

Direct Continuous Flow Synthesis of Two Difficult Polypeptides Using β -Cyclodextrins

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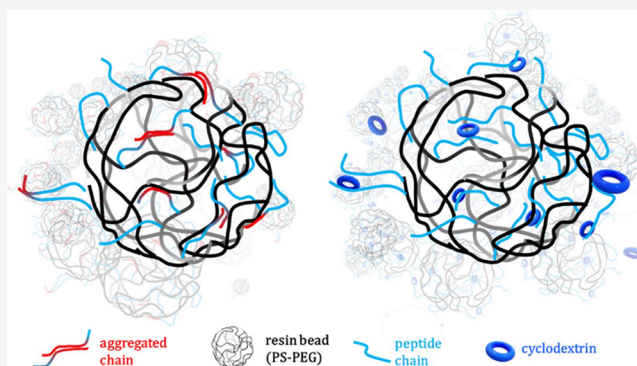


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ABSTRACT: This study focuses on investigating the use of β -cyclodextrin (β -CyD) derivatives as additives in continuous flow peptide synthesis, with particular emphasis on challenging sequences such as the Jung-Redemann decapeptide and the 42-residue amyloid β polypeptide [$A\beta(1-42)$]. The efficacy of the OH-free β -CyD and two of its derivatives (Ac- β -CyD and HP- β -CyD) is compared with alternative, state-of-the-art synthetic methods, including the widely used and recently improved pseudoproline monomer technique, e.g., Ser(Ψ Pro). Our results show that the use of β -CyD as an additive results in a significant (8–19%) increase in the purity of the crude polypeptide compared to that determined by our reference method. The chromatograms determined by LC-MS were deconvoluted to estimate the more precise purity of the crude products, and we found that the improvement is greater when the free OH β -CyD is used and moderate when the acetyl- β -CyD or the 2-hydroxypropyl- β -CyD derivatives are used. We have found that the free CyD gives an improvement comparable to that achieved with the Ser(Ψ Pro) derivative.



INTRODUCTION

Today, solid phase peptide synthesis (SPPS), introduced by Merrifield, is routinely used to synthesize short polypeptides of up to ~ 30 residues.¹ However, alternative techniques must be developed for the successful synthesis of longer polypeptides. (i) One approach, pioneered by Kent in 1994, is native chemical ligation (NCL), in which oligopeptide fragments are ligated and coupled to each other.² (ii) The other approach is stepwise synthesis, in which the polypeptide chain is built residue by residue. However, challenges that arise during sequence elongation, such as the incomplete removal of the Fmoc protecting group or the incomplete acylation of the free amino group of the N-terminal residue, pose problems. An inherent difficulty with SPPS is the aggregation of the nascent polypeptide chains on the resin. Various strategies have been developed to overcome this problem and prevent or disrupt specific aggregation during stepwise synthesis. Some of these methods involve modifications of the polypeptide backbone, such as the use of DmB (dimethoxybenzyl), HmB (2-hydroxy-4-methoxybenzyl), or HnB (2-hydroxy-5-nitrobenzyl) protecting groups, or the incorporation of pseudoproline dipeptides.^{3,4} Another approach is the depsipeptide method, in which the peptide backbone is extended on one of the side chains of the Thr or Ser residues.⁵ In addition, additives such as chaotropic agents have been used to prevent aggregation during synthesis.⁶ These strategies, involving backbone modifications or the use of additives, aim to alleviate the aggregation problem

encountered during the stepwise synthesis of longer polypeptides, which have a strong tendency to aggregate.

The resilient aggregation propensity observed during the synthesis of $A\beta(1-42)$ and Jung-Redemann decapeptide (JR 10-mer) polypeptides has led to these sequences being termed “difficult sequences”.⁷ Several approaches for overcoming this problem have been explored.⁹ Some methods use pseudoproline dipeptides,^{9,10} depsipeptides,^{11,12} or co-solvents such as dimethyl sulfoxide (DMSO) and anisole.^{6,13} Bacsá et al. used RAM ChemMatrix resin and employed microwave (MW) heating as part of their synthetic strategy on a manual peptide synthesizer, to make $A\beta(1-42)$.¹⁴ Collins et al. also used MW-induced heating to develop their “high-efficiency SPPS” method, which was also explored for the synthesis of JR 10-mers, in addition to $A\beta(1-42)$. These approaches often use specific residues or unusual protecting groups or involve incomplete O-acylation and additional steps such as the O–N acyl shift (depsipeptide method). Although there are numerous methods for the synthesis of $A\beta$ peptides, most of them use batch synthesis techniques. As an alternative, flow

