = 128

TGG --> G + TG TG m/e = 177

GLT --> GL + LT GL m/e = 143

GLA --> GL + LA

ALG --> AL + LG

The dipeptides were identified on the basis of their $[M-NH_2-COH]^+$ or $[M-45]^+$, produced by reaction with Ninhydrin (15). Measurement of spot intensity as a function of time would give kinetic data,

which will be the subject of future studies with larger peptides. These mechanisms are unremarkable, indicating that microwave-induced hydrolysis of peptides on TLC plates is equivalent to other microwave-based hydrolysis methods. The presence of degredation products was not seen, however, we did not specifically evaluate this issue with peptides containing "sensitive" amino acids.

We regard the ability to separate and detect hydrolyzed amino acids in the same medium in which they were formed a potential benefit. However, the general difficulties of quantitation from TLC plates places this technique at a disadvantage relative to pre-column hydrolysis/derivatization methods. However, the ability to perform acid hydrolysis on several samples simultaneously is a clear advantage. These may be discrete samples, spotted onto the plate, or individual components from a complex protein mixture, separated on an interfaced miniature LC column. In addition, the ability to speed the hydrolysis with microwave irradiation/heating is also an attribute. Finally, microwave "processing" of TLC plates is generally an attractive option for thin layer chromatographic analyses.

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