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Incorporation of Pseudoproline Derivatives Allows the Facile Synthesis of Human IAPP, a Highly Amyloidogenic and Aggregation-Prone Polypeptide

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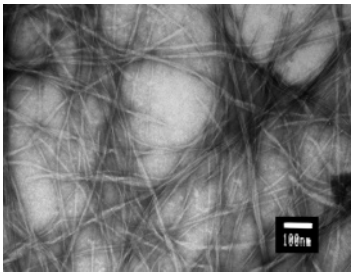
ABSTRACT



The efficient Fmoc solid-phase peptide synthesis of the 37-residue human Amylin and its amyloidogenic 8–37 fragment was achieved using pseudoproline (oxazolidine) dipeptide derivatives. Syntheses of $hAmylin_{8-37}$ using Fmoc amino acids produced only traces of the desired peptide. Incorporation of pseudoproline dipeptides produced the desired product with high yield and allowed for the synthesis of the full length peptide. The crude material was pure enough to allow formation of the Cys-2 to Cys-7 disulfide by air oxidation.

Human islet amyloid polypeptide (IAPP) or Amylin is coproduced with insulin in the islet β-cells of the pancreas[1−4] and acts as a hormone involved in the regulation of carbohydrate metabolism. The mature 37-residue Amylin polypeptide has a Cys-2 to Cys-7 disulfide bridge and an amidated C-terminus. Under normal conditions, Amylin is co-secreted with insulin into the circulation as a soluble monomer and excreted from the body by the kidney.[5,6] Amylin is the major protein component of amyloid plaque deposits in pancreatic islets of type II diabetic patients.[7] Synthetic amyloid fibrils are toxic to the insulin-producing β-cells. These findings indicate that Amylin fibril formation in the pancreas may contribute to islet cell dysfunction and death in type II diabetes mellitus.[8] The sequence of the 37-residue human Amylin and its highly amyloidogenic 8−37 fragment are shown in Figure 1.

There are no reports of the high level expression of human Amylin, and its hydrophobic sequence has proven to be very

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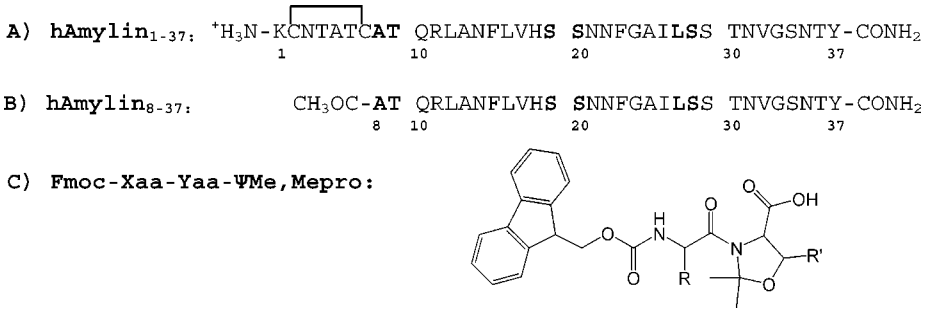

Figure 1. Primary sequence of human islet amyloid polypeptide. (A) Full length sequence (hAmylin$_{1-37}$) with Cys-2 to Cys-7 disulfide bridge and free N-terminus. (B) The 8−37 sequence (hAmylin$_{8-37}$) with acetylated N-terminus. All peptides have an amidated C-terminus. Residues are numbered according to their position in full length Amylin. Residues in bold indicate sites of Fmoc-Xaa-Yaa-$\Psi^{Me,Me}$pro substitution. (C) Molecular structure of Fmoc-pseudoproline (oxazolidine) dipeptide derivatives. **1**: Yaa. Ala: R = H, Xaa. Thr: R′ = CH_3. **2**: Yaa. Ser: R = H, Xaa. Ser: R′ = H. **3**: Yaa. Leu: R = isobutyl, Xaa. Ser: R′ = H.