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solid phase peptide synthesis (SPPS) has become popular due to its time efficiency and effectiveness.^{15–28} Tickler and colleagues also used a continuous flow Fmoc SPPS approach to synthesize the A β (1–42) peptide, achieving a crude cleavage product purity of 45%.²⁹

In the context of the self-aggregation tendency of A β (1–42) in its native form, the inhibitory effect of CyD derivatives on A β fibrillization has recently been described in the literature. Ren and colleagues identified 2-hydroxypropyl- β -CyD (HP- β -CyD) as a promising inhibitor of A β aggregation in vitro.³⁰ Danielsson et al. measured the K_d values of β -CyD and various A β fragments, providing experimental evidence for the involvement of their aromatic residues in the interaction with β -CyD.³¹ However, the millimolar binding strength may not be strong enough [e.g., A β (1–40) $\times$ β -CyD K_d = 4.7 mM] to be relevant.^{31–33} The focus of these studies was primarily on β -CyD, a cyclic oligosaccharide composed of seven glucopyranose rings, all linked together by an α -(1,4) glycosidic linkage. This structural arrangement forms a truncated cone shape, the interior of which is less polar than water and shelters apolar molecules such as the aromatic side chains of amino acid residues. Each sugar moiety within β -CyD has three free hydroxyl groups, resulting in a total of 21 hydroxyl functional groups on the exterior part of β -CyD^{34–40} (Figure 1).

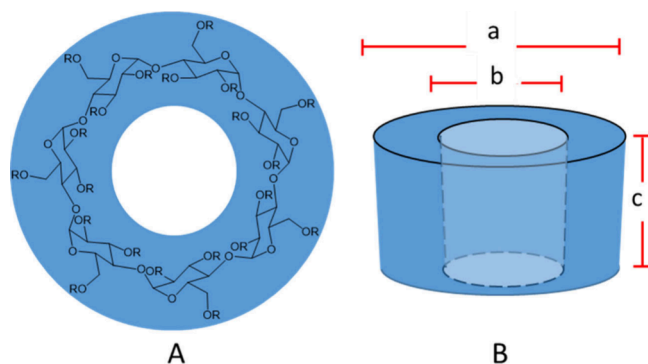


Figure 1. Schematic structure of β -CyD derivatives. (A) Top view of β -CyD ($R = H$), acetyl- β -CyD ($R = \text{acetyl}$), and HP- β -CyD ($R = 2$ -hydroxypropyl). (B) Side view of the truncated cone shape of a β -CyD with its representative dimensions represented as a (outer diameter, 15.3 Å), b (inner diameter, 7.8 Å), and c (depth, 7.8 Å), giving a total internal volume of ~ 372 Å³.

Approximately 74 protein structures with cyclodextrins have been crystallized from water and/or buffer and deposited in the Protein Data Bank (PDB). Most of them form inclusion or axial complexes with CyDs (e.g., the CyD-Phe261 complex of PDB entry 5e6z). Similarly, of the 20 different CyD and oligopeptide complexes found in the Cambridge Structural Database (CSD), most are axial complexes. However, a few equatorial or lid type CyD complexes were found in both the PDB and CSD. For example, CyD forms a 1:1 lid type complex with Trp296 (α NH) of protein B. thaitaomicron (PDB entry 3ck8) and Thr49 (α NH) of protein aconitase (PDB entry 1ACO). Protein α -amylase B (PDB entry 3bcd) forms several lid complexes with CyD involving residues Ala237 (α CO), Lys198 (ϵ NH₂), and Met176 (α CO).^{41–44} An inclusion lid type complex was also found in the CSD, which was assigned to the CyD-hemikis[1-butyl-3-(*p*-toluenesulfonyl)-urea] clathrate hydrate (CSD:buxui).⁴⁵ On the basis of these data, the following conclusions were drawn. Both axial and

equatorial complexes can be formed with CyD, with the mode of interaction correlating with the type of solvent used for crystallization. In highly polar water, the inclusion or axial type complexes are prominent, whereas in the less polar and aprotic dimethylformamide (DMF), the equatorial or lid complexes are more abundant.^{46,47} These considerations lead us to our working hypothesis.

