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Efficient Microwave-Assisted Synthesis of Human Islet Amyloid Polypeptide Designed to Facilitate the Specific Incorporation of Labeled Amino Acids

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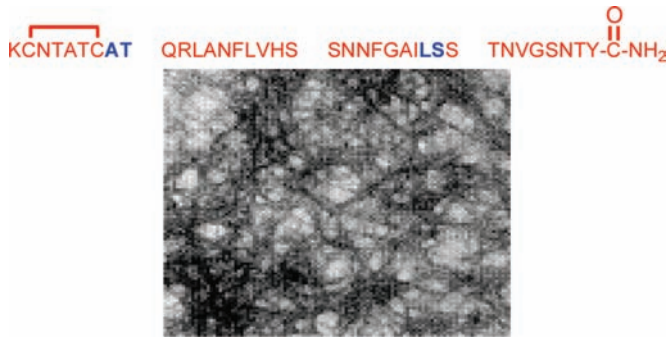
ABSTRACT



A cost-efficient, time-reducing solid-phase synthesis of the amyloidogenic, 37 residue islet amyloid polypeptide (IAPP) is developed using two pseudoprolines (highlighted blue in sequence) in combination with microwave technology. A yield twice that obtained with conventional syntheses is realized. The utility of this protocol is demonstrated by the synthesis of a $^{13}C^{18}O$-labeled Ser-20 IAPP variant, a prohibitively expensive and chemically challenging site to label via other protocols. TEM analysis shows the peptide forms normal amyloid (abstract image).

Human islet amyloid polypeptide (IAPP or amylin) is a highly amyloidogenic 37 residue peptide that is stored with insulin and cosecreted from the β-cells of the pancreas.[1] The bioactive form contains an amidated C-terminus and a disulfide bond between two cysteine residues located at positions 2 and 7. IAPP forms amyloid deposits in the islets of the pancreas during type 2 diabetes, a process that is thought to contribute to the decline in β-cell mass observed

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in type 2 diabetes.[1a,b,2] Islet amyloid formation has been implicated as a potentially complicating factor in islet cell transplantation.[3] These considerations have led to broad interest in the polypeptide. Biophysical and biochemical investigations of amyloid formation by IAPP are active areas of research, but the peptide is very difficult to synthesize and expresses poorly. Thus, there is interest in improving methods for the preparation of IAPP. Of particular importance are effective and economical protocols that are compatible with the incorporation of labeled amino acids and nongenetically coded amino acids for IR, fluorescence, and FRET studies. Such derivatives have proved very useful in a range of biophysical and biochemical studies; however, the required amino acids can be expensive or difficult to prepare, and it is essential that effective solid-phase peptide synthesis protocols be utilized for their incorporation. Exploiting recent advancements in microwave solid-phase peptide synthesis and utilizing pseudoproline dipeptide derivatives, we demonstrate a cost-effective, rapid synthesis of IAPP that doubles the yield obtained from conventional synthesis in under half the time and cost.

The hydrophobicity of IAPP, combined with a significant number of β-branched amino acids, leads to difficulties in the solid-phase peptide synthesis (SPPS) of the peptide. IAPP has been successfully synthesized via Fmoc chemistry by the incorporation of three pseudoproline dipeptide derivatives, together with the double coupling of 20 amino acids.[4] Pseudoprolines induce significant kinks within the backbone of the growing chain, much like proline, remove hydrogen bond donors, and disrupt secondary structure, thus aiding in the prevention of aggregation. Standard TFA cleavage procedures regenerate the native structure of the peptide.[5] The use of pseudoproline dipeptides and double coupling of the pseudoprolines, β-branched residues, and the residues immediately following the pseudoprolines and β-branched residues leads to a synthesis scheme which we have found typically generates 40−50 mg of pure peptide from a 0.25 mmol scale synthesis using PAL-PEG-PS resin.[4] Kelly and co-workers modified this procedure to significantly reduce the number of double couplings and obtained yields on the order of 20 mg of pure peptide from a 0.1 mmol scale synthesis.[6] Both of these methods used pseudoprolines at positions 8−9, 19−20, and 27−28.