difficult to synthesize by solid-phase peptide synthesis (SPPS). During SPPS, the excessive hydrophobicity of the growing protected polypeptide chain gives rise to low coupling yields and undesirable side products. Previously, we observed that synthesis of highly aggregation-prone fragments of Amylin by Fmoc-SPPS was easier when proline substitutions were incorporated. These observations encouraged us to explore new developments in Fmoc-SPPS. Recently, Hmb-protected amino acids and pseudoproline dipeptides have been proposed for the synthesis of difficult aggregation prone sequences.[9,10] Comparative studies indicate that pseudoprolines can allow for faster and more complete coupling.[11] These Fmoc-Xaa-Yaa-$\Psi^{Me,Me}$pro units are formed through a cyclocondensation reaction of Ser, Thr, or Cys residue side chains. This reaction links the side chains of these amino acids to their respective N-terminal peptide nitrogen atoms,[10] similar to that of the proline residue. As a result, the incorporation of pseudoproline (oxazolidine) dipeptides induces significant kinks in the backbone of the growing polymer-bound polypeptide chain and removes hydrogen bond donors, thus disrupting secondary structures such as β-sheets and preventing aggregation during chain assembly. Upon completion of the peptide synthesis, the native structure of the peptides is completely regenerated upon cleavage and deprotection using standard TFA methods. Here we test the utility of Fmoc-protected pseudoproline (oxazolidine) dipeptide derivatives (Fmoc-Xaa-Yaa-$\Psi^{Me,Me}$pro) in the synthesis of Amylin sequences. The molecular structure of Fmoc-Xaa-Yaa-$\Psi^{Me,Me}$pro is shown in Figure 1. We report the successful synthesis of the 37-residue human Amylin polypeptide (hAmylin$_{1-37}$) by Fmoc-SPPS, as well as the improved synthesis of its aggregation prone 8−37 fragment (hAmylin$_{8-37}$) using Fmoc-Xaa-Yaa-$\Psi^{Me,Me}$pro.

All peptides were synthesized using standard reaction cycles on an automated ABI 433A peptide synthesizer. Three different strategies were used for the synthesis of hAmylin$_{8-37}$:

(A) double coupling of β-branched residues and those directly following β-branched residues, using only Fmoc-protected amino acids; (B) double coupling of all residues using only Fmoc-protected amino acids; and (C) incorporation of three oxazolidine pseudoproline dipeptide derivatives, with double coupling of β-branched residues, pseudoproline dipeptide derivatives, and residues following either of these. Three pseudoproline dipeptide derivatives were chosen for these syntheses, since previous studies have demonstrated that Fmoc-Xaa-Yaa-$\Psi^{Me,Me}$pro units have only a local effect and that multiple use of these units leads to enhanced coupling yields throughout a difficult peptide synthesis.[10,12] The first derivative, Fmoc-Ala-Thr($\Psi^{Me\ Me}$pro)-OH **1**, was substituted for residues Ala-8 and Thr-9. This derivative could likely have been omitted since it represents the last two residues coupled to the growing chain. We ultimately wanted, however, to synthesize the full length hormone, and thus we incorporated this derivative in the 8−37 sequence to test if it would lead to any difficulties in coupling, which it did not. The second derivative, Fmoc-Ser-Ser($\Psi^{Me,Me}$pro)-OH **2**, was inserted in place of Ser-19 and Ser-20; and the third derivative, Fmoc-Leu-Ser($\Psi^{Me,Me}$pro)-OH **3**, replaced residues Leu-27 and Ser-28. These positions were chosen in accordance with published protocols, which suggest that the optimal placement of Fmoc-Xaa-Yaa-$\Psi^{Me,Me}$pro is before regions of hydrophobic residues, spaced at least five to six amino acids apart throughout the sequence.[10,13] The UV detector traces from the synthesizer (not shown) and the analytical HPLC traces of crude products (Figure 2) show that strategy C was the most successful for the assembly of hAmylin$_{8-37}$.

