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Hot or not—the influence of elevated temperature and microwave irradiation on the solid phase synthesis of an affibody

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ABSTRACT

Despite the advances of solid phase peptide synthesis (SPPS) the synthesis of long peptides is still challenging. Microwave irradiation and conventional heating are considered to improve the efficiency of SPPS. It has been shown that conventional heating and heating by microwave irradiation improves the efficiency of solid phase synthesis of peptides that are prone to aggregation as compared to the synthesis at room temperature. In this Letter, the influence of elevated temperature and microwave irradiation on the homogeneity of the synthesis product of a 58-mer peptide affibody has been compared. A detailed analysis by high resolution HPLC and LC–MS mass spectrometry using a high-mass resolution Orbitrap Exactive mass spectrometer was performed. This study revealed that neither thermal heating nor microwave heating improves the yield and purity of the crude product as compared to the synthesis at room temperature. In contrast, the formation of undesirable side products rather increased by microwave irradiation. These results indicate that neither heating nor microwave enhancement of solid phase synthesis does allow a significant improvement of peptide sequences with a low aggregation potential.

The achievements in the field of molecular medicine provide fascinating alternatives to common therapy strategies. However, the high molecular weight of many therapeutic and diagnostic targeting molecules—obviously an intrinsic property of highly selective drugs—complicates their production and therefore dramatically interferes with their transfer into clinical use. Recently it has been shown that several proteinaceous drugs that are obtained by recombinant synthesis can be reduced in size to improve their pharmacokinetic behavior.[1] Fortunately, this goes along with the possibility of chemical synthesis and results in new tasks for medicinal chemistry: to provide small proteins for the clinical application.

Solid phase peptide synthesis has come a long way since its introduction by Merrifield in 1963.[2] The research during the last decades in this area was focused on improving the yield and minimizing the side reactions by mainly finding new types of solid supports,[3,4] protection groups,[5,6] and coupling reagents[7–10] as well as improving the synthesis strategy.[11,12] Today most short and medium length peptides can be synthesized by standard procedures in high yield. However, the methods of SPPS still fail in some cases, in particular for long or difficult peptides. The outcome of a peptide synthesis is determined by the efficiency of the coupling

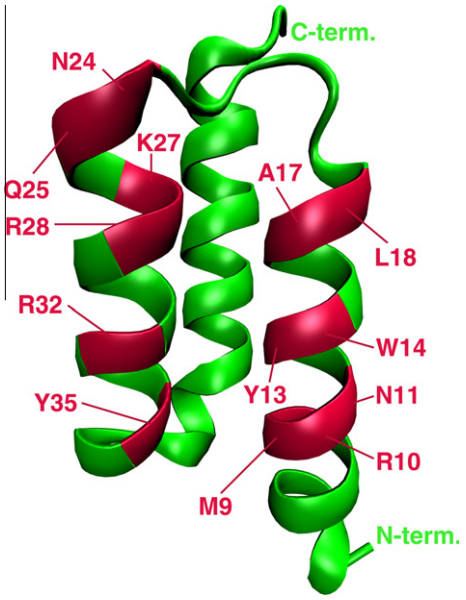

Figure 1. Three-dimensional structure of the affibody ZHer2:342. The structure was automatically modeled at Swiss Model Workspace[26] and visualized with Visual Molecular Dynamics (VMD). Randomizable amino acids are painted in red.

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and deprotection steps and the amount of side reactions that occur within the whole coupling and deprotection process.

Recently, most of the work on improving SPPS focuses on the synthesis of the so-called 'difficult sequences'. The fascinating possibility of a microwave irradiation-driven enhancement seemed to be obvious for many researchers. In fact systematic studies have shown that the 'microwave effect' observed is simply a misinterpretation of the acceleration of the synthesis by the elevated temperature in the microwave experiments.[13,14] In this work the effects of elevated temperature and microwave irradiation of a long peptide were compared using an affibody as the model system.

Affibody molecules are peptides with antibody-like properties which consist of a 58 amino acid scaffold derived from the Z domain of the staphylococcal protein A.[15] By randomization of 13 superficial amino acids, a peptide library was generated from which binding proteins have been selected by phage display (Fig. 1).[16,17] They recently gained attraction due to the ease of production and modification by SPPS in contrast to antibodies.[18–22] The affibody $Z_{HER2:342}$ is a very promising peptide for breast cancer detection. It binds with a high affinity ($K_D \sim 22$ pmol/L)[23] to the Her2 epitope that is overexpressed in tumors and can be radiolabeled for in vivo imaging.[24] In order to optimize the yield and the purity for clinical purposes according to good manufacturing practice (GMP), the synthesis parameters have to be optimized. This paper scrutinizes the question of whether elevated temperature or microwave irradiation leads to better synthesis results than room temperature of the long peptide sequences.

