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Report

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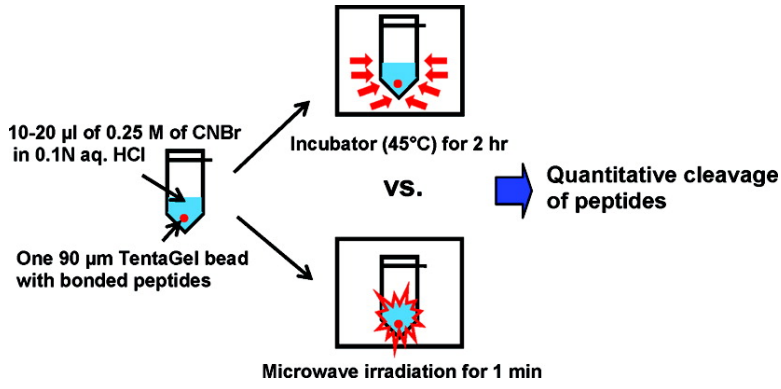

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Rapid Microwave-Assisted CNBr Cleavage of Bead-Bound Peptides

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Large libraries of peptides, cyclic peptides, and other molecules are standard tools for the discovery of drugs, molecular probes, and affinity reagents. In particular, one-bead-one-compound (OBOC) libraries,[1] prepared by the split-and-mix method,[2] provide access to a broad chemical space with a minimum of reagents. Once such a library has been screened against the target of interest, the chemical identity of the library elements on the hit beads is identified. For peptide libraries and their variants, mass spectrometry (MS) based peptide sequencing provides the most rapid method for such analysis. OBOC libraries are constructed in a number of ways to facilitate MS analysis,[3−5] but one common feature is that the peptide must be cleaved from the bead prior to being introduced into the mass spectrometer. While a number of chemical[6] and photochemical[7] cleavage strategies have been developed, the most common strategy is to incorporate a CNBr-cleavable methionine-linker group at the C-terminus of the peptide.[8] CNBr cleavage has also been widely used in proteomics to cleave proteins.[9] With such chemistry, up to 100 beads from an OBOC peptide library can be sequenced in a 24 h period.[10] A large fraction of that time, however, is devoted to the CNBr cleavage step. Standard literature protocols describe CNBr cleavage as requiring between 12 and 24 h, using 20−30 μL of 0.25 M CNBr in 70% aqueous formic acid at room temperature.[11] Although the CNBr cleavage time may be reduced to 2−4 h at elevated temperatures (~47 °C), significant side-products may be generated.[12] All reports that we have found that describe CNBr cleavage chemistry from single beads have used the same conditions as for proteomics, although the two chemical processes are not necessarily equivalent.

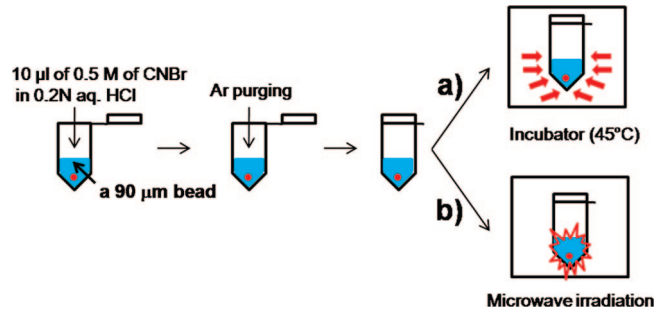
Microwave-assisted organic reactions have been widely performed with great effects on increasing reaction rates and improving yields and selectivity.[13,14] However, the "specific" or "nonthermal" microwave effects[13c,14a] are still in dispute.[15,16] In principle, molecular rotation is induced by the interaction of molecular dipoles with the microwave fields leading to heat generation.[13a] Reactions involving molecules with significant dipole moments may be more influenced by microwave irradiation, showing a great improvement in kinetic rates and product yields. Proteins and peptides have large dipole moments, and thus associated reactions have the potential for significant improvements via microwave assistance.[17] In particular, microwave irradiation has been successfully applied to solid-phase peptide synthesis, increasing the peptide coupling reaction rate and not generating appreciable racemization.[18,19] Recently, microwave irradiation was also used for the rapid generation of combinatorial peptide libraries.[20]

Here, we report on the optimization of single-bead CNBr cleavage conditions and demonstrate that the reaction time may be reduced to 1 min through the use of microwave-assisted cleavage in an aqueous medium. We further show that microwave-assisted cleavage yields very pure cleaved peptides from single beads. We performed MS analysis of various peptides of known sequence that were cleaved using a variant of the typical CNBr cleavage chemistry, as well as a rapid, microwave-assisted protocol. The peptides ranged in length from 5-mers to 8-mers and were prepared to represent the natural and non-natural stereoisomers of all amino acids except cysteine and methionine. The MS analysis yielded comparable results from both cleavage methods. Thus, microwave-assisted CNBr cleavage of peptides in water is significantly more efficient than the standard protocol.[21]

Results and Discussion. The standard protocol for CNBr cleavage of peptides from single beads is to use 20−30 μL of 0.25 M CNBr in 70% aqueous formic acid for 12−16 h at room temperature. We first investigated elevated temperature (45 °C) and reduced reaction times (2−4 h). Franz et al. reported that such reaction conditions generated a byproduct that exhibited a mass shift of +28 amu relative to the parent mass. To reduce such side products, other acidic media such as 70% TFA[22] and HCl[23,24] were tested; 0.1−0.2 N $HCl_{(aq)}$ showed the cleanest reaction by MS analysis (Scheme 1).

