

ABSTRACT

Human amylin₁₋₃₇ and its 1-13 fragment were synthesized with and without pseudoprolinamide dipeptides. Thallium (III) trifluoroacetate (Tl(tfa)₃), a mild oxidant, was used to cyclize the peptides by forming a disulfide bridge from Cys-2 to Cys-7. Based on our model studies, incorporation of a pseudoprolinamide dipeptide decreases the amount of time necessary for the crude linear amylin₁₋₁₃ to cyclize on the resin. Without pseudoprolinamide dipeptides, the crude linear hAmylin₁₋₃₇ was not pure enough to undergo the cyclization reaction. Incorporating pseudoprolinamide dipeptides into the peptide sequence increases the purity of the crude linear hAmylin₁₋₃₇ peptide, and allowed cyclization [2]. Following the cyclization studies, the synthesis time of the linear hAmylin₁₋₃₇ was systematically reduced from 58 hours to 8.5 hours by shortening the reaction times. Syntheses were performed on the *Prelude*TM peptide synthesizer from Protein Technologies, Inc. *Single-Shot*TM deliveries were used to deliver pseudoprolinamide dipeptides and the thallium (III) reagent without waste, and the use of an on-resin oxidation technique allowed the entire process to be fully automated on the *Prelude*TM.

INTRODUCTION

Currently, over 150 million people worldwide are affected by type-II diabetes, which is caused by chronic insulin resistance and a progressive decline in pancreatic beta-cell function [3]. Human islet amyloid polypeptide (IAPP) (Figure 1) or amylin, is a 37-residue major protein contributor to the amyloid deposits found in the pancreases of type-II diabetes patients [4,5]. Amylin contains a disulfide bridge from Cys-2 to Cys-7 and has an amidated C-terminus. During peptide synthesis, amylin is prone to aggregation because of excessive hydrophobic tendencies. This causes low coupling yields, incomplete couplings and side products. The addition of pseudoprolinamide dipeptides to the peptide increases the purity of difficult peptides by decreasing the aggregation [6].

In 2005, Abedini and Raleigh reported that they used Mutter's dimethylloxazolidine dipeptide (pseudoprolinamide dipeptide) derivatives to obtain crude linear hAmylin₁₋₃₇ successfully. They cyclized the peptide between Cys-2 and Cys-7 with air oxidation after the peptide was cleaved from the resin. However, it took about 24 hr to fully react and form the disulfide bridge [2]. Our internal studies [7] and Yajima *et al.* [8] report that Tl(tfa)₃, a mild oxidant, sometimes gives better yields and purities of the desired disulfide products, with respect to methods using I₂ or air oxidation. We synthesized linear hAmylin₁₋₃₇ and used Tl(tfa)₃ for the on-resin disulfide bridge formation. We used the model peptide, hAmylin₁₋₁₃, to study how a pseudoprolinamide dipeptide adjacent to Cys-7 affects on-resin disulfide formation.

As far as we know, there are no reports relating the synthesis of long, difficult peptides incorporating pseudoprolinamide dipeptides followed by on-resin disulfide bridge formation and the effect of pseudoprolinamide dipeptides on on-resin disulfide formation.



Figure 1: Sequence of hAmylin₁₋₃₇

EXPERIMENTAL

Linear peptide synthesis: The peptides were synthesized on a Protein Technologies, Inc., *Prelude*TM peptide synthesizer at the 40 μmol scale using a 5-fold excess of Fmoc-amino acids (200 mM) relative to the Fmoc-Rink MBHA resin (0.47 mmol/g, Polymer Laboratories). Deprotection was performed using 20% piperidine/DMF. Coupling was performed using 1:1:2 amino acid/HCTU/NMM in DMF. The side chain protecting groups utilized included t-butyl for serine, and tyrosine; trityl for asparagine, glutamine and histidine; pentamethylidihydrobenzofuran-5-sulfonyl for arginine; t-butylxycarbonyl for lysine and threonine; and acetamidomethyl for cysteine. Pseudoprolinamide dipeptides were delivered by the *Prelude*TM's *Single-Shot*TM delivery feature that gives a zero dead volume by delivering the entire contents of the bottle to the reaction vessel. Amino acids, HCTU, and 20% piperidine were obtained from Protein Technologies, Inc. Pseudoprolinamide dipeptides were provided by Novabiochem.

Cyclization and Cleavage: On-resin linear bis(S-Acm)-protected peptide was treated with 1.2 eq of Tl(tfa)₃ in DMF at room temperature followed by DMF and DCM washes [9]. Cleavage was performed with 95:2.5:2.5 TFA/anisole/EDT.

Analysis: Crude peptides were precipitated with ether and dissolved in 5% MeCN and 95% HPLC grade water. Crude linear bis(S-Acm)-protected peptides and cyclized peptides were analyzed on a Varian Microsorb-MW 300Å 5μm C18 column, 250 x 4.6 mm over 60 minutes using a gradient of 5-95% aqueous MeCN with 0.1% TFA at 1 mL/min. Detection was at 214 nm.

