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De Novo Sequencing of Peptides on Single Resin Beads by MALDI-FTICR Tandem Mass Spectrometry

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An efficient approach in combinatorial chemistry is the synthesis of one-bead-one-compound peptide libraries. In contrast to synthesis and functional screening, which is performed in a largely automated manner, structure determination has been frequently laborious and time-consuming. Here we report an approach for de novo sequencing of peptides on single beads by matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance (MALDI-FTICR) tandem mass spectrometry, using a resin with a photolinker for solid-phase peptide synthesis. Upon sorting out single beads, an efficient sample preparation on the MALDI target was developed that enables fragmentation upon irradiation of the bead-matrix mixture with the ultraviolet (UV)-MALDI laser, with enhanced yield of sequence-specific fragment ions at increased laser energy. This approach is illustrated by sequence determinations of two peptides from a library with sequences varying in a single amino acid; the feasibility with tandem-MS procedures and fragment ion assignment was ascertained by sustained off-resonance irradiation/collision induced dissociation (SORI/CID) and infrared multiphoton dissociation (IRMPD) fragmentation.

Combinatorial chemistry was introduced into the repertoire of drug discovery tools in the late 1980s and is now an established field of research. The development of the split-mix synthesis of peptides has probably provided the highest impact [1–3]. This method provides fast access to solid-phase bound peptides libraries in which each resin bead carries a single product, the so-called one-bead-one-compound (OBOC) libraries [4]. Following functional screening of an OBOC library, the positive beads are isolated and analyzed. While synthesis and screening of OBOC libraries is usually rapid and widely employed, the structural characterization of positively tested beads is frequently a bottleneck of the approach [5], particularly with regard to the small sample amounts (typically 0.1–5 nmol) available, depending on loading density and size of the resin beads [6]. In the case of peptide libraries with a free N-terminus, Edman microsequencing is frequently used [2, 4]; however, this method, although fully automated, has limitations in speed (only 3–4 peptide sequences per day), the requirement of a free N-terminus, and sequence determination of modified peptides such as glycopeptides. Alternative approaches are based on coding or tagging methods [7–11] which, however, do not provide a characterization of the active component itself, but rather the "reaction history" of the beads. Mass spectrometry for structure analysis of peptide libraries is amenable to small amounts of peptides, owing to its high specificity and sensitivity well below the detection limits of alternative methods such as nuclear magnetic resonance (NMR) spectroscopy [6, 12].

Two principal approaches can be used to identify active beads of peptide libraries by mass spectrometry. (1) Cleavage of the peptide from the resin followed by MS analysis in solution. Amino acid sequences have been determined both by electrospray ionization (ESI)- tandem-MS [13, 14] and matrix-assisted laser desorption/ionization (MALDI)- tandem-MS [15]. For the analysis of chemically truncated peptides, both ladder synthesis [16] and ladder sequencing by partial Edman degradation [17–19] have been employed. (2) A second approach is the direct on-bead analysis without prior cleavage. Here, photolinkers are widely used, which can be cleaved by the laser beam during MALDI ionization. Fitzgerald et al. [20] and Gerdes and Waldmann [21] have used this approach to determine molecular masses of peptides and small organic compounds from solid-phase libraries. For sequencing of peptides, the direct on-bead method has been also used in conjunction with base-catalyzed cleavage by ammonia [22] and with the ladder synthesis [23], in which,

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however, the one-bead-one-compound principle is lost. Here, we report a de novo sequencing method for peptides from single resin beads by high-resolution MALDI-Fourier transform ion cyclotron resonance (FTICR) tandem mass spectrometry, using a photolinker and optimized procedure for laser-induced cleavage from the resin.

Experimental

Solid-Phase Peptide Synthesis (Figure 1)

Peptide libraries and peptides **1** and **2** were synthesized on Hydroxyethyl-Photolinker NovaSyn TG resin **3** (Novabiochem, Läufelfingen, Switzerland). The first amino acid (Fmoc-Lys(Boc)-OH) was manually coupled by the symmetrical anhydride method to the resin-bound product **5** (Figure 1). The loading density of the first coupling product **5** was determined to be 0.18 mmol g^{-1}. Peptide synthesis was performed on an automated peptide synthesizer (ABI-433A; Applied Biosystems, Foster City, CA, USA) using the 20-μmol (3 mL RV) 2.1.0 chemistry file. Fmoc deprotection was performed by treatment with a 22% piperidine solution in N-methylpyrrolidone (NMP) (3 × 3 min). Peptide coupling was carried out with 5 mol-equivalent of Fmoc- amino acid using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), and diisopropylethylamine (Hünig's base) (DIPEA) as coupling reagents (35 min). After completion of synthesis, the peptide-loaded resin was extensively washed with CH_2Cl_2 and dried in vacuo.