Strategy A failed, resulting in a crude analytical HPLC trace with multiple overlapping peaks. Characterization of these multiple products by MALDI-TOF MS revealed

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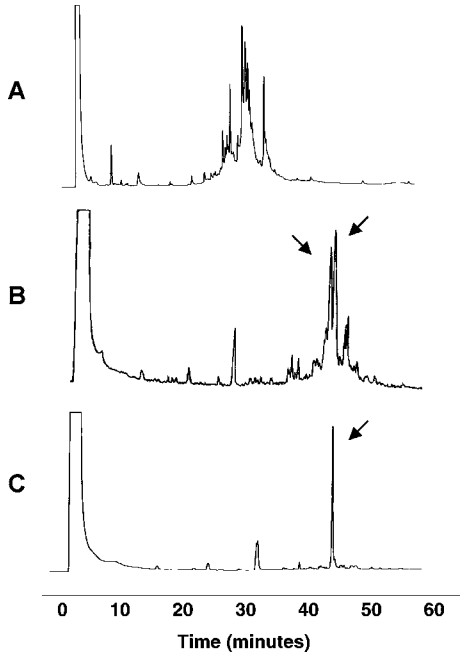

Figure 2. Reversed-phase HPLC traces of crude samples of hAmylin$_{8-37}$ obtained after synthesis using three different strategies: (A) ordinary double coupling of β-branched residues and those directly following β-branched residues, using only Fmoc-protected amino acids; (B) ordinary double coupling of all residues using only Fmoc-protected amino acids; and (C) double coupling strategy incorporating three oxazolidine pseudoproline dipeptide derivatives. All HPLC traces were run at a gradient of 0−90% buffer B in 90 min. Arrows indicate elution positions of desired peptide.

multiple fragments of the target peptide sequence eluting simultaneously. Separation of these deletion peptides was never completely achieved by HPLC. Strategy B was plagued by poor coupling as detected by the UV traces. This strategy produced the desired sequence, after two failed attempts. The analytical HPLC trace of the crude material indicated several overlapping peaks. Characterization of these peaks by MALDI-TOF MS revealed three main products: the first product was the correct peptide [m/z = 3226], the second corresponded to a deletion peptide with a mass 112 Da too low, and the third was a deletion peptide with a mass 114 Da too low. Furthermore, the correct peptide eluted at the same gradient positions as the two deletion peptides. The high level of impurities produced by strategy B also resulted in very poor solubility of the crude material, increasing the difficulty of purification by HPLC. Strategy C was the most successful in producing the desired peptide. The UV detector traces suggested efficient coupling. The analytical HPLC trace of the crude material revealed one major peak (Figure 3).

Characterization of this product by MALDI-TOF MS revealed the desired peptide [m/z = 3226]. The high purity of the product dramatically increased the solubility of the crude material, making purification by HPLC very simple and effective. We found that repeatedly dissolving the crude peptide in 20% acetic acid and lyophilizing significantly

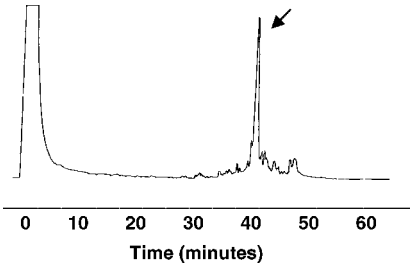

Figure 3. Reversed-phase HPLC trace of crude hAmylin$_{1-37}$ obtained after synthesis using the pseudoproline dipeptide strategy. The HPLC trace was run at a gradient of 0−90% buffer B in 90 min. Arrow indicates elution position of the desired peptide.

improved its solubility. The purity of the final product was estimated by analytical HPLC using two different buffer systems and was found to be greater than 90% pure after one HPLC step.

Encouraged by the results of the hAmylin$_{8-37}$ synthesis, we tested the pseudoproline dipeptide strategy on the full length hAmylin$_{1-37}$. The same conditions were employed, including the type, number and placement of Fmoc-Xaa-Yaa-$\Psi^{Me,Me}$pro units (Figure 1). Synthesis of hAmylin$_{1-37}$ using the pseudoproline dipeptide strategy produced the desired peptide on the first attempt. The UV detector traces suggested efficient coupling and no aggregation. The analytical HPLC trace of the crude material indicated one major peak (Figure 3). Characterization of this product by electrospray MS confirmed that it was the desired peptide [m/z = 3905]. The pseudoproline dipeptide strategy significantly increased the purity and solubility of the crude hAmylin$_{1-37}$ product, making purification by HPLC trouble-free.

