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Fast Fmoc synthesis of hAmylin$_{1-37}$ with pseudoproline assisted on-resin disulfide formation

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Abstract: Human amylin (1–37) and the (1–13) fragment were synthesized with and without pseudoproline dipeptides. Thallium (III) trifluoroacetate, a mild oxidant, was used to cyclize the peptides by forming a disulfide bridge from C^2 to C^7. On the basis of our model studies, incorporation of a pseudoproline dipeptide decreases the amount of time necessary for the crude linear amylin (1–13) to cyclize on the resin. Without pseudoproline dipeptides, the 1–37 crude linear amylin was not pure enough to undergo the cyclization reaction. Following the cyclization studies, the synthesis time of the linear human amylin (1–37) was systematically reduced from 58 h to 8.5 h by shortening the reaction times. Cyclization and cleavage times were also reduced to 1.5 h. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: fast Fmoc SPPS; human amylin$_{1-37}$; pseudoproline dipeptide; disulfide bridge

INTRODUCTION

Human islet amyloid polypeptide (IAPP) or amylin is a peptide containing 37 amino acids (KCNTATCATQR-LANFLVHSSNNFGAILSSTNVGSNTY–NH_2, C^2–C^7). It contains a C^2–C^7 disulfide bridge and an amidated *C*-terminus. hAmylin$_{1-37}$ is co-secreted with insulin in the islet β-cells of the pancreas and functions as part of the endocrine system and contributes to glycemic control [1]. It was isolated and characterized from islet amyloid deposits in patients with insulinoma [2] or type-II (noninsulin-dependent) diabetes [3].

In 2005, Abedini and Raleigh reported [4] the synthesis of hAmylin$_{1-37}$ incorporating Mutter's pseudoproline dipeptide (dimethyloxazolidine dipeptide) derivatives to successfully obtain crude linear hAmylin$_{1-37}$. They formed a disulfide bridge between C^2 and C^7 with air oxidation. However, it took about 24 h to fully react to form the disulfide bridge.

Serine and threonine-derived pseudoproline dipeptides serve as reversible protecting groups for serine and threonine and prove to be versatile tools for overcoming some intrinsic problems in SPPS. The presence of a pseudoproline dipeptide within a peptide sequence results in the disruption of β-sheet structures (considered to be a source of intermolecular aggregation during chain elongation), thus increasing solvation and coupling/deprotection kinetics in SPPS [5]. There are reports in the literature on the successful synthesis [4–9] of long peptides or difficult peptides with pseudoproline dipeptides. In addition, there is a report in the literature demonstrating that pseudoproline dipeptides aid in cyclization reactions. In 2002, Schmiedeberg and Kessler [10] reported that the ring-closing metathesis reaction (RCM) of a peptide containing ten amino acid residues did not take place at all. Only the introduction of the secondary structure-disrupting reversible backbone protection Ser($\Psi^{Me,Me}$pro) and subsequent optimized reduction and purification protocols were able to generate a full set of RCM cyclized peptides. To the best of our knowledge, there is currently no literature on the synthesis of long difficult peptides incorporating pseudoproline dipeptides, followed by on-resin disulfide bridge formation and how the pseudoproline dipeptides affect the on-resin disulfide bridge formation.

Our internal studies and report of Yajima *et al.* [11] showed that thallium (III) trifluoroacetate [$Tl(tfa)_3$] is a mild oxidant that sometimes gives better yields and purities of desired disulfide products, with respect to methods using I_2 or air oxidation. We synthesized linear hAmylin$_{1-37}$ similar to Abedini and Raleigh's report [4] and used $Tl(tfa)_3$ for the on-resin disulfide formation. A model study was designed to synthesize hAmylin$_{1-13}$ to study how incorporating pseudoproline dipeptides adjacent to C^7 affect on-resin disulfide formation.