Therefore, our working hypothesis was that the noncovalent and reversible binding capabilities of CyDs to bind to the peptide backbone could be advantageous in the synthesis of polypeptides with a high aggregation propensity. However, the low-affinity binding strength of CyDs could be strong enough to prevent aggregation of the polypeptide chains but not too strong to cause irreversible binding and thus block chemical synthesis by protecting the free -NH₂ group of the N-terminus. We therefore carried out experiments with CyD derivatives with “medium” sized cavities, such as β -CyD, as well as β -CyD derivatives with an increased cavity depth, such as Ac- β -CyD and HP- β -CyD. If they interact with each other, they must affect the yield obtained, which is more pronounced when synthesizing difficult sequences. In other words, in the synthesis of polypeptides with a strong tendency to aggregate, the beneficial effect of using CyDs could be amplified and become more evident. To test our hypotheses, it seems sufficient to use CyDs for the synthesis of a shorter and a longer sequence, both of which are difficult to synthesize. Therefore, the 10-residue JR 10-mer and the 42-residue A β (1–42) were synthesized, and the composition of the crude product was monitored and analyzed as a function of the CyD derivatives and conditions used.

SYNTHETIC METHODS

Our continuous flow peptide synthesizer was used for all syntheses. The efficiency, cost, and environmental friendliness of the amino acid coupling are at the heart of our synthesis. We refer to this complex approach as smart peptide chemistry in flow (SPF).^{26,28,48} In this study, we adopted the SPF method for the synthesis of target peptides in the presence of CyDs. Our aim was to investigate the potential of CyD derivatives as aggregation inhibitors (Figure 2).

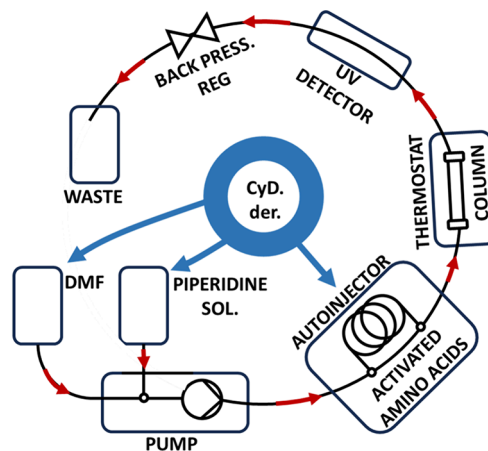


Figure 2. Schematic diagram of continuous flow peptide synthesis (SPF). For a uniform concentration of the cyclodextrin derivatives, 0.01 mmol/mL (15 mg/mL), CyD must be present (i) in the eluent (DMF), (ii) in the solution used for Fmoc deprotection (30% piperidine/DMF), and (iii) in the solution of the activated amino acids (NMP).

In the case of method 1, the reference method used in SPF synthesis, all amino acids were coupled using protocol A, in which the coupling of all of the amino acid residues follows the same protocol (Figure 3A). Previous results suggest that DMSO has the potential to

CyD was used in method 5, Ac- β -CyD in method 6, and HP- β -CyD in method 7. Method 8 is a combination of methods 3 and 5, in which amino acids K, R, Q, N, and E in A β (1–42) and W, F, and T in the JR 10-mer were coupled using protocol C, with the addition of β -CyD.

RESULTS AND DISCUSSION

To test the impact of CyD derivatives using SPF synthesis, we selected two difficult sequences: JR 10-mer and A β (1–42). We established a baseline to compare our results after performing the reference synthesis (method 1) for both peptides. Note that our reference method (method 1) gives a crude product ratio of 47%, which is very similar to or slightly better than the value of 42% first reported by Collins et al. The yield improved to 67% crude product when they fully optimized their method.⁵⁰ The difficulty of synthesizing the JR 10-mer even in a variable bed flow reactor (VBFR) was demonstrated by Sletten et al. Using a conventional approach, they achieved the target decapeptide using DMSO and pseudoproline dipeptide Fmoc-L-Thr(tBu)-L-Thr(Ψ Pro)-OH.⁵¹ Manne and co-workers also used monomers of two Thr(Ψ Pro) residues at positions 4 and 8 to improve the synthesis of the JR 10-mer.⁵²

The result of our reference method, method 1, shows a purity for A β (1–42) of ~53% (Table 1). Using DMSO as a chaotropic agent was not effective enough as it reduced the crude purity of both the JR 10-mer (41%) and A β (1–42) (40%). Using method 3, when protocol C is applied to selected amino acids [K, N, Q, R, E, and V39 for A β (1–42) and W, F, and T for the JR 10-mer] and thus the residence time for these selected residues is controlled, a significant increase in the crude peptide purity was obtained: 57% (+16%) for the JR 10-mer and 75% (+25%) for A β (1–42). When monomeric pseudoproline Ser(Ψ Pro) was used (method 4), the purity increased in both cases: 59% for the JR 10-mer and 59% for A β (1–42). It should be noted that the effect of pseudoproline on backbone disruption affects only the five or six residues in the vicinity (Figure 3B). Below we compare the positive effect and applicability of CyD to the results obtained using the reference method presented above.

