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An Improved Synthesis of Pentapeptide (SCAP1e) Using Solid-Phase Method with Reduced Resin Loading Time

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Abstract

SCAP1e is an alanine-rich-pentapeptide, analog of SCAP1 an antioxidant peptide. The previous solid-phase synthesis on this peptide resulted in low yield of the product (7%) due to the aggregation that might occur during synthesis. The aim of the research was to resynthesize this peptide using the similar method but with a slight modification in the resin loading step. SCAP1e was successfully synthesized through solid-phase method with an improved yield 87.9% and high purity (90.2%) based on the HPLC analysis. The reduction of resin loading value from 1.2 mmol/gram to 0.63 mmol/gram has made a good impact for the improvement of the synthetic yield.

Keywords: Solid-phase peptide synthesis; resin loading; aggregation; SCAP1; SCAP1e.

1. Introduction

Solid-phase peptide synthesis (SPPS), introduced by Merrifield in 1960s, is an effective and efficient method to prepare peptides [1][2]. SPPS can be defined as a process in which amino acid bound by its C-terminus to an insoluble polymer. Elongation of the peptide takes place on the insoluble polymer. This method has made peptide synthesis simple, rapid, and easily subject to automation. However, the method is not without problems. Aggregation is one of the issues potentially emerged during the synthesis [3]. An inter- or intra-molecular β-sheet interactions is the cause of the aggregation.

Alanine-rich peptides were known to be prone of aggregation [4]. Most of the time, aggregation happens in the synthesis, resulting in low yield of the synthetic peptides. Antioxidant pentapeptide SCAP1 (Leu-Ala-Asn-Ala-Lys) isolated by Umayaparvathi et al. (2018) from the hydrolysate of oyster, has two alanines in the structure [5]. The synthetic effort of SCAP1has been made by Sabana et al. (2020) using solid-phase method with Fmoc strategy obtaining the synthetic SCAP1 with 8.28 % yield [6]. The low yields of the products were also seen in the synthesis of SCAP1 analogues SCAP1e (Leu-Ala-Tyr-Ala-Lys) and SCAPf (Leu-Ala-Trp-Ala-Lys) with the synthetic yields of subsequent 7% and 10% [7]. The reasons for the low yield of the synthetic product were described by Sabana et al. (2020) and Maharani et al. (2020) due to the presence of two alanines, the selection of incorrect protecting group of asparagine, and the higher resin loading value [6][7]. Trityl-protected Asn was found to contribute highly in the process of aggregation than the unprotected Asn [8]. These three factors were thought to be the major cause of aggregation that eventually resulted in low yield of

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final product. In Fmoc-based solid-phase synthesis, one of the main issues during elongation of the peptide is aggregation. According to Paradís-Bas (2015), aggregation can be caused by several factors, including the nature of the amino acid's side chain, the amino acid side chain protection group, the Nα-substituent in the amide bond and the type of solvent [9]. Chan and White (2000) mention that the presence of highly Ala, Val, Ile, Asn, Gln in the peptide structure increase the potency of aggregation [4]. Aggregation on the peptidic resin is the reason of incomplete deprotection and coupling [10]. A huge steric hindrance makes penetration of reagents during deprotection and coupling step reduced and makes the peptides contaminated by peptides with similar structures. A common main resistance of difficult peptides and also the sequence of aggregates in solution is their solubility. The peptide sequence and most importantly, the amino acid composition, plays a key role in terms of secondary structure, thus directly influencing molecular solubility [9]. RP-HPLC of insoluble peptides is a major problem resulting in low peptide loading into the column, poor resolution and low yield [11]. In addition, hydrophobic and aggregation-prone peptides are very difficult to characterize by mass spectrometry because of their poor ionization [12].

The reduction of resin loading time at the initial stage of solid-phase synthesis has been suggested to minimize potency of aggregation. In the synthesis of SCAP1e, the resin loading value obtained was 1.2 mmol/g and described as good because Chan and White (2000) explained that a good loading resin value is in between 0.20-1.30 mmol/g [4]. The aim of the research is to improve the yield of the synthesis of SCAP1e by decreasing the time of resin loading.

The paper describes an improved synthesis of SCAP1e using solid-phase method.

2. Experimental

2.1. *Materials and Instruments*

All the compounds were analyzed by analytical RP-HPLC Waters 2998 Photodiode Array Detector, LiChrospher 100 RP-18 5 µm column. 1H- and 13C-NMR spectra were recorded on Agilent NMR 500 MHz (1H) and 125 MHz (13C) using deuterated solvent. Mass spectrometry spectra were recorded on Waters HR-TOF-MS Lockspray. Loading resin absorbance was measured on TECAN Infinite pro 200 UV-Vis Spectrophotometer.