High-quality MS data were obtained by using 0.1 N aqueous HCl for 2−4 h at 45 °C without producing any noticeable side products (see Supporting Information, S-Figure 2). The oxidation of tryptophan (W)[25] was significantly reduced by purging the reaction vessels with Ar for a short time before and after the addition of the reaction

Scheme 1. CNBr Cleavage of Peptides Bound to a Single Bead (a) for 4 h at 45°C and (b) for 1 min Under 30 W Microwave Irradiation



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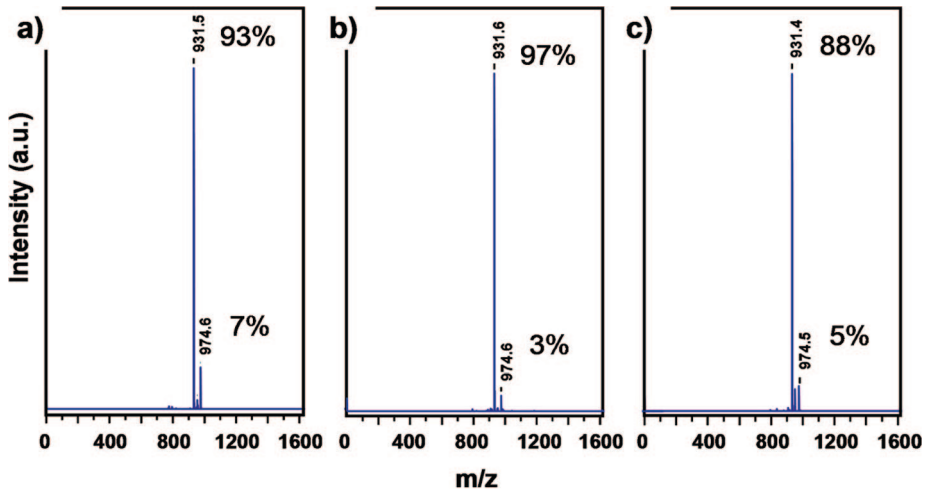

Figure 1. MS spectra of the peptide HLYFLRM* (M* = homoserine lactone, MW = 931.5), following CNBr cleavage using 3 protocols. (a) Microwave-assisted cleavage in water, with a 1 min microwave exposure. (b) Microwave-assisted cleavage in water, with a 4 min microwave exposure. (c) Cleavage at 45 °C in H_2O/MeCN for 4 h. Adjacent to each peak is the percent of the total intensity.

medium. Consequently, the final optimized conditions were 5−20 μL of 0.25 M CNBr in 0.1 N aq HCl for 2−4 h at 45 °C. This protocol was utilized as a comparison against the microwave-assisted protocol and for quantifying the amount of peptide cleaved from single beads.

Microwave-assisted acceleration was performed using identical chemical conditions (Scheme 1), but with 1−4 min of microwave irradiation replacing the 45 °C heating. Figure 1 shows MS data of the peptide HLYFLRM* (M* = homoserine lactone) from a single 90 μm TentaGel S Amino bead, in which the CNBr-cleavable M (methionine) is at the C-terminal. Ten percent of the cleaved peptide from a single bead was used as a sample for MALDI-MS. Data are shown for rapid microwave-assisted CNBr cleavage for 1 and 4 min (Figure 1a and b) using a household microwave oven. This process was calibrated against a CEM Discover microwave reactor (see Supporting Information, S-Figure 7). With the CEM reactor, a 1 min, 30 W exposure produced results identical to the 1 min microwave irradiation from the household microwave oven. Figure 1c is a plot of MS data from a peptide cleaved from a single bead using the above-described protocol of 4 h CNBr cleavage at 45 °C. In each plot, the major peak represents the primary mass of the peptide HLYFLRM* (931 amu). The data indicate that, at least for this peptide, the microwave-assisted and thermal cleavage processes are equivalent and that neither method adversely affects the peptide.

A calibrated optical fiber thermometer was used to measure the temperature of a 1 min irradiation of 20 μL of water within the same microvessels used for bead cleavage. For both the 30 W Discover reactor and for the household microwave oven, ΔT was 13−14 °C during the course of the exposure. Thus, 45 °C was obtained when starting at 31 °C, and 39 °C was obtained when starting at 26 °C (see Supporting Information, S-Figure 8).