RESULTS

To test the effects of pseudoprolinamide dipeptides on on-resin disulfide bridge formation, hAmylin₁₋₁₃ was synthesized with and without a pseudoprolinamide dipeptide, then cyclized with Tl(tfa)₃. The first peptide was synthesized with Fmoc-Ala-Thr-Ψ^{Me}Me⁺pro at A⁸. The second peptide was synthesized with Fmoc-Ala-OH and Fmoc-Thr(Boc)-OH at A⁸ and T⁹, respectively. Each linear protected peptide was treated on-resin with Tl(tfa)₃ at room temperature, and the cyclization reaction was monitored [10] (Figure 2). The hAmylin₁₋₁₃ containing the pseudoprolinamide dipeptide cyclized to completion in 45 min, while the hAmylin₁₋₁₃ without the pseudoprolinamide dipeptide did not cyclize to completion even after 120 min.

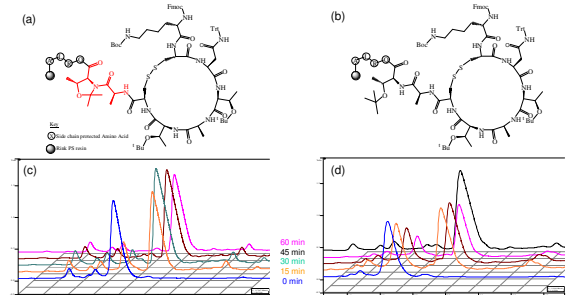


Figure 2: Structure of hAmylin₁₋₁₃ (with all of amino acids in the L-configuration) (a) with the pseudoprolinamide and (b) without the pseudoprolinamide. Disulfide bridge formation progress (c) with the pseudoprolinamide and (d) without the pseudoprolinamide.

After determining that the presence of a pseudoprolinamide dipeptide actually promoted the cyclization reaction, hAmylin₁₋₃₇ was synthesized with Fmoc-Ala-Thr-Ψ^{Me}Me⁺pro (A⁸T), Fmoc-Ser-Ser-Ψ^{Me}Me⁺pro (S¹⁵S) and Fmoc-Leu-Ser-Ψ^{Me}Me⁺pro (L²⁷S). A second hAmylin₁₋₃₇ peptide was synthesized without pseudoprolinamide dipeptides for comparison purposes. The purity of the hAmylin₁₋₃₇ without pseudoprolinamide dipeptides was so poor it could not be cyclized (Figure 3a). With pseudoprolinamide dipeptides, however, the linear hAmylin₁₋₃₇ was considerably more pure (Figure 3b). Cyclization of the peptide containing pseudoprolinamide dipeptides was easily accomplished on-resin with Tl(tfa)₃, and went to completion within 10 minutes (Figure 4). Close-ups of the HPLC's show the retention time shifts from 25.7 minutes to 26.8 minutes upon cyclization, and the mass spectrometry data show a change in mass from 4047 m/z to 3905 m/z upon cyclization, thus verifying the cyclization occurred.

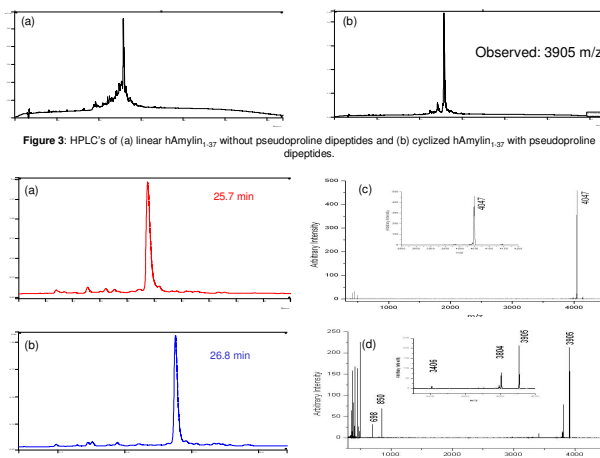


Figure 3: HPLC's of (a) linear hAmylin₁₋₃₇ without pseudoprolinamide dipeptides and (b) cyclized hAmylin₁₋₃₇ with pseudoprolinamide dipeptides. Mass spectra of (c) linear and (d) cyclized hAmylin₁₋₃₇.

The initial synthesis of hAmylin₁₋₃₇ used coupling times of 30 min x 2 [12] and deprotection times of 3 min followed by 20 min, giving a total synthesis time of 58 hours (Table 1 and Figure 5a). Based on previous studies where ⁶⁵-⁷⁴ACP [13] and G-LHRH [14] were synthesized with reduced reaction times, linear hAmylin₁₋₃₇ was synthesized with deprotection times reduced to 1 min x 2 and coupling times reduced to 2.5 min x 2. It was found that pseudoprolinamide dipeptides did not require additional reaction times, and the total synthesis time was reduced from 58 hours to 8.5 hours without reducing the crude product purity (Table 1 and Figure 5).