Before launching the experiments with CyD to test its positive outcome on the quality of the raw products, we had to ensure that CyD is present at the same concentration throughout the overall synthetic cycle (Figure 3). During the synthesis, the concentrations of all CyD derivatives were kept fixed (0.01 mmol/mL or 15 mg/mL). This concentration cannot be considerably exceeded, as higher CyD concentrations would have resulted in a solution that was too viscous

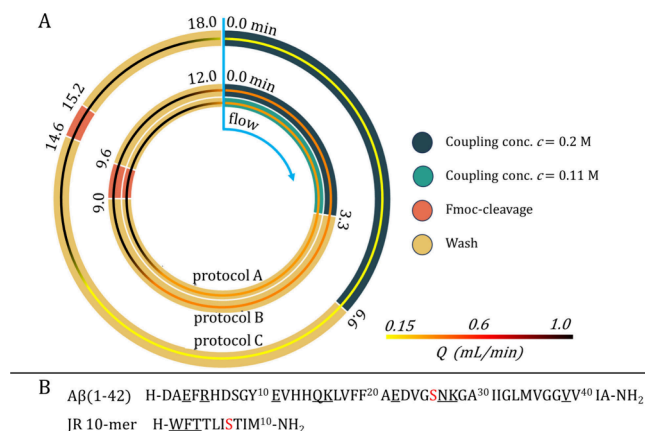


Figure 3. (A) Circle diagram of SPF coupling protocols. Each circle represents a protocol type of different length. Protocols a and b are 12 min, while protocol C is the longest with a total time of 18 min. Each circle is divided into coupling, washing, and deprotection times, as indicated. The inner thin circles represent the flow rate (Q) in milliliters per minute. Bright yellow indicates a rate of 0.15 mL/min, orange a rate of 0.6 mL/min, and black a rate of 1 mL/min. (B) Primary sequences of A β (1–42) and the JR 10-mer. The underlined amino acids were coupled with protocol C in method 3. The red S indicates Ser(Ψ Pro) in method 4. These protocols used to acylate a pseudoproline monomer were tested in our previous work.^{28,49} More detailed protocols can be found in Table S1.

disrupt the H-bonds formed during polypeptide aggregation on the resin. Therefore, in method 2, protocol A was used with the addition of DMSO to the eluent [80:20 (v/v) DMF/DMSO]. Method 3 is based on residence time control (RTC), in which most residues are coupled using protocol A, except for residues K, R, Q, N, and E, which carry bulky side chain protecting groups, and for sterically hindered V39 and V40 in A β (1–42), and the last three amino acids W, F, and T in the JR 10-mer, because their depletion is mainly observed. These latter residues were coupled by using protocol C to increase the coupling efficiency (Figure 3).

To avoid chain aggregation, the suggestion is to use either Ser or Thr masked as monomeric pseudoproline residues, Ser(Ψ Pro) or Thr(Ψ Pro), respectively, because they bend the polypeptide backbone like Pro. Method 4 uses protocol A for coupling and employs Ser(Ψ Pro) at position 26, with protocol B for the subsequent Gly25 in the case of A β (1–42) (Figure 3B). Methods 5–7 all use protocol A for the coupling of all residues with CyD derivatives. Specifically, β -

Table 1. Comparison of the Different SPF Synthesis Methods and the Resulting Crude Product Purities for the JR 10-mer and A β (1–42) Polypeptides^a

method	protocol(s)	additive	crude purity (%)		chromatograms/MS spectra
			JR 10-mer	A β (1–42)	
1	A	—	47	53	Figure 4A,B and Figures S1 and S9
2	A	20% DMSO	41	40	Figures S2 and S10
3	A and C	—	57	75	Figure 4C,D and Figures S3 and S11
4	A and B	Ser(Ψ Pro)	59	59	Figures S4 and S12
5	A	HO- β -CyD (free)	55	72	Figure 4E,F and Figures S5 and S13
6	A	Ac- β -CyD	53	48	Figures S6 and S14
7	A	HP- β -CyD	49	52	Figures S7 and S15
8	A and C	HO- β -CyD (free)	56	73	Figures S8 and S16

^aAll improved yields are compared to those of method 1 (shown in bold), which is used as the reference SPF method.