MATERIALS AND METHODS

Reagents

All protected natural amino acids, 1H-Benzotriazolium 1-[*bis*(dimethylamino)methylene]-5-chloro-,hexafluorophosphate (1-),3-oxide (HCTU) and 0.4 M NMM in DMF were provided by Protein Technologies Inc. (Tucson, AZ). Fmoc-Rink amide MBHA resin was purchased from Peptides International

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(Louisville, KY), Fmoc-Ala-Thr-$\Psi^{Me,Me}$pro–OH, Fmoc-Ser-Ser-$\Psi^{Me,Me}$pro–OH and Fmoc-Leu-Ser-$\Psi^{Me,Me}$pro–OH were provided by Novabiochem (San Diego, CA). Solvents were purchased from VWR (West Chester, PA) and used without further purification. $Tl(tfa)_3$, anisole and ethanedithiol (EDT) were purchased from Sigma-Aldrich (St Louis, MO) and TFA was purchased from Spectrum (Gardena, CA).

Peptide Synthesis

Linear peptide synthesis. Peptides were synthesized on a Protein Technologies, Inc *Prelude* peptide synthesizer at a 40-μmol scale using a fivefold excess of Fmoc-amino acids (200 mM) relative to the Fmoc-Rink amide MBHA resin (0.47 mmol/g). Deprotection was performed using 20% piperidine/DMF. Coupling was performed using 1 : 1 : 2 amino acid/HCTU/NMM in DMF. DMF top washes (0.5 min) were performed between deprotection and coupling steps. Pseudoproline dipeptides were delivered by the *Prelude*'s *Single-Shot* feature that delivers the entire contents of an amino acid vial to a selected reaction vessel without priming. This is useful in avoiding the addition of expensive reagents since nothing is wasted.

Cyclization. Linear bis(S-Acm)-protected peptides on the resins were treated with 1.2 equivalents of $Tl(tfa)_3$ in DMF for 30 min followed by DMF and DCM washes.

Cleavage. Cleavage was performed with 95 : 2.5 : 2.5 TFA/anisole/EDT.

Peptide Analysis

Crude linear peptides and cyclized peptides were analyzed on a Varian ProStar HPLC on a Microsorb-MW 300-5 C18 column, 250 × 4.6 mm over 60 min using a gradient of 5–65% aqueous MeCN with 0.1% TFA at a flow rate of 1 ml/min. Detection was at 214 nm. Mass measurements were carried out on a Bruker Reflex III MALDI-TOF instrument using a matrix of sinapinic acid and reflectron acquisition in the positive mode.

RESULTS AND DISCUSSION

As a model study, two $hAmylin_{1-13}$ peptides (KCNTATCATQRLA–NH_2, disulfide bridge: C^2–C^7) were synthesized. One linear bis(S-Acm) protected peptide was synthesized using Fmoc-Cys(Acm)–OH, Fmoc-Ala–OH and Fmoc-Thr(^{t}Bu)–OH for the C^2, C^7, A^8 and T^9 positions. The other linear bis(S-Acm) protected peptide was synthesized using Fmoc-Cys(Acm)–OH and Fmoc-Ala-Thr-$\Psi^{Me,Me}$pro for the C^2, C^7, A^8 and T^9 positions. Both the linear bis(S-Acm) protected peptides were treated with $Tl(tfa)_3$ at room temperature, and the reaction was monitored [12,13]. As Figure 1 illustrates, the incorporation of pseudoproline dipeptides allowed $hAmylin_{1-13}$ to completely cyclize in 60 min., whereas without a pseudoproline dipeptide, $hAmylin_{1-13}$ did not cyclize to completion even after 120 min.