All amino acids, Fmoc-L-Leu-OH, Fmoc-L-Ala-OH, Fmoc-L-Lys(Boc)-OH, Fmoc-L-Tyr(t-Bu)-OH, coupling reagents 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazole (HOAt), and 2-chlorotrityl chloride resin (0.972 mmol/g) were purchased from GL-Biochem Ltd., China. Dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), piperidine, *N*,*N*-diisopropylethyl amine (DIPEA), acetaldehyde, p-chloranil, and trifluoroacetic acid used in the synthesis were in analytical grade.

2.2. *Methods*

The procedure of synthesis is similar to the protocol applied for the synthesis of SCAP1e reported by Maharani et al. (2020) [7]. However, the resin loading time was reduced from overnight to four hours.

2.2.1. Resin loading

To the 2-chlorotrityl resin (0.4 g, 0.45 mmol) was added DCM (10 mL) and a solution of Fmoc-L-Lys(Boc)-OH (0.67 mmol) that was previously treated with dry dichloromethane (10 mL) and DIPEA (1.20 mmol). The mixture was shaken for 4 h.

To calculate resin loading value, 1 mg of resin was placed in the effendorf tube and then added by 300 µL of 25% of piperidine in DMF and leaved for 1 h. Then, the mixture was added by 2700 µL of 25% of piperidine in DMF and centrifuged for 5 minutes. The absorbance of the solution was then measured using UV spectrometer at wavelength of 290 nm. The resin loading value was calculated based on the formula of:

$$Resin\ loading = \frac{(A290\ nm - Aref) \times V}{5800 \times 0.7 \times 10^{-3}}$$

Methanol: DCM:DIPEA (15:80:5) (5 mL) was then added and the mixture was shaken for 10 min. The latter step was undertaken in two cycles. The resin was then filtered and washed successively with dichloromethane, dimethylformamide and dichloromethane. The resin was dried in vacuo for 30 min to obtain dry resin-Lys(Boc)-NHFmoc.

2.2.2. Fmoc deprotection

Resin-Lys(Boc)-NHFmoc was shaken with 20% piperidine in DMF (10 mL) for 30 min to give resin-Lys(Boc)-NH_2. Then, the resin was filtered and washed with dichloromethane, DMF and dichloromethane, successively. The exposed primary amine was tested by the addition of a chloranil test solution into the resin beads.

2.2.3. HATU/HOAt-mediated coupling

To the dry resin-Lys(Boc)-NH_2 (0,63 mmol) having been swollen by dichloromethane:DMF (1:1, 5 mL) was added Fmoc-Ala-OH (2 eq.) that was previously treated with HATU (2 eq.), HOAt (2 eq.), and DIPEA (8 eq.) in DCM:DMF (1:1) to give resin-Lys(Boc)-Leu-NHFmoc. The resin was shaken for 24 h. The resin was then filtered and washed with DCM, DMF and DCM, successively. To a small portion of resin beads was added a chloranil test solution for the reaction confirmation.

2.2.4. Elongation of peptide

Elongation of the peptide was carried out through a repetitive protocol of Fmoc deprotection and HATU/HOAt coupling of subsequent Fmoc-Tyr(tBu)-OH, Fmoc-Ala-OH, and Fmoc-Leu-OH to give resin-Lys(Boc)-Ala-Tyr(tBu)-Ala-Leu-NH_2.

Cleavage of linear pentapeptide from chlorotrityl resin

To the resin-Lys(Boc)-Ala-Tyr(tBu)-Ala-Leu-NH_2 was added a cleavage cocktail of 95% of trifluoroacetic acid in water. The yellow resin turned bright red. It was shaken for 1 h and then filtered. The resin was washed with further cleavage cocktail (5 mL x 2) and dry DMF (5 mL x 2). The combined organic layers were evaporated and the resulting residue was analysed by analytical RP-HPLC. The product was analysed by HR-TOF-MS and NMR.