Advances in microwave technology coupled with solid-phase peptide synthesis have led to reports of syntheses of difficult, hydrophobic peptides at greater yields than reported using conventional synthesis.[7] Microwave energy allows for rapid heating at the molecular level, driving the coupling and deprotection reaction rates forward while reducing aggregation. A solid-phase microwave-assisted synthesis of IAPP on the 0.1 mmol scale has recently been reported which avoided the use of pseudoproline derivatives.[8] However, the protocol involved double coupling residues 1 to 17 and 24 to 37 while triple coupling residues 18 through 23. In all, 80 couplings were required. Furthermore, 10 equiv of amino acid was used at 14 positions including all of the residues that were triple coupled. Thus, while the approach avoids the use of pseudoprolines, it consumes large amounts of amino acids and solvents, is time-consuming, and can be prohibitively expensive to incorporate costly labeled or noncanonical amino acids.

Here we demonstrate a much more rapid and cost-effective synthetic strategy by combining a modified pseudoproline approach with microwave heating. Only two pseudoprolines were required, together with thirteen double couplings. Only 5 equiv of Fmoc amino acids was used for each step, leading to a considerable savings in time and cost. The utility of the method is demonstrated by preparing normal human IAPP and by the incorporation of $^{13}C^{18}O$-labeled Ser at position-20, a region of the chain which is prone to low coupling efficiencies. The ability to label this segment of the polypeptide chain is important because it has been proposed to be a critical initiation site for amyloid fiber growth.[9] Incorporation of labeled residues into this region will provide the spectroscopic markers required to test this conjecture. Unfortunately, the introduction of labeled residues at this site is prohibitively expensive using existing protocols since it would require either the preparation and double coupling of a labeled pseudoproline or the use of a triple coupling with 10 equiv of amino acid per coupling. The approach described here incorporates the label using a single coupling with 5 equiv of amino acid.

IAPP was synthesized with a CEM Liberty 12-Channel Automated Peptide Synthesizer using standard reaction cycles. The sequence of IAPP and the coupling scheme is shown in Figure 1. Use of a PAL-PEG-PS resin afforded an amidated C-terminus, and HBTU was used as the coupling

Figure 1. Primary sequence of IAPP, illustrating the coupling scheme used for the microwave solid-phase peptide synthesis. Residues that were double coupled are underlined. The pseudoproline residues are colored blue, and all other double coupled residues are underlined.

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agent. Two pseudoproline dipeptides were chosen for the synthesis: Fmoc-Ala-Thr($\Psi^{Me,Me}$Pro)-OH replaced residues Ala-8 and Thr-9, and Fmoc-Leu-Ser($\Psi^{Me,Me}$Pro)-OH replaced residues Leu-27 and Ser-28. A preliminary synthesis involving single coupling of all residues and no pseudoprolines was attempted but lead to a large mixture of deletion peptides. Double couplings were performed for the pseudoprolines, for the residues following each pseudoproline, for Arg-11, and for every β-branched residue except for Thr-36 (Figure 1). In total, 11 residues in addition to the two pseudoprolines were double coupled, and 5 equiv of amino acid was used for all couplings. The microwave power settings and conditions for the deprotection and coupling steps are listed in Table 1 (see also Supporting Information).

Table 1. Microwave Settings and Times for the Coupling and Deprotection Steps

	step	power (W)	temperature (°C)	reaction time (s)
single couple	(1)	35	75	300
double couple	(1)	35	75	300
	(2)	35	75	300
Cys and His couple	(1)	0	50	120
		15	50	240
Arg couple	(1)	0	75	1,500
		15	75	300
	(2)	35	75	300
deprotection	(1)	40	75	30
	(2)	40	75	180

Cys and His residues are known to be susceptible to racemization if coupling reactions are conducted at elevated temperatures. A maximum temperature of 50 °C was set for the His and Cys couplings to reduce the possibility of racemization. The synthesis was complete in less than 24 h.