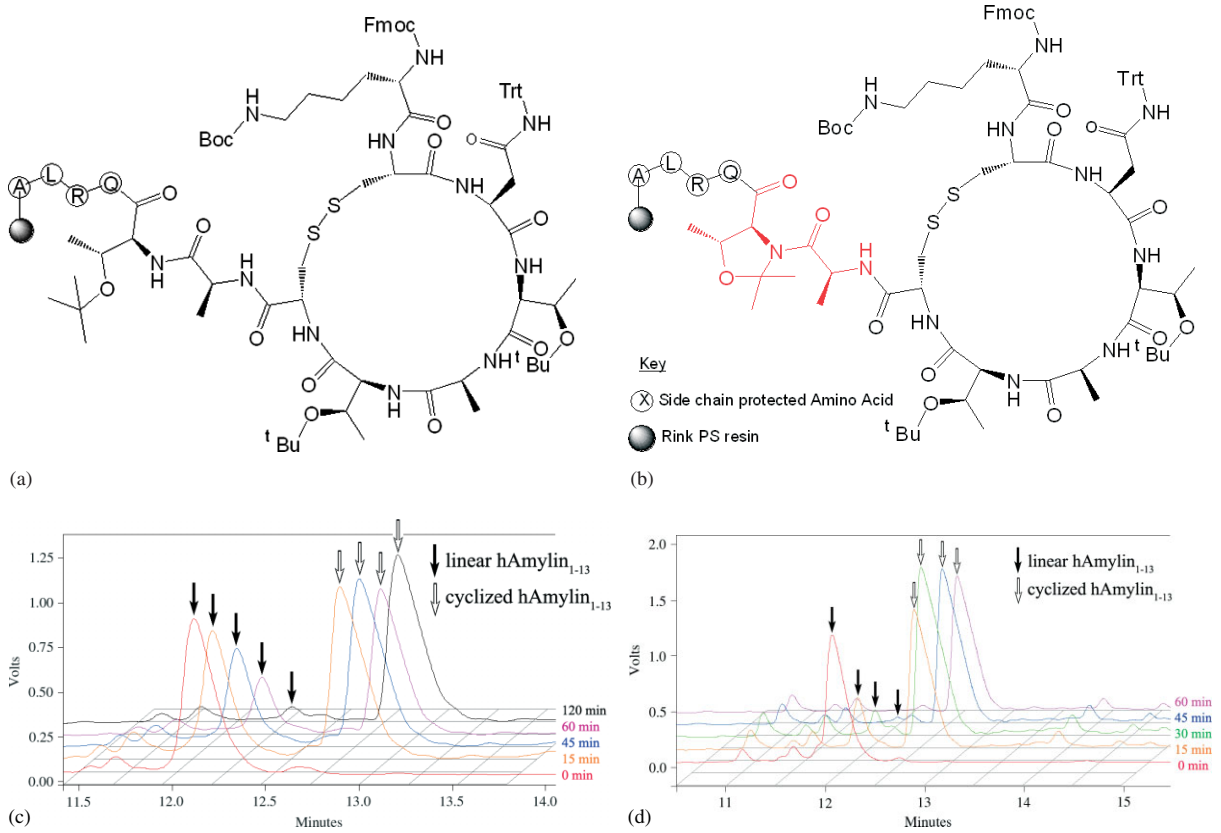

Figure 1 On-resin structure of cyclic $hAmylin_{1-13}$: (a) without pseudoproline dipeptide incorporation and (b) with pseudoproline dipeptide incorporation (shown in red). Progress of disulfide bridge formation (c) without pseudoproline dipeptide incorporation and (d) with pseudoproline dipeptide incorporation.

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After observing fast and successful disulfide bridge formation with a pseudoproline dipeptide in our model studies, linear hAmylin$_{1-37}$ was synthesized with pseudoproline dipeptides as Abedini and Raleigh reported [4], except Fmoc-Cys(Acm)–OH was used instead of Fmoc-Cys(Trt)–OH to allow the on-resin cyclization. Fmoc-Ala-Thr-$\Psi^{Me,Me}$pro–OH, Fmoc-Ser-Ser-$\Psi^{Me,Me}$pro–OH and Fmoc-Leu-Ser-$\Psi^{Me,Me}$pro–OH were used for $A^{8}T^{9}$, $S^{19}S^{20}$, and $L^{27}S^{28}$ respectively (Figure 2).