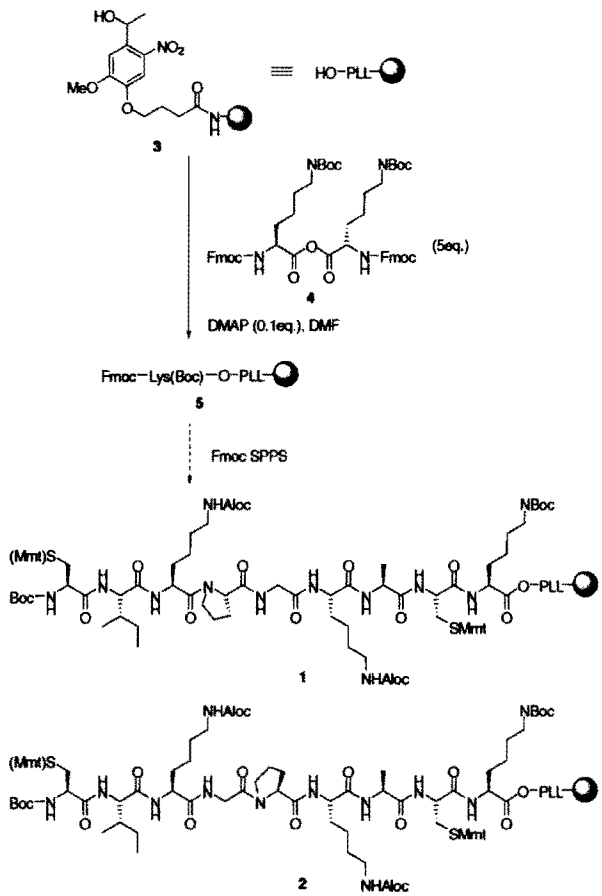


Figure 1. Solid phase peptide synthesis (SPPS) of peptides 1 and 2. Aloc = allyloxycarbonyl; Boc = tert-butyloxycarbonyl; DMAP = 4-(dimethylamino)pyridine; Fmoc = 9-fluorenylmethyloxycarbonyl; Mmt = monomethoxytrityl; PLL = photo labile linker.

Sample Preparation for Mass Spectrometry

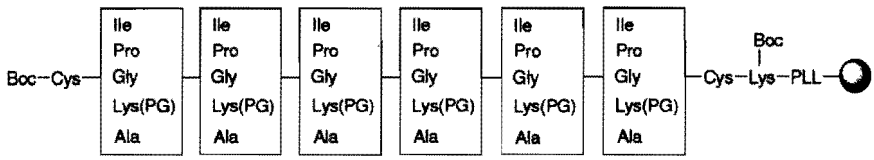
Resin beads were placed in a Petri dish and immersed in acetonitrile/0.1% aqueous trifluoroacetic acid (TFA) (2:1). Single beads were then separated under a microscope and placed onto the MALDI target. After smashing and homogenizing the single beads with a spatula, beads were covered with the matrix solution, 2,5-dihydroxybenzoic acid (DHB) in acetonitrile/0.1% aqueous TFA (2:1).

MALDI-FTICR Mass Spectrometry

Spectra were obtained on an APEX II-FTICR mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 7 T superconductive magnet and a cylindrical Infinity ICR cell. The pulsed nitrogen laser of the SCOUT-100 MALDI source was operated at 337 nm. For each scan, 15–20 laser shots with a total laser power of 64%–70% were used, and desorbed ions were accumulated in a hexapole ion guide and transferred into the ICR cell. The scanned mass range was *m/z* 100–1500, using an ionization pulse length of 2500 μs; 32–64 scans were accumulated for one spectrum. Ions of interest were isolated using stored waveform inverse Fourier transformation (SWIFT) with a correlation sweep according to 100–1500 *m/z* and an ejection safety belt of 500 Hz. MS/MS experiments were carried out using pulsed Ar gas (80–150 ms) and either radio frequency (rf) excitation at the frequency of the parent ion sustained off-resonance irradiation/collision induced dissociation (SORI/CID), or irradiation for 0.3–0.65 s with a CO_2-laser (Synrad, Mukilteo, WA) at 10.6 μm infrared multiphoton dissociation (IRMPD).