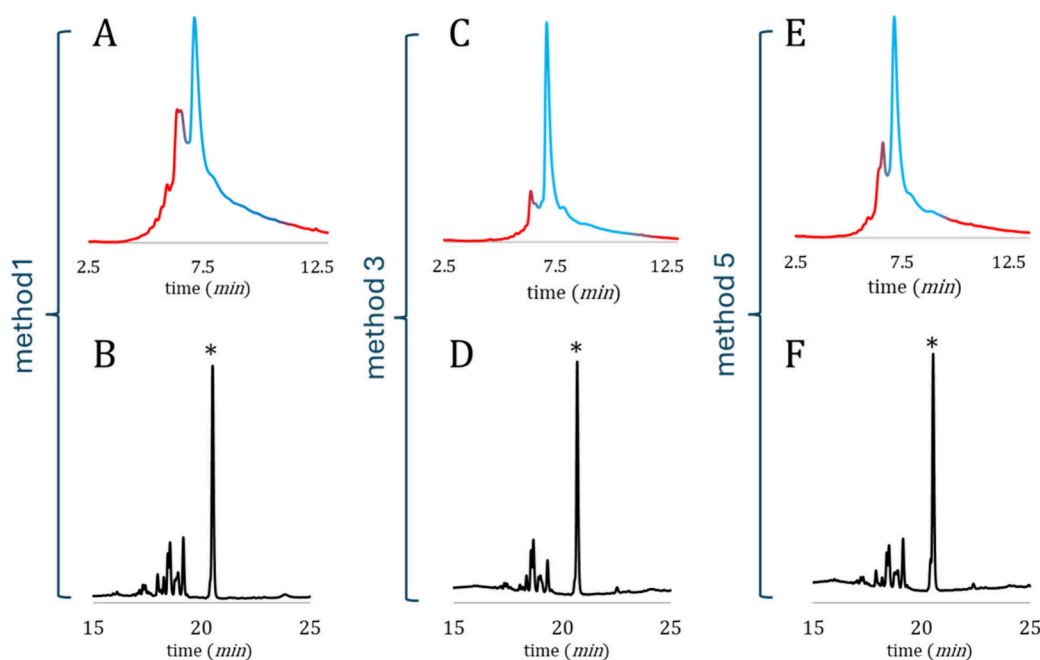


Figure 4. UV chromatograms (recorded at 220 nm) of the crude polypeptides SPF synthesized by method 1 (reference method): (A) $A\beta(1-42)$, (B) JR 10-mer (RTC, method 3), (C) $A\beta(1-42)$, (D) JR 10-mer (using free β -CyD, method 5), (E) $A\beta(1-42)$, and (F) JR 10-mer. The blue line indicates the elution of target molecule $A\beta(1-42)$, while the asterisk indicates the target polypeptide in the case of the JR 10-mer.

to handle safely with the autoinjector or the pump. Moreover, in an overly viscous medium, diffusion of the reagents would have been too slow on the resin. Upon application of these conditions associated with CyD, methods 5–8, the quality of the crude JR 10-mer increased from ~47% (reference method 1) as follows. The raw product purity increased just slightly if using method 7 (HP- β -CyD) (49%) and more upon application of method 5 or 6 (β -CyD or Ac- β -CyD), 55% and 53%, respectively. Upon application of method 8, a combination of method 3 (using protocol C for the last three amino acids, Trp, Phe, and Thr) and method 5 (using β -CyD) gave results similar to those of method 3 (56%). Very similar results were obtained when we used the same methods during the synthesis of $A\beta(1-42)$. Method 5 (β -CyD) improved the yield of the synthesis to 72% compared to that of the reference (53%). Surprisingly, methods 6 and 7 resulted in crude product purities of 48% and 52%, respectively. Using combined method 8, we achieved a crude peptide purity (73%) that was 20% higher than that of the reference.

We concluded that the use of free hydroxyl β -CyD can significantly improve the raw product purity for both the JR 10-mer and $A\beta(1-42)$ from 47% to 55% and from 53% to 72%, respectively. The best results for both peptides were also obtained from methods 3–5 and 8. This means that the use of β -CyD can be as successful as the most advanced methods such as the use of Ser(Ψ Pro) (method 4) or the use of RTC (method 3 of Table 1). However, the use of the residence time control (method 3) increases the SPF cycle time by 50% (from 12.0 to 18.0 min) and also requires a larger amount of reagents. This makes the RTC approach slightly slower and less environmentally friendly. Using methods 5–7, in which the three β -CyD derivatives were probed as useful additives, without changing the standard SPF protocol, we observed the following improvements. The enhanced amount of the crude $A\beta(1-42)$ product indicates that while the use of the OH-

protected β -CyD is beneficial, the use of the free OH-containing β -CyD is clearly the best [72% (Table 1)].