The peptide was cleaved from the resin and deprotected using standard TFA procedures with 1,2-ethanedithiol, anisole, and thioanisole as scavengers. The crude, reduced peptide was analyzed using analytical HPLC and MALDI-TOF mass spectroscopy (Figure 2). The analytical HPLC trace revealed a major peak centered at approximately 45 min, corresponding to a 75% purity as judged by the integrated area of the peaks detected at 220 nm. The mass spectrum revealed an intense peak at 3904.9 Da which corresponds to the expected *m*/*z* for reduced IAPP (3905.3 Da). The mass spectrum also revealed three deletion peptides of masses 1481.7, 1743.0, and 2982.9 Da.

The disulfide bond between Cys-2 and Cys-7 was formed by dissolving the crude peptide in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/mL and allowing it to oxidize as previously reported.[10] We feel this is the safest method to use as on-resin cyclization, or the use of powerful oxidants can generate unwanted byproduct, over oxidation of the cysteine to sulfonic acid, modification of the C-terminal Tyr, or, depending on the conditions, aggregation of the peptide. HCl was used as the counterion instead of the more commonly employed TFA as TFA can interfere with infrared studies. TFA-based HPLC buffers usually provide higher-resolution separations than buffers which use HCl as the counterion. The effective purification achieved here with the HCl system reflects the high efficiency of the synthetic protocol, which leads to crude material that is already relatively pure. Pure, oxidized IAPP was generated at a nearly 15% yield (~95 mg) based on the weight of the crude material. The yield calculated based on the mass of the resin used was approximately 10%. This yield is higher than reported for the alternative 0.1 mmol microwave protocol despite requiring no triple couplings, far fewer double couplings, and avoiding the use of 10 equiv of amino acid. The levels of racemization were 0.95% and 1.36% D-enantiomer for the His and Cys residues, respectively, measured via GC-MS (Supporting Information). The value for Cys reflects the total amount detected per IAPP molecule. These levels are below those reported for other peptides synthesized by microwave synthesis.[7a]

IAPP synthesized by this approach behaved similarly to IAPP synthesized with other protocols, forming amyloid on the same time scale and forming fibrils of identical morphology. Figure 4 displays a thioflavin-T fluorescence kinetic experiment conducted with the microwave synthesized peptide. The time course is identical to that observed previously, and the transmission electron microscopy (TEM)

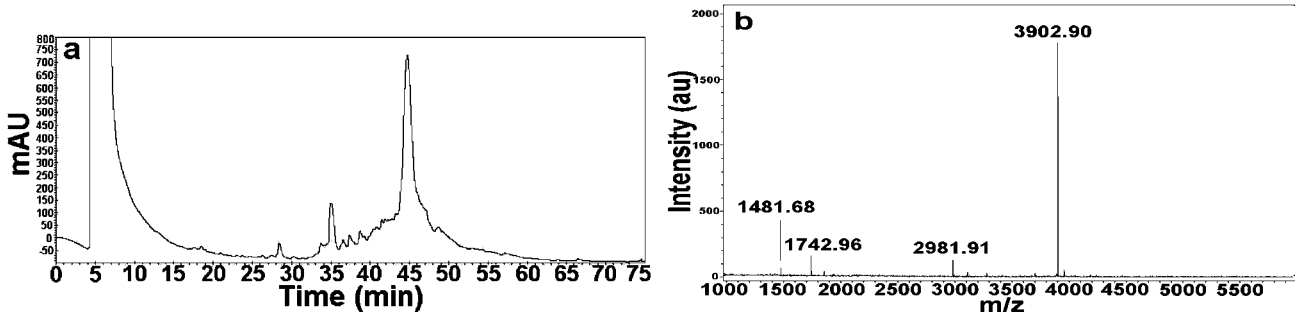

Figure 2. (a) Reversed-phase analytical HPLC trace and (b) MALDI spectrum of crude, reduced IAPP. The HPLC was run on a C18 Vydac analytical column at a flow rate of 1 mL/min with a gradient of 0−90% buffer B (80% acetonitrile, 0.045% HCl) over 90 min.