The high purity of the hAmylin$_{1-37}$ crude product allowed the direct oxidation to the Cys-2 to Cys-7 disulfide without prior purification. To establish the initial oxidation state of the crude peptide, the HPLC retention time of a sample of crude material treated with DTT was compared to that of untreated crude peptide. The analytical HPLC trace of both samples displayed a major peak with the same retention time. Characterization of these products by electrospray MS revealed identical molecular weights for both samples [m/z = 3905], which correspond to reduced hAmylin$_{1-37}$. Thus as expected, the crude peptide was reduced. Formation of the intramolecular disulfide was achieved by air oxidation at pH 8.5. Aliquots from the mixture were monitored every few hours by analytical HPLC (Figure 4). Two major peaks appeared within 2 min of sample preparation. The first peak eluted at 27.5 min (a) and the second eluted at 32 min (b). The first peak had the retention time expected for the reduced peptide. This was confirmed by electrospray MS. Electrospray MS indicated that the second peak corresponded to the oxidized peptide. Full conversion to the oxidized state was achieved after 24 h.

The ability to effectively oxidize hAmylin$_{1-37}$ in the crude form further demonstrates the utility of the pseudoproline strategy and simplifies the synthesis by eliminating the need

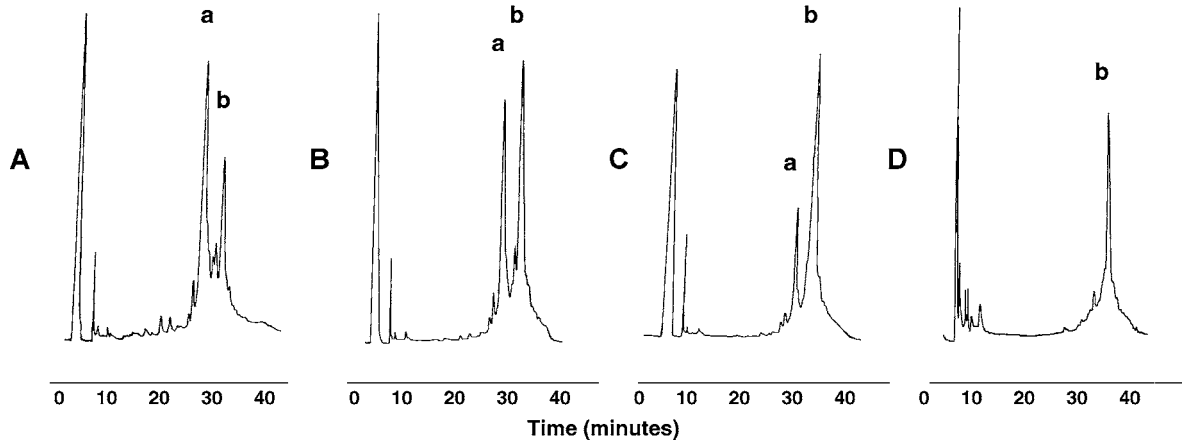

Figure 4. Reversed-phase HPLC traces of crude hAmylin$_{1-37}$ as a function of time during air oxidation in 43mm Tris 0.86M GuHCL after (A) 2 min, (B) 1 h, (C) 3 h and (D) 24 h. All HPLC traces were run at a gradient of 30−50% buffer B in 50 min. (a) Elution position of the reduced peptide. (b) Elution position of the oxidized peptide. HPLC traces are not normalized relative to each other.

to purify before oxidation. The oxidized material was purified by reversed-phase HPLC using the same gradient. The purity of the final product was tested by analytical HPLC using two different solvent systems and was found to be greater than 90% pure after a single HPLC step.

Using the pseudoproline dipeptide strategy, we have successfully synthesized the difficult full length hAmylin$_{1-37}$ sequence, and the highly aggregation prone 8−37 fragment. The crude products were more soluble than material obtained with conventional approaches, facilitating HPLC purification. Crude material could be oxidized to form the intramolecular Cys-2 to Cys-7 disulfide without prior purification. Fmoc based chemistry is widely employed and the necessary pseudoproline derivatives are commercially available, thus this strategy should prove generally useful to workers interested in producing Amylin and Amylin analogs.

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Supporting Information Available: Experimental details, abbreviations, and analytical HPLC traces of pure final products using two different buffer systems. This material is available free of charge via the Internet at http://pubs.acs.org.

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