Both DMSO and CyD are used to reduce or break the H-bonds formed between the aggregated polypeptide chains during the SPF synthesis. Upon comparison of the results obtained using 20% DMSO (method 2) with those obtained using CyD (methods 5–7), it appears that the use of free β -CyD works better! As suggested above, the temporary binding of CyDs to the main chain of the polypeptide could partially prevent or disrupt unwanted chain associations by forming lid-like temporary complexes. In contrast to the use of the expensive pseudoproline derivatives (method 4), which form kinks in the nascent polypeptide chain and thus break the unwanted alignment of the polypeptides, termed “internal inhibition”, the use of CyD as an additive prevents or disrupts amyloid formation via an “external” inhibition mechanism. We found that this inhibitory effect of applied free β -CyD is impressive and can improve the crude product ratio by $\leq 72\%$ (Table 1). However, we found that this positive effect of CyD and that of RTC (method 3) were not additive. The 8% improvement measured for the JR 10-mer [47% $\rightarrow$ 55% (Table 1)] and the 19% improvement obtained for $A\beta(1-42)$ (53% $\rightarrow$ 72%) in crude product purities cannot be exceeded when synthetic method 5 (using CyD-free) is combined with method 3 [RTC (Table 1)], as method 8 gives an additional increase of only ~1%.

The raw products obtained from both the reference method (method 1) and the fully optimized method (method 8) were purified by using column chromatography. After lyophilization, we observed that the purity of the peptides was similar but their isolated yields differed. The original method yielded 0.7 mg of the purified peptide (8.75% yield), whereas the optimized method yielded 1 mg of the purified peptide (12.5% yield). These results show that our optimized method improved the isolated yield by 42%.

CONCLUSION

The aim of this study was to investigate the applicability of β -CyD derivatives as additives during continuous flow peptide synthesis, particularly for challenging sequences such as the JR 10-mer and $A\beta(1-42)$ polypeptides. The efficacy of β -CyD derivatives in improving the pure product ratio within the crude product was analyzed (Table 1). Compared to the more expensive and demanding approach of incorporating pseudo-proline derivatives [e.g., monomeric Ser(Ψ Pro)], the crude product purity of both the JR 10-mer and $A\beta(1-42)$ improved by ~8–19%, especially when the free OH-containing β -CyD was used instead of its derivatives (Figure 5 and Table 1). The

exact molecular mechanism behind the improved product purity should be further investigated, but our working hypothesis suggests that CyD derivatives may act as aggregate disruptors, like DMSO, but more effectively. Upon comparison of the positive effect of β -CyD derivatives, the improvement in product purity is significant. Therefore, it is plausible that the free hydroxyl groups of β -CyD interact with the peptide chain, form a lid-like supramolecular complex, and disrupt aggregation. In conclusion, we have shown that the use of CyD derivatives improves the purity of the crude product, even in the case of the very difficult and challenging $A\beta(1-42)$ sequence, and is particularly effective when free OH-CyD is used to disrupt aggregation during SPF.

EXPERIMENTAL SECTION

Chromatogram Integration. Integration of the JR 10-mer is straightforward due to the baseline separation of the peaks. Given the inherent complexity of purifying polypeptide $A\beta(1-42)$, several published studies have relied solely on the crude peptide purity determined by high-performance liquid chromatography (UPLC) as the primary measure of synthesis efficiency. This approach is well-documented in the comprehensive review by Kasim et al.⁸ Therefore, the challenging purification of the current difficult sequences is also beyond the scope of this study. Consistent with other literature data, we used the integrated chromatograms of the crude polypeptides to report on coupling and synthetic success.

In the case of $A\beta(1-42)$, the presence of “peak tailing” in the chromatogram indicates self-aggregation, which complicates accurate crude product estimation using a single fitted Gaussian curve. Therefore, UPLC-MS chromatograms for $A\beta(1-42)$ were analyzed by using Thermo XCalibur and FreeStyle. Peak integration was based on an automated peak detection and integration (PPD) algorithm, which deconvolves chromatograms into Gaussian curves representing different polypeptides. When Gaussian curves at different retention times yield identical MS spectra, they are combined to account for the fact that they contain the same peptide. The crude purity assessment of the polypeptide is dependent on this integration method. A detailed example is shown in Figure 6. All UHPLC-UV-MS chromatograms and MS spectra are available in Figures S1–S16.