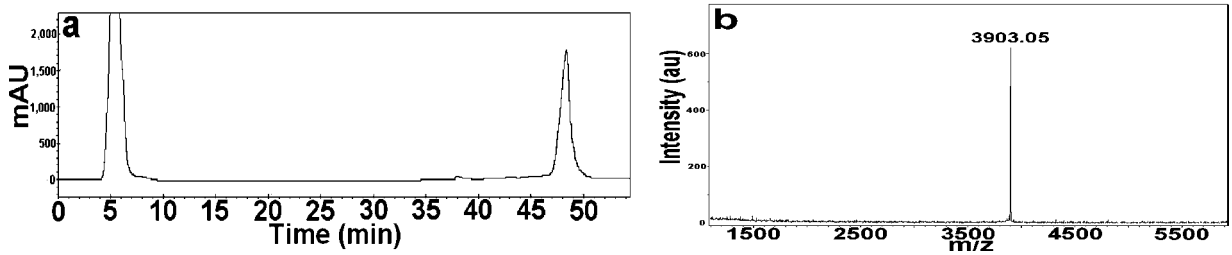

Figure 3. (a) Reversed-phase analytical HPLC trace and (b) MALDI mass spectrum of pure, oxidized IAPP. The HPLC was run on a C18 Vydac analytical column at a gradient of 0−90% buffer-B (80% acetonitrile, 0.045% HCl) over 90 min. The pure peptide elutes at 48 min, corresponding to 48% buffer-B.

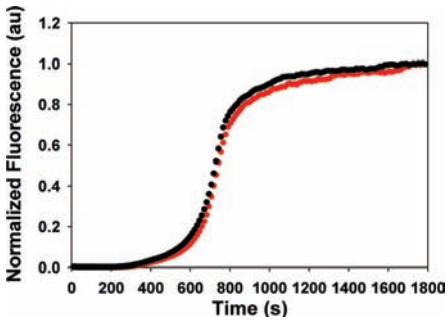

Figure 4. Thioflavin-T monitored kinetics of IAPP synthesized by microwave (red) and conventional (black) synthesis.

images of the final reaction products reveal dense mats of fibrils with the standard morphology (see abstract image).[11]

We also prepared a variant of IAPP labeled at Ser-20 with $^{13}C^{18}O$. This site has been implicated as part of a critical initiation site for fiber growth.[9] The ability to label at or near this site is important for future spectroscopic studies, but would be prohibitively expensive using either our original method, which required a pseudoproline at positions 19−20, or using the previously reported microwave-assisted protocol which requires triple coupling with 10 equiv per coupling. Fmoc and *t*-butyl protected 1-$^{13}C^{18}O$ backbone labeled Ser was prepared adopting the approach of Seyfried and co-workers (Supporting Information).[12] $^{13}C^{18}O$-Ser IAPP was synthesized using the same protocol as employed for the unlabeled peptide, but using a 0.1 mmol scale. Approximately 50 mg of pure material was obtained, a 13% yield based on the amount of resin used (Supporting Information).

In summary, a protocol for the synthesis of IAPP with a yield comparable to or better than other strategies, but at a considerably reduced time and cost, has been developed, by combining microwave-assisted methods with the incorporation of only two strategically placed pseudoproline dipeptide derivatives. The protocol allows the cost-effective incorporation of labeled residues into critical regions of the polypeptide chain. The use of this approach is applicable to other variants of IAPP and to other hydrophobic and aggregation-prone peptides.

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Supporting Information Available: Description of the microwave temperature control and experimental methods for the preparation of 1-$^{13}C^{18}O$ Fmoc-*O*-*tert*-butyl-L-Ser. This material is available free of charge via the Internet at http://pubs.acs.org.

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