Reported coupling times for difficult and long peptides with pseudoproline dipeptides were normally 45–120 min [5,6,14]. Therefore, we started with a coupling time of 30 min $\times$2 and a deprotection time of 3 min followed by 20 min, resulting in a total synthesis time of 58 h (Figure 2(b)). We have previously reported [15,16] a fast Fmoc synthesis of $^{65-74}$ACP (H–VQAAIDYING–OH) and G-LHRH (H–GHWSYGLRPG–NH_2). They were synthesized with a 0.5 min $\times$2 deprotection time and a 1 min $\times$2 coupling time. On the basis of the $^{65-74}$ACP and G-LHRH results, linear hAmylin$_{1-37}$ was synthesized again, but this time with coupling times reduced to 2.5 min $\times$2, and deprotection times reduced to 1 min $\times$2. The washes were reduced from 6 to 3 washes, reducing the total synthesis time from 58 h to 8.5 h. Shorter reaction times minimized the time for side reactions to occur, resulting in a higher purity peptide (Figure 3). Initially, there was a concern that pseudoproline dipeptides would require longer coupling times than regular amino acids since the kinetics of their reactions are unknown. However, it was found that both regular amino acids and pseudoproline dipeptides could be coupled in a short time. A summary of the synthesis and reaction times is illustrated in Table 1.

After the successful fast synthesis of hAmylin$_{1-37}$, the linear hAmylin$_{1-37}$ was cyclized using the previous

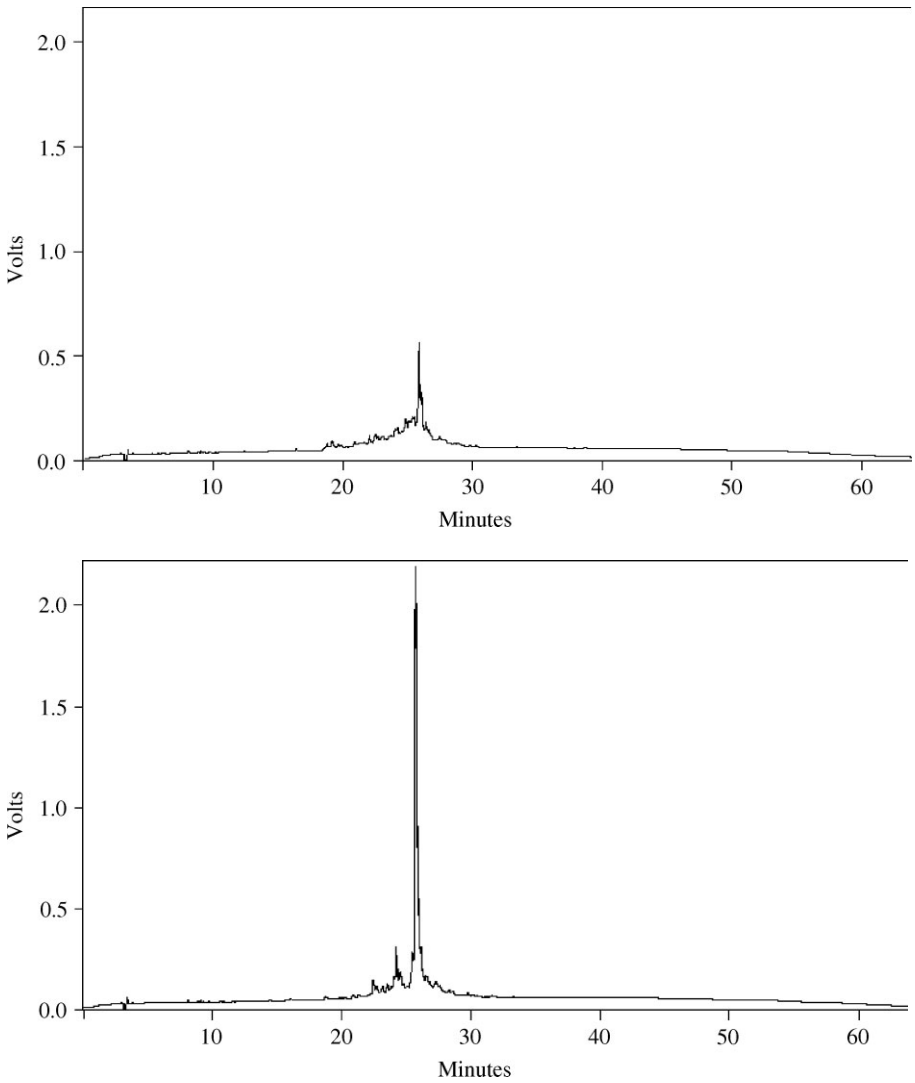

Figure 2 HPLCs of crude hAmylin$_{1-37}$ (a) without pseudoproline dipeptides and (b) with pseudoproline dipeptides synthesized in 58 h.