3. Result and Discussions

SCAP1e was successfully synthesized with an improved yield. The synthesis was carried out on 2-chlorotrityl chloride resin with standard Fmoc strategy. Fmoc-L-Lys(Boc)-OH was attached on the resin, following a simple SN1 reaction. The reaction was shaken for four hours resulting in resin loading value of 0.63 mmol/g with a sharp reduction in the loading value compared with the previously reported synthesis (1.2 mmol/g). The following step was to cap the unreacted resin that does not bind the first amino acid with a cocktail of dichloromethane: methanol: DIPEA. The capping process can prevent attachment of the next amino acid to the active site of the resin. Deprotection of the Fmoc group using 20% piperidine in DMF was then undertaken. The Fmoc group elimination is initiated by the abstraction of hydrogen atom at the fluorene group with the basic piperidine, resulting in cyclopentadiene intermediate [4]. The reaction resulted in the free amino group of the lysin residue that was ready for coupling with the second amino acid Fmoc-L-Ala-OH. HATU/HOAt was employed to activate the carboxylic acid group of the Fmoc-amino acid before coupled to the amino group of the lysin residue on the resin. The active ester and the resin-Lys-NH_2 were condensed to form amide bond. The elongation step involved a repetitive protocol of Fmoc deprotection and coupling reaction to subsequent Fmoc-L-Tyr(t-Bu)-OH, Fmoc-L-Ala-OH, and Fmoc-L-Leu-OH to result in resin-Lys(Boc)-Ala-Tyr(tBu)-Ala-Leu-OH. The pentapeptide was then released from the resin using 95% TFA in water. Water is required to scavenge carbocations eliminated during the step. The route of the synthesis can be seen at Scheme 1.

Crude pentapeptide was analysed using analytical RP-HPLC with eluent acetonitrile:water (0% - 100%), flow rate 1 ml/minutes for 30 minutes. The chromatogram of the analytical RP-HPLC showed the high purity of the synthetic product (90.2%) (Figure 1) with 87.9% yield. The significant increase of the yield of the synthesis was explained due to the half reduction of the resin loading value from the previous 1.2 mmol/gram to 0.6 mmol/gram. The later value is very effective to diminish the potency of aggregation during the synthesis. Sletten et al. (2019) mentions that in addition to using a more polar solvent, lowering the value of loading resin to spread out the elongation peptides is considered effective to inhibit aggregation [13]. The first synthesis of SCAP1e by Maharani et al. (2020) required multiple purification of the crude to finally obtained the peptide in 7% yield [7]. Aggregation has made the purification step even more difficult because the impurities consists of other peptides with similar properties.

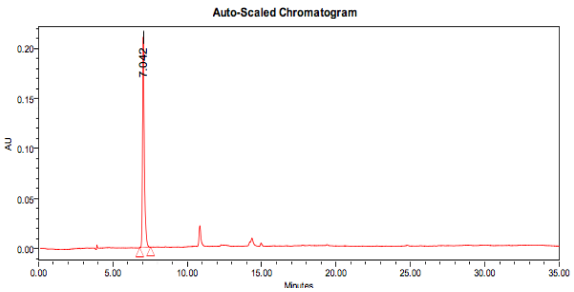

Fig. 1. Chromatogram of analytical RP-HPLC of SCAP1e.

Without any purification, the crude pentapeptide was directly characterized by using HR-TOF-MS and NMR. The mass spectrum showed a correct molecular ion peak of $[M+H]^+$ *m/z* 565.3358 (calculated *m/z* 565.3358 for $C_{27}H_{45}N_6O_7$).

1D (^{1}H-, ^{13}C-NMR, DEPT) and 2D (HSQC dan HMBC) NMR were applied to confirm the consistency of NMR data with the structure. The NMR data showed that SCAP1e has been successfully synthesized. HMBC correlations (Figure 2) between δ_H 0.97 ppm and δ_C 17.9 ppm dan 41.6 ppm, δ_H 1.36 ppm and δ_C 50.4 ppm and 174.2 ppm, δ_H 2.90 ppm and δ_C 128.7 ppm and 130.0 ppm, δ_H 3.05 ppm and δ_C 128.7 ppm and 130.0 ppm, δ_H 4.34 ppm and δ_C 17.9 ppm and 174.2 ppm, δ_H 6.68 ppm and δ_C 116.5 ppm, 128.7 ppm, and 157.6 ppm, and δ_H 7.07 ppm and δ_C 130.0 ppm and 157.6 ppm strongly confirmed the position of the residue in the structure.

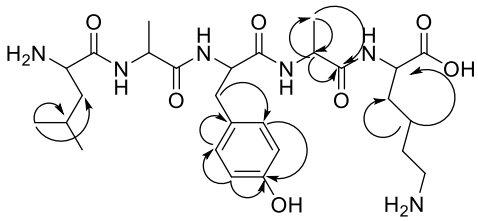

Fig. 2. HMBC correlation of SCAP1e.