The crude product obtained from methods 1 and 8 was purified as follows. Eight milligrams of the crude peptide was dissolved in 8 mL of distilled water and filtered through a 0.45 μ m PTFE filter. The filtered solution was then injected onto a Jupiter 10 μ m Proteo 90 Å

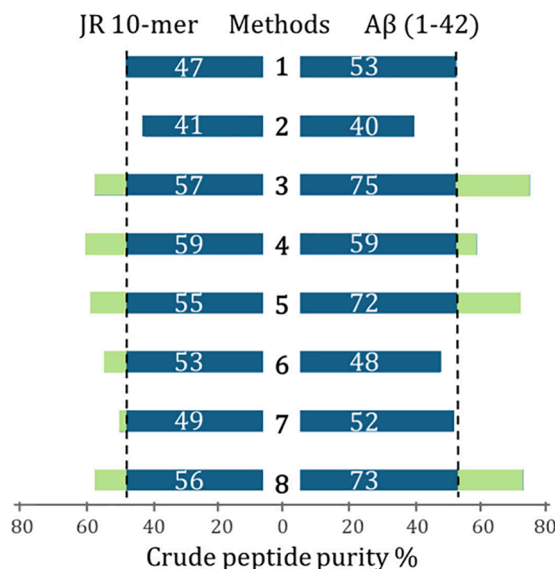


Figure 5. Column plots illustrating the improvement in crude product purity achieved during the SPF synthesis of the JR 10-mer and $A\beta(1-42)$ as a function of the method (1–8) applied. The dashed black line represents the yields obtained by method 1 (reference method) for the JR 10-mer (47%) and $A\beta(1-42)$ (53%). The green bars indicate an improved conversion percentage compared to that of method 1.

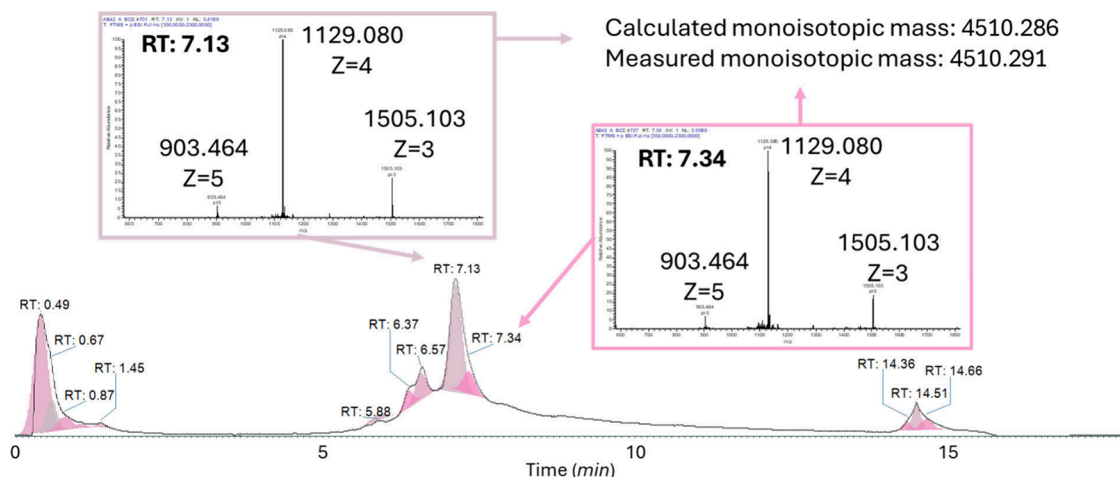


Figure 6. Deconvolution of the $A\beta(1-42)$ chromatogram into Gaussian curves using the PPD algorithm (ref) with the MS spectra of two Gaussian curves, namely, those of RT = 7.13 and RT = 7.34. As the MS spectra reveal and confirm the chemical identity of the polypeptides measured at two slightly different retention times (7.13 and 7.34 min, respectively) and thus deconvolve into two Gaussian curves, their integrals can be added together as they represent the same $A\beta(1-42)$ polypeptide. This SPF synthesis was performed according to method 5.

C12 column (250 mm $\times$ 10 mm). The following solvent gradient was used for purification: 100% solvent A and 0% solvent B from 0 to 15 min and 5% solvent A and 95% solvent B for 62.5 min.

It is important to note that during the purification process partial oxidation of the methionine at position 35 of A β (1–42) was observed. This resulted in the presence of a minor peak that could not be completely eliminated. However, as this oxidation occurred after purification, we included this minor component in the final purity calculations. In addition, in the case of the JR 10-mer peptide, the main side products originated mainly from des-W, des-F, and des-WFT peptides.