Results and Discussion

The peptide library shown in Scheme **1** contains 5^6 (15,625) members. This library was designed for use as a scaffold library for the multivalent presentation of carbohydrate epitopes attached to lysine side chains [24]. The cysteine residues can be used for conformational restriction via disulfide cyclization, while the C-terminal lysine residue facilitates the generation of a positive charge. The library contains isobaric sublibraries of up to 6!/2! = 360 compounds. Thus, peptide analysis by molecular mass determination would be insufficient for characterization, requiring individual sequence determination.



Scheme 1. Hypothetical peptide library containing 15,625 peptides (PG, polyvalent glycosyl) [24].

The two isobaric peptides **1** and **2** of the library, which can only be distinguished by sequencing, were randomly selected and synthesized, and single beads separated both from a sub-library synthesized and from individual batches (Figure 1). The least diversity was generated by permutation of two neighboring positions (Pro, Gly) in the amino acid sequence. The protecting groups were chosen with regard to a possible application as scaffolds. To simulate an unnatural modification of amino acids, the protecting groups were not removed prior to mass spectrometric analysis. A nitrobenzyl linker [25] was used for attachment to the resin, which is photocleavable at the wavelength of the UV-MALDI laser (337 nm). Starting with the hydroxyethyl photolinker NovaSyn TG resin **3**, the first amino acid was coupled by addition of the anhydride **4**, and peptide synthesis was performed with an automated peptide synthesizer using standard Fmoc chemistry. The building block Boc-Cys(Mmt)-OH for attachment of the N-terminal amino acid was synthesized in two steps from cysteine hydrochloride with an overall yield of 92%, by first introducing the Mmt-group according to Barlos et al. [26] followed by Boc-protection with di-tert-butyl dicarbonate.

The development of a suitable sample preparation procedure was found to be the critical step for the MALDI-MS analysis and sequence determination. Four matrix systems were evaluated for their MALDI-FTICR-MS performance [27, 28]: (1), 2,5-DHB in acetonitrile/water (2:1); (2), 2,5-DHB in acetonitrile/0.1% aqueous TFA (2:1); (3), α-cyano-4-hydoxycinnamic acid (HCCA) in acetonitrile/water (2:1); and (4), HCCA in acetonitrile/0.1% aqueous TFA. In addition, several pretreatment protocols for the resin beads were compared (swelling in the matrix containing solvent; smashing; and combinations of both procedures). Of the procedures tested, best results were obtained by swelling the beads in acetonitrile/0.1% aqueous TFA, placing single resin beads on the MALDI target under a stereo microscope, followed by smashing the beads on the MALDI target, and subsequent addition of the matrix (DHB in acetonitrile/0.1% aqueous TFA). This procedure provided the most homogeneous matrix-analyte mixture, and highest fragment ion abundances. Efficient solubilization attained with initial swelling in an organic/aqueous acidic solvent mixture *prior* to the addition of matrix was found to be the main feature.

Figure 2 shows MALDI-FTICR mass spectra obtained from single beads of the peptides **1** and **2**. The most abundant ion signals were found for the protonated peptide ions upon loss of the acid-labile Mmt and Boc groups, which were cleaved by the acidic matrix conditions. Moreover, a series of sequence-specific b- and y″-fragment ions were found, which are formed by cleavage in the matrix or by laser-induced cleavage. Since the time between generation and detection of the ions in MALDI-FTICR-MS is ~100-fold longer compared with MALDI-time of flight (TOF)-MS, laser-induced dissociation is substantially increased. Accordingly, enhanced fragmentation was found by increasing the laser energy used for release of the peptide from the solid-phase, while only minimal fragmentation was found at low (<ca. 60%) laser energy. The b- and y″-fragment ions for peptide **1** (Figure 2a) provided a near-complete sequence determination with sequence-specific fragment ions lacking only two peptide bonds at the C-terminal side of proline, reducing the number of 360 possible isobaric members of the library to only six (CIUPGUACK, CIUPUGACK, CIUGUPACK, CIUGPUACK, CIUUPGACK, CIUUGPACK) [where U = Lys(Aloc)]. The N-terminal peptide bond of Pro residues is labile, which has been described as the proline-effect [29–31], and has been ascribed to its steric lability, in contrast to the proton affinity of proline assumed previously [31], leading to either b- or y″-fragment ions, depending on the charge localization of the peptide chain. In the case of peptide **1**, the most abundant fragment ion was found by dissociation of the peptide bond between the sterically demanding amino acid residues Lys(Aloc) (U) and proline (y_6''). For analysis of the sequence-specific fragment ion formation, the $[M + H]^+$ peptide ions were isolated and subjected to SORI-CID and IRMPD fragmentation, which ascertained the sequence assignment CIUPGUACK. In contrast, attempts to enhance the fragmentation by further increasing the laser power (>ca. 70%) produced a series of polymeric ion signals with *m/z* differences of 44 Da originating from the polyethylene glycol chain of the solid TentaGel, and led to increased background ions.