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Table 1 Summary of hAmylin$_{1-37}$ synthesis and reaction times

Synthesis	Coupling time (min ×2)	Deprotection time	Number of washes	Cycle time[a] (min)	Total synthesis time[a] (h)	Retention time (min)
Control	30	3 min, 20 min	6	94	58	25.7
Reduced	2.5	1 min × 2	3	15	8.5	25.8

[a] Peptide was synthesized on *Prelude* and cycle time and total synthesis time were determined.

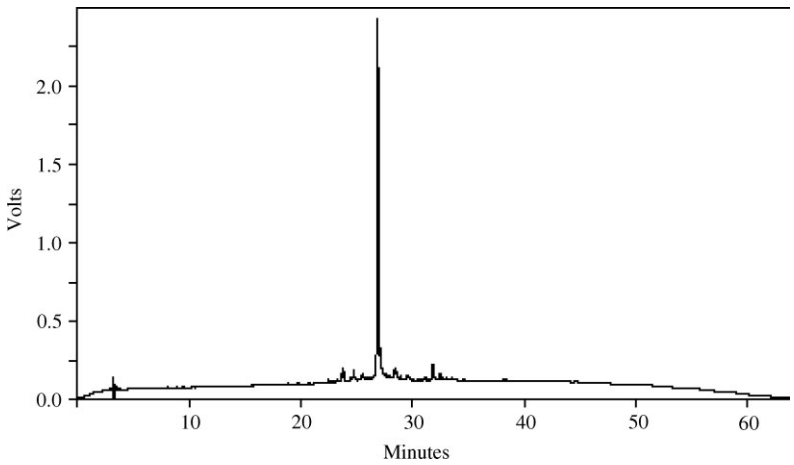

Figure 3 HPLC of linear hAmylin$_{1-37}$ synthesized in 8.5 h.