The HPPS-4000 flow peptide apparatus used, manufactured by METALON Ltd. (Budapest, Hungary), is a modified version of a standard Jasco LC-4000 series HPLC system. The modification involves the addition of an extra valve to the PU-4180 HPLC pump to allow recirculation and control of solvent flow, such as in the cleavage mixture used in peptide synthesis. ChromNAV2 version 2.04.06 was used to fully automate the process. Reagent injection was performed by an autosampler from 2 mL sample vials placed in the sample rack. The poly(ether ether ketone) (PEEK) column material was used as a fixed bed reactor tube for the resin. DMF was used as the solvent and eluent. For all syntheses, ~150 mg of Fmoc-Rink amide TentaGel resin (0.24 mmol/g) was loaded into the PEEK column. All peptides were synthesized using the Fmoc/Bu strategy. CyD derivatives were provided by CycloLab Ltd. (Budapest, Hungary). HP- β -CyD is a randomly substituted ether derivative of β -CyD with an average degree of substitution (DS) of 4.5. This compound is highly soluble in both water and methanol, is amorphous, is a noncrystallizable composite, and is a complicated isomeric mixture.⁵³ Ac- β -CyD is also a randomly substituted β -CyD ester derivative with an average DS of 7.0. The compound is highly soluble in water and methanol and appears as an amorphous solid. The distribution of acetyl groups is usually random on the primary and secondary edges of the β -CyD molecule but may also depend on the synthetic route used. The Tentagel S resin was purchased from Rapp Polymere GmbH (Tübingen, Germany), and Fmoc-Ser(ΨMe,MePro)-OH was purchased from Iris Biotech GmbH (Marktredwitz, Germany).

The reagent solutions were injected into the resin-filled column. For Fmoc deprotection, the deprotection solution consisted of 30% (v/v) piperidine in DMF. DMF was used for the washing steps during the synthesis. In the vials, the protected amino acids were dissolved with the coupling reagent *N*-methyl-2-pyrrolidone (NMP). The activating agent (DIC) was added just before coupling and was injected with the autosampler. During the synthesis, the pressure was varied between 5 and 6 MPa by using a back pressure regulator.

After peptide synthesis, the resin was washed with dichloromethane (DCM) and then dried under a vacuum. Cleavage from the resin was performed with (A) a 95:2.5:2.5 (v/v) TFA/H₂O/TIS mixture or (B) 5 mL of TFA, 250 μ L of H₂O, 250 μ L of thioanisole, 125 μ L of EDT, 60 μ L of TIS, and 250 mg of phenol with stirring for ~4.5 h at room temperature. The solution was then filtered, and the TFA was removed from the solution using a rotary vacuum evaporator. The peptide was washed with diethyl ether, dried under vacuum, solubilized in water or a water/acetonitrile solution, and lyophilized.

Crude peptides were dissolved in 0.6% NH₄OH in water to prevent aggregation. Slight aggregation was observed as “peak tailing” during liquid chromatography, which is considered typical for A β peptides.⁵⁴ The crude cleavage products were analyzed by ultraperformance liquid chromatography coupled with ultraviolet (UV) detection and mass spectrometry (UPLC-UV-MS). UPLC-UV-MS analysis was performed on a Thermo Scientific (Bremen, Germany) Q Exactive Focus, high-resolution, and high-mass accuracy hybrid quadrupole-orbitrap mass spectrometer. UHPLC separation was performed on a Dionex 3000 UHPLC system using a Waters ACQUITY Premier Peptide BEH C18 column (Waters, 2.1 mm $\times$ 150 mm, 130 Å) with gradient elution consisting of 0.3% (v/v) NH₄OH in water (eluent A) and 10% (v/v) eluent A in acetonitrile (eluent B) with a gradient from 5% to 90% B over 16 min. The column temperature was 60 °C. The flow rate was 0.4 mL/min, and UV absorbance was detected at λ values of 220 and 280 nm. The data were analyzed using Thermo

XCalibur. Peak integration for A β (1–42) was based on an automated peak detection and integration (PPD) algorithm. Crude products of JR 10-mer syntheses were analyzed by MS (Waters ACQUITY RDa detector) and UPLC (Waters ACQUITY UPLC H-Class PLUS System) using UNIFI 1.9.13.9 (Waters Corp., Milford, U.K.).

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.joc.4c01589>.

UPLC-UV-MS chromatograms and MS spectra for products JR 10-mer and A β (1–42) produced by methods 1–8 (PDF)

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Notes

The authors declare no competing financial interest.

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