$Z_{Her2:342}$ (H-VENKFNKEMRNAYWEIALLPNLNNQQKR AFIRSLYDD PSQSANLLAEAKKLNDAQAPK-NH_2) was obtained by solid phase peptide synthesis using Fmoc-chemistry on a Tentagel resin (R RAM, Rapp Polymere Tübingen, Germany) with a loading of 0.19 mmol/g.[25] Three different conditions were applied: synthesis in an automated Applied Biosystems 433A peptide synthesizer (a) at room temperature, (b) at 60 °C using a self-constructed heating chamber with a temperature-controlled fan heater provided for a constant temperature during the synthesis, and (c) in a CEM liberty peptide synthesizer with microwave irradiation. Cleavage from the resin was performed with 95:2.5:2.5 TFA/water/triisopropylsilane for 2 h at room temperature and subsequent precipitation with cold ether. The precipitate was dried and dissolved in 50% acetonitrile in water. Analysis was performed by reversed-phase high-performance liquid chromatography (RP-HPLC) at 60 °C on a Zorbax Stable Bond C18 1.8 μm, 4.6 × 150 mm column (Agilent) with a gradient of 5–60% B over 120 min (flow 250 μl/min; solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in acetonitrile).

HPLC analysis of the crude product obtained by Fmoc synthesis of $Z_{Her2:342}$ at room temperature revealed the formation of several side products (Fig. 2B). The Fmoc cleavage pattern during the synthesis (Fig. 2A) shows that the peptide does not aggregate and cause any difficult sequence. However, a relatively high gradual decrease of the Fmoc cleavage value was observed. In order to examine with minuteness the crude synthesis product, a detailed analysis using high resolution LC–MS analyzes was performed.

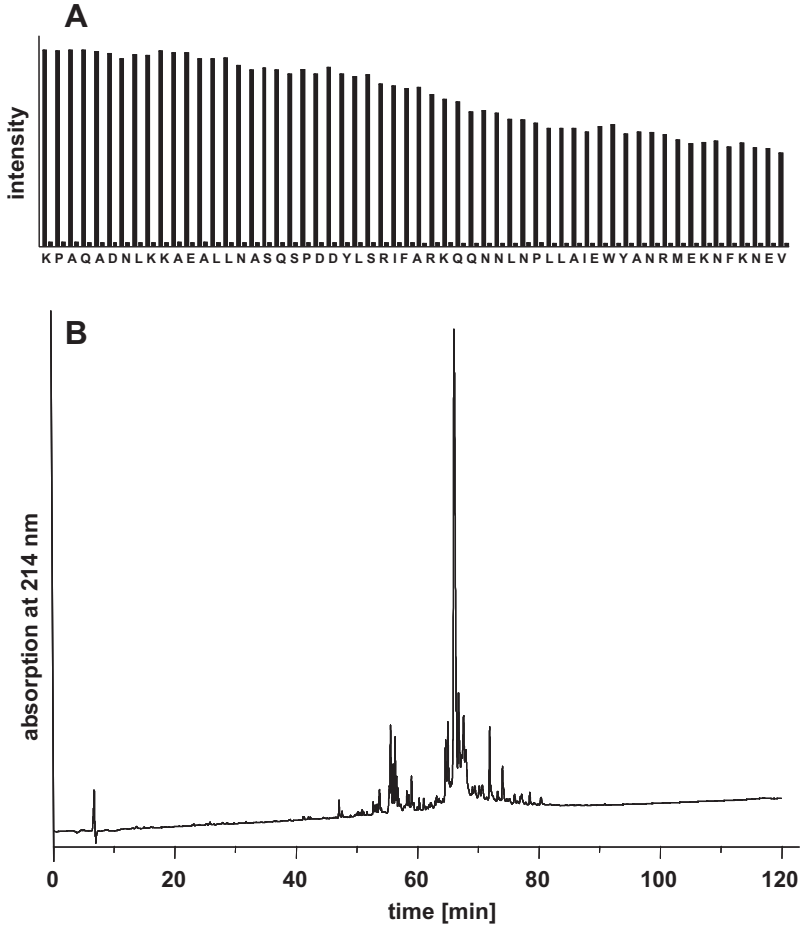

Figure 2. (A) UV measurement (Fmoc cleavage pattern) of the peptide synthesizer during the synthesis of $Z_{Her2:342}$. (B) HPLC of the crude $Z_{Her2:342}$ products obtained by conventional synthesis at room temperature. Although no difficult sequence can be observed in (A), a relatively high decrease of the Fmoc cleavage value is due to incomplete coupling steps which is confirmed by HPLC in (B).