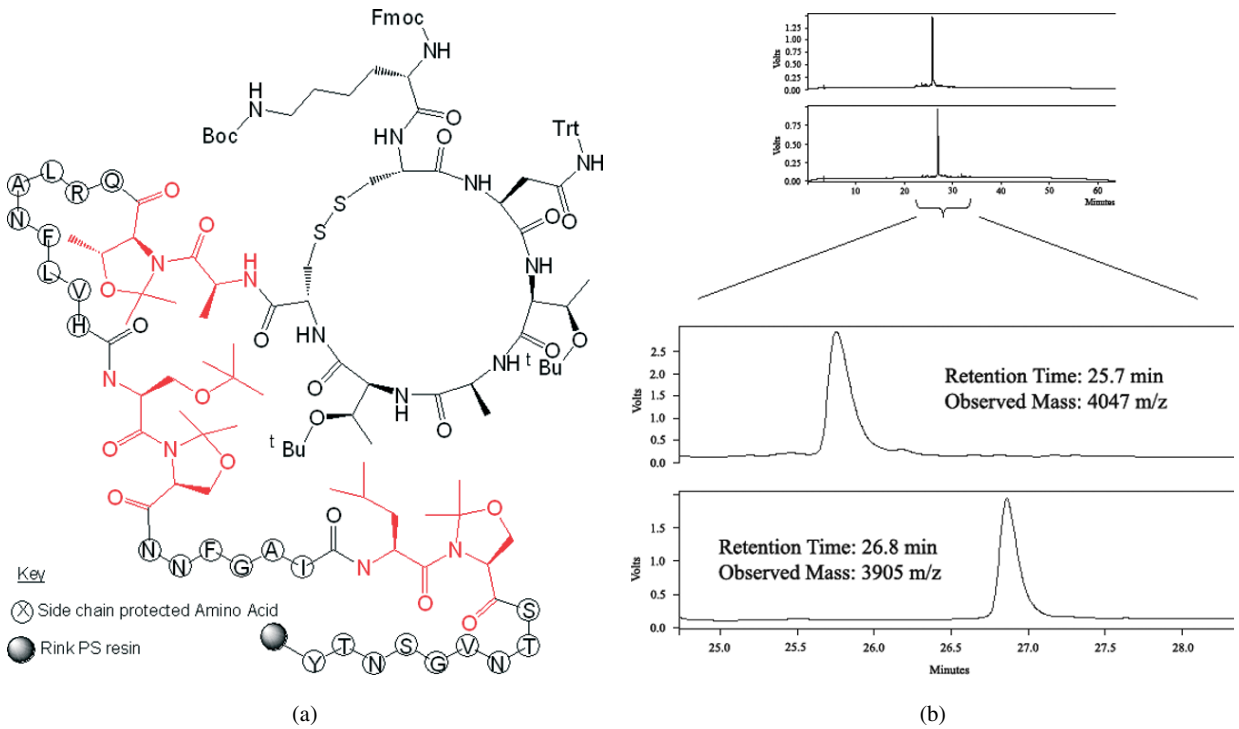

Figure 4 (a) On-resin structure of hAmylin$_{1-37}$ with pseudoproline dipeptides (b) HPLC comparison of linear (top) and cyclized (bottom) hAmylin$_{1-37}$.

cyclization method. Cyclization with Tl(tfa)$_3$ was completed within 10 min and gave good crude hAmylin$_{1-37}$. The HPLCs of the linear and cyclized peptide (Figure 4) were compared and confirmed complete cyclization. The

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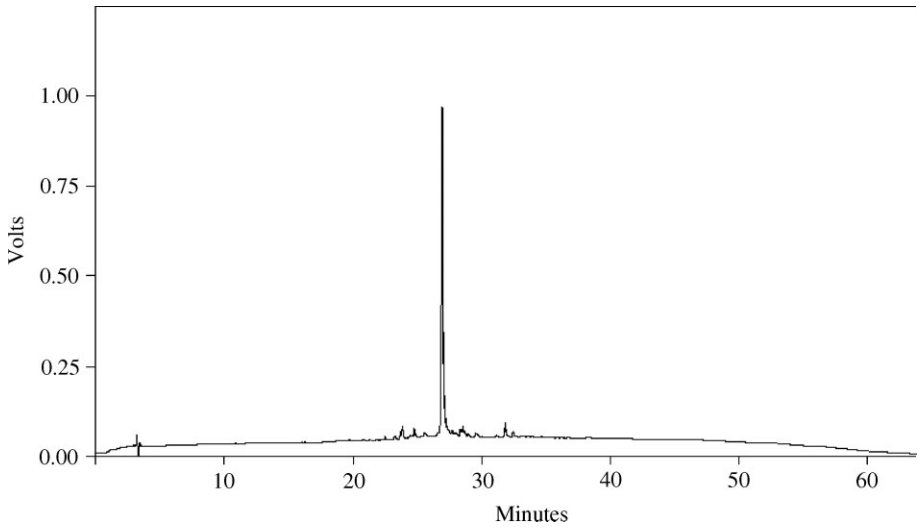

Figure 5 HPLC of cyclized hAmylin$_{1-37}$ after 1 h cleavage.

retention time shifted after cyclization from 25.7 to 26.8 min. The observed mass also changed from 4047 to 3905 m/z.

Previous peptides were cleaved with TFA cocktail for 3 h. However, we had confidence that cleavage could be completed in under 3 h. Cleavage was monitored, and Figure 5 shows that 1 h was enough to remove the peptide from the resin and remove the side chain protecting groups.

CONCLUSION

Our model study shows that hAmylin$_{1-13}$ containing a pseudoproline dipeptide cyclized faster than without the pseudoproline dipeptide. This is because the peptide disaggregated with a pseudoproline dipeptide is more flexible and more accessible to cyclization than without the pseudoproline dipeptide. Our study also shows that linear hAmylin$_{1-37}$ was successfully synthesized and cyclized with the incorporation of pseudoproline dipeptides. Unnecessary coupling, deprotection and wash times have been shortened dramatically, and pseudoproline dipeptides did not require additional reaction time. Linear hAmylin$_{1-37}$ was synthesized in 8.5 h, while oxidative cyclization and cleavage took 1.5 h. We therefore synthesized hAmylin$_{1-37}$ in 10 h on the *Prelude*.

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