Table 1: Summary of hAmylin₁₋₃₇ synthesis to reduce time from 58 hr to 8.5 hr.

Synthesis	Deprotection Time	Coupling Time	# of Washes	Cycle Time	Total Synthesis Time
Control	3 min & 20 min	30 min x 2	6	94 min	58 hr
Reduced Time	1 min x 2	2.5 min x 2	3	15 min	8.5 hr

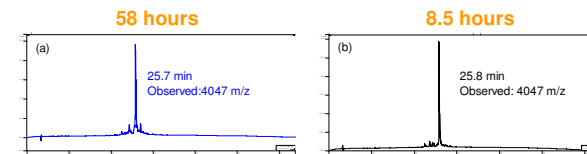


Figure 5: HPLC's of linear hAmylin₁₋₃₇ synthesized in (a) 58 hours and (b) 8.5 hours

After reducing the synthesis time, the cleavage time was minimized to 1 hour, resulting in a total synthesis time of 10 hours for the cleaved and cyclized hAmylin₁₋₃₇ peptide.

CONCLUSIONS

- Incorporating pseudoprolinamide dipeptides into hAmylin₁₋₁₃ improved the kinetics of its on-resin cyclization with Tl(tfa)₃.
- Pseudoprolinamide dipeptides do not require additional reaction time to couple.
- Linear hAmylin₁₋₃₇ was synthesized in 8.5 hr.
- Cleaved and cyclized hAmylin₁₋₃₇ was produced in 10 hr on the *Prelude*TM.

REFERENCES

- [1] K. Page, C. Hood, H. Patel, G. Fuentes, M. Menakuru, and J.H. Park, On-Resin Disulfide Bridge Formation with Pseudoprolinamide Dipeptides and Fast Fmoc Synthesis of hAmylin₁₋₃₇. Submitted for publication, 2007.
- [2] A. Abedini, D.P. Raleigh. *Org. Lett.*, 7, 693-696 (2005).
- [3] G.J.S. Cooper, A.C. Willis, A. Clark, R.C. Turner, R.B. Sim, K.B.M. Reid. *Proc. Natl. Acad. Sci. USA*, 84, 8628-8632 (1987). (b) P. Westermark, C. Wernstedt, E. Wilander, D.W. Hayden, T.D. O'Brien, K.H. Johnson. *Proc. Natl. Acad. Sci. USA*, 84, 3881-3885 (1987). (c) P.C. Butler, J. Chou, W.B. Carter, Y.N. Wang, B.H. Bu, D. Chang, J.K. Chang, R.A. Rizza. *Diabetes*, 39, 752-755 (1990).
- [4] A. Abedini and D.P. Raleigh. *Biochemistry*, 44, 16284-16291 (2005).
- [5] A. Clark, C.A. Wells, I.D. Buley, J.K. Cruickshank, R.L. Vanhegan, D.R. Matthews, G.J. Cooper, R.R. Holman, R.C. Turner. *Diabetes Res.*, 9, 151 (1988).
- [6] T. Wöhr, F. Wahl, A. Neftzi, B. Rohwedder, T. Salo, X. Sun, M. Mutter. *J. Am. Chem. Soc.*, 118, 9218-9227 (1996).
- [7] Protein Technologies Inc. Application Note AN-0001, 2007.
- [8] N. Fujii, A. Otake, S. Funakoshi, K. Beesho, T. Watanabe, K. Akaji, H. Yajima. *Chem. Pharm. Bull.*, 35, 2339-2347 (1987).
- [9] M.C. Munson, G. Barney. *J. Am. Chem. Soc.*, 115, 10203-10210 (1993).
- [10] Chan, W.C., and White, P.D. *Fmoc Solid Phase Peptide Synthesis*. (Oxford University Press, 2000), p. 103.
- [11] P. White, J.W. Keyte, K. Bailey, G.J. Bloomberg. *Peptide Sci.*, 10, 18-26 (2004).
- [12] Innovations 3/04, Novabiochem, <http://www.embiosciences.com/html/NBC/pseudoprolinamide1.htm>.
- [13] G. Fuentes, C. Hood, K. Page, H. Patel, J.H. Park, M. Menakuru. *Fast Conventional Fmoc Synthesis of ⁶⁵-⁷⁴ACP on the Symphony[®] and PreludeTM*. Poster Presented at the 2006 European Peptide Symposium, http://www.peptideinstruments.com/images/images/29EPS_P559.pdf
- [14] G. Fuentes, C. Hood, K. Page, H. Patel, J.H. Park, M. Menakuru. *PreludeTM to the Future of Peptide Synthesis... Now!*, Poster, Protein Technologies, Inc., <http://www.pti-instruments.com/images/Prelude.pdf>