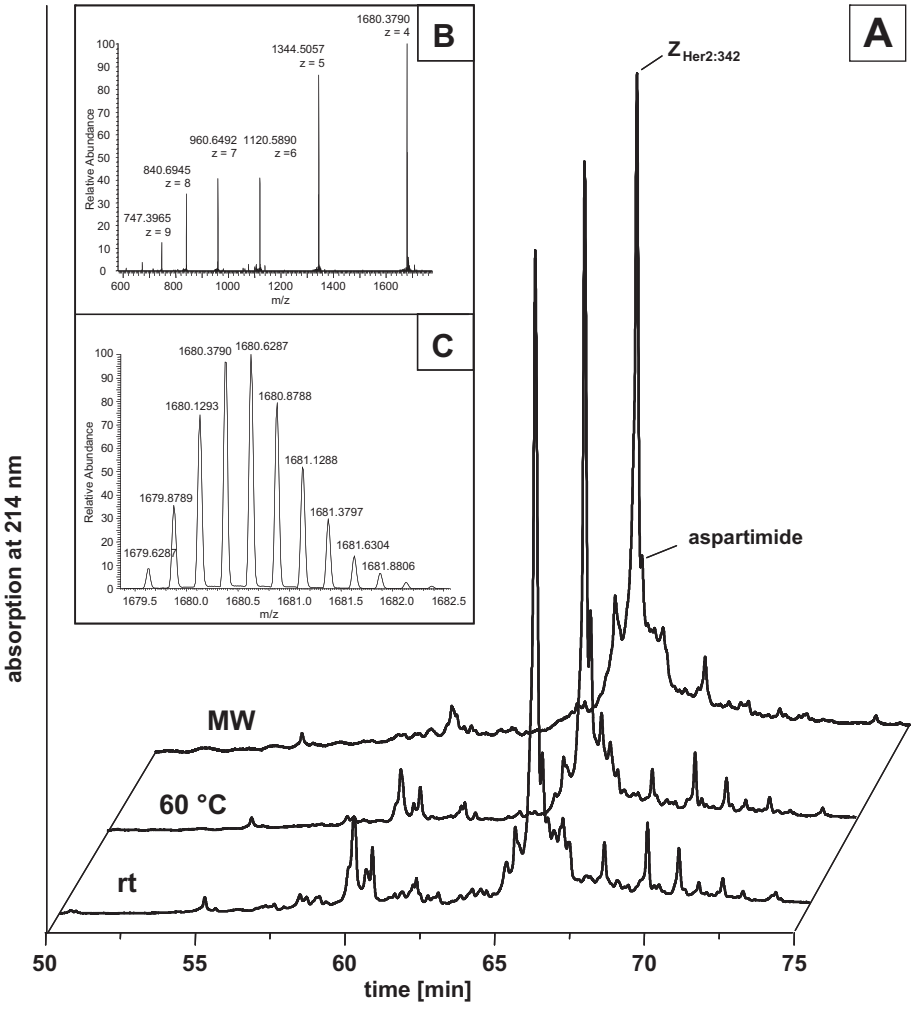

Figure 3. (A) HPLC analysis of the crude $Z_{Her2:342}$ products obtained by conventional synthesis at room temperature (rt), 60 °C and microwave-assisted synthesis (MW). (B) Mass spectrum of the HPLC main peak (conventional synthesis at room temperature). (C) Isotopic pattern of the product peak ($z = 4$).

The effects of the different reaction conditions on the side product formation of $Z_{Her2:342}$ are shown in Figure 3. By using conventional heating to 60 °C the overall side product formation decreased only slightly as compared to room temperature. The main peak of the product obtained by microwave synthesis broadens significantly in comparison to the conventional synthesis at room temperature or at 60 °C (Fig. 3). Mass spectrometric analysis revealed that the microwave-assisted synthesis led to significantly more deletion sequences than the conventional synthesis at room temperature or 60 °C (data not shown). In addition, synthesis at higher temperatures increased the amount of aspartimide and related side products.

Our results show that heating does not allow a significant improvement of the affibody synthesis. This may be explained by the fact that this peptide is not prone to aggregate in the solid phase synthesis process. Similar or better results can be obtained by conventional Fmoc solid phase synthesis at room temperature. The standard synthesis using optimized conditions (Rink amide resin with low loading, large excess of the amino acids) present the most effective technique to synthesize this peptide.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.08.096.

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25. Fmoc-protected amino acids with the following side-chain protecting groups were used: *tert*-butyl (*t*Bu) for Asp, Glu, Ser, Thr, and Tyr, *tert*-butyloxycarbonyl (Boc) for Lys and Trp, trityl (Trt) for Asn, Gln, and His and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg. The synthesis cycle of the peptide synthesizer consisted of: (1) Fmoc cleavage: 20% piperidine/DMF, (2) NMP washings, (3) coupling: Fmoc-AA-OH/HBTU/DIPEA/mmol peptidyl-resin 10:9:20:1, 8 min, (4) NMP washings. The synthesis cycle of the microwave synthesizer consisted of: (1) Fmoc cleavage: $2 \times 20\%$ piperidine/0.1 M HOBt/DMF, (2) DMF washings, (3) coupling: Fmoc-AA-OH/PyBOP/DIPEA/mmol peptidyl-resin 10:9:20:1, 4 min, in the case of Arg additional HOBt and double coupling were used, (4) DMF washings.
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