The MALDI-FTICR-MS of a single resin bead of peptide **2** is shown in Figure 2b. Upon removal of the acid labile protecting group, the $[M + H]^+$ ion of the peptide was observed as the most abundant. The sequence specific fragment ions of **2** provided almost complete sequence determination, leaving only two of the 360 possible library sequences (CIUGPUACK and CIUGUPACK). As in the case of peptide **1**, isolation of $[M + H]^+$ and subsequent SORI-CID fragmentation

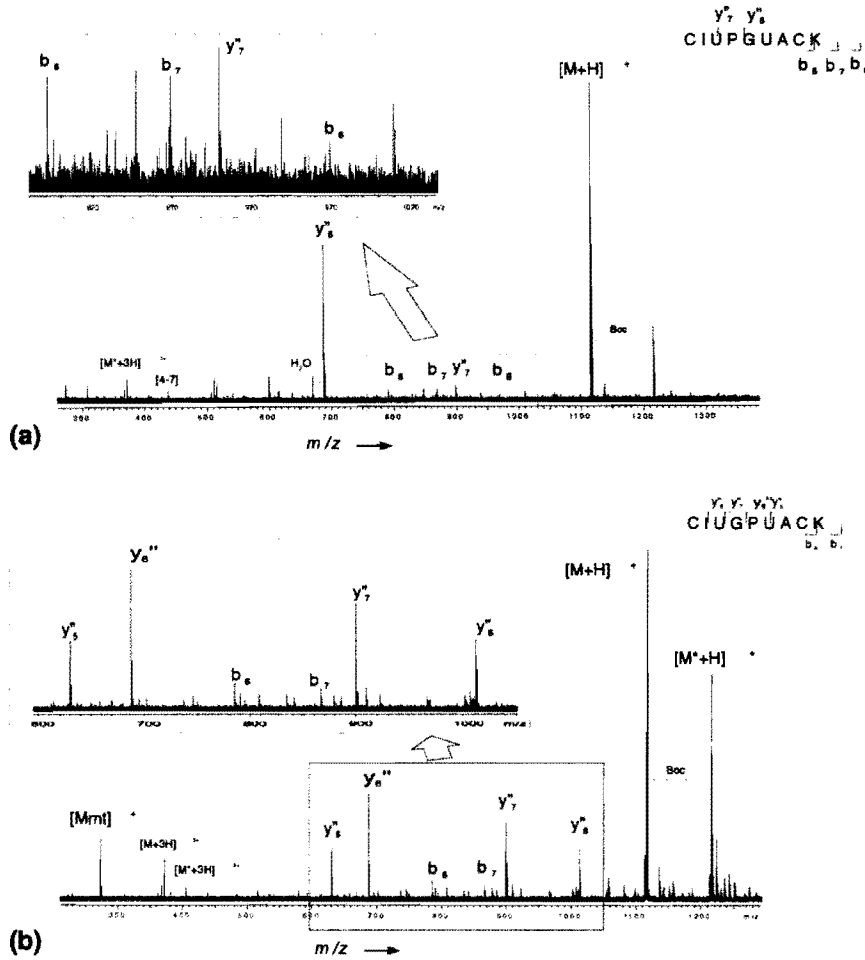


Figure 2. MALDI-FTICR Mass spectra obtained from single resin beads of solid-phase bound peptides **1** (**a**) and **2** (**b**). The molecular mass M corresponds to the peptides without the acid-labile protecting groups shown in the upper right corner (one-letter code, U = Lys(Aloc)). Corresponding y"- and b- fragments are indicated.

provided the missing y_4"-fragment ion to complete the sequence assignment.

Conclusions

We present here the application of MALDI-FTICR-MS using direct laser-induced fragmentation as an efficient and sensitive approach for de novo sequence characterization of peptide libraries from single beads. Using a photolabile linker and an optimized smashing and cleavage procedure for resin beads, peptides can be directly desorbed for MALDI-MS analysis, without artificial cleavage from resin beads. The problem of incomplete sequence coverage due to stability differences of peptide bonds can be effectively reduced by direct laser-induced peptide fragmentation, and sequence determinations are ascertained by tandem-MS using SORI/CID or IRMPD fragmentation. Thus, although no complete de novo-sequencing might be obtained for any library component, the combined photochemical fragmentation-MALDI-MS approach presented here effectively provides the molecular characterization of single bead peptide libraries.

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