We quantified both processes using liquid chromatography (LC) of cleaved peptides. To enhance the UV absorbance for LC analysis, we introduced 5-(dimethylamino)naphthalene-1-sulfonyl (Dansyl) group[26] at the N-terminal of the benchmark peptide HLYFLR. The reaction proceeded smoothly in the presence of *N*,*N*-diisopropylethylamine (DIEA) to elaborate the corresponding sulfonamide. The resulting resin was treated with TFA for removal of protective groups. The dansyl group attached at the N-terminal of the peptide HLYFLRM*, as evidenced by MALDI-MS analysis which yielded a parent mass at 1064 amu (see Supporting Information, S-Figure 3). To obtain sufficient amount of peptide for HPLC quantification, peptides were separately released under both thermal conditions and microwave conditions with ~ 320 μg of the resin, respectively.

We first investigated the amount of the cleaved peptides after 30 min, 1 h, and 2 h under the thermal conditions. The 2 h thermal reaction led to nearly complete cleavage of peptides from beads. The 30 min and 1 h thermal reactions resulted in ~36% and ~70% cleavage of peptides, respectively (see Supporting Information, S-Figure 6). Then, samples of identical quantity for the thermal and microwave reaction conditions were prepared. The amount of the released peptide under 1 min microwave conditions was comparable to both 2 min microwave cleavage and 4 h thermal (45 °C) cleavage (Figure 2). One minute of microwave reaction led to effectively complete cleavage of peptides.

We used Scheme 1 to cleave more than one hundred beads from OBOC peptide libraries, ranging in length from 5-mers to 8-mers (not including methionine) and containing all of the amino acids excepting cysteine and methionine (methionine was used only for the cleavage at the C-terminal), as well as both natural and unnatural stereoisomers. The 9-mer peptide library were synthesized with −FLRM in the C-terminal. The peptide sequences were obtained by de novo sequencing methods with their MS/MS data. Some of the obtained sequences were confirmed by synthesizing peptides with known sequence and obtaining their MS/MS data. Representative MS data from the peptides of known sequence are presented in the Supporting Information, S-Figure 9, including 4 peptides with −FKRM* in the C-terminal. After evaporation of the reaction medium, just 10% of the released peptides were consumed for MALDI-MS sampling after mixing with a matrix (CHCA) solution. Most of the released

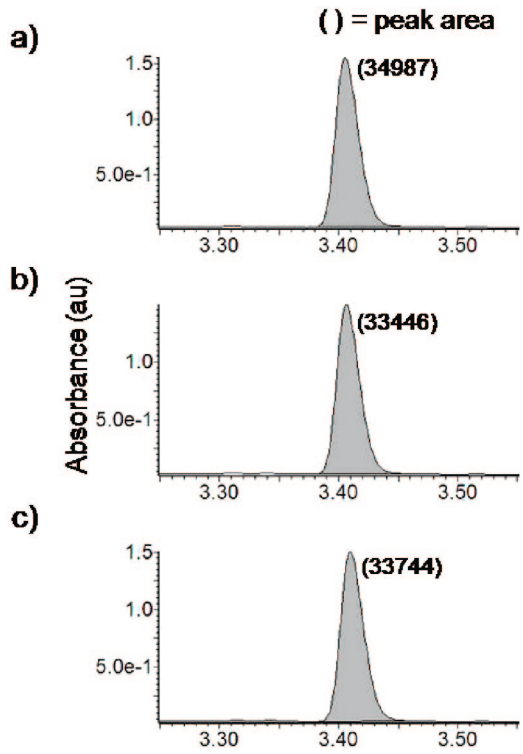

Figure 2. HPLC spectra of (a) CNBr microwave-assisted cleavage for 2 min, (b) CNBr microwave-assisted cleavage for 1 min, and (c) CNBr cleavage for 4 h at 45 °C, which was separately shown to lead to complete cleavage. All peaks exhibit a cleaved peptide retention time of 3.40 or 3.41 min, and the integrated peak area for all three plots is equal to within 5%, indicating that for the 1 min microwave-assisted cleavage reaction, the reaction has proceeded to completion.

peptides from the beads showed high quality MS spectra, which can lead to high quality MS/MS sequence information.

Conclusions. We have demonstrated an efficient microwave-assisted CNBr-based cleavage of peptides from single beads. One minute cleavage times were demonstrated for 5-mer and 8-mer peptides containing both the D- and L-stereoisomers of 18 out of the 20 amino acids. Released peptides exhibited high purity, as measured by both mass spectrometry and HPLC. This simple and rapid CNBr cleavage protocol should be useful in accelerating the screening of one-bead-one-compound peptide libraries. We have not tested whether this technique can be used to accelerate the site-specific cleavage of proteins.

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Supporting Information Available. Detailed experimental methods and supplemental data as referred to in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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