The analysis result of the obtained NMR data was presented in Table 1.

4. Conclusion

SCAP1e has successfully synthesized through Fmoc-based synthesis on 2-chlorotrytil resin. The reduction in the resin loading value has significantly increase the yield from 7% to 87.9 %. The synthetic SCAP1e was obtained in more than 90% purity.

5. Conflicts of interest

There is no conflict of interest.

6. Funding sources

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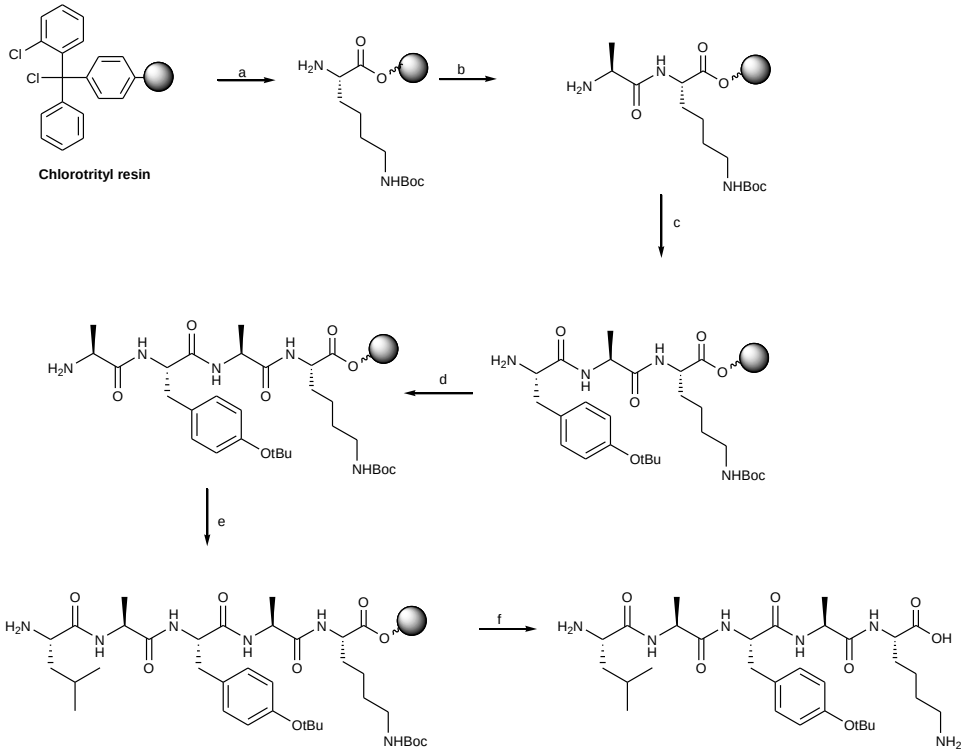

Scheme 1 Route of synthesis of SCAP1e. (a) (1) Fmoc-L-Lys(Boc))-OH, DIPEA, dichloromethane, 4h, rt, (2) dichloromethane/MeOH/DIPEA (8:1.5:0.5), (3) 20% piperidine in DMF, (b) Fmoc-L-Ala-OH, HOAt, HATU, DIPEA, dichloromethane: DMF (1:1), 4 h, rt (2) 20% piperidine in DMF, (c) (1)Fmoc-L-Tyr(OtBu)-OH, HOAt, HATU, DIPEA, dichloromethane: DMF (1:1), 4 h, rt, (2) 20% piperidine in DMF, (d) Fmoc-L-Ala-OH, HOAt, HATU, DIPEA, dichloromethane: DMF (1:1), 4 h, rt, (2) 20% piperidine in DMF, (e) Fmoc-L-Leu-OH, HOAt, HATU, DIPEA, dichloromethane: DMF (1:1), 4 h, rt, (2) 20% piperidine in DMF, (f) 95% TFA in water

Table 1: ^{1}H- and ^{13}C-NMR data of SCAP1e

Residue	^{1}H -NMR (δ_H in ppm)	^{13}C (δ_C in ppm)
Lysine		
CO		174,6
α-CH	4,31 – 4,39	50,6
β-CH_2	1,47 – 1,50	23,7
γ-CH_2	1,47 – 1,50	27,9
δ-CH_2	1,86 – 1,89	32,2
ω-CH_2	2,85 – 2,94	40,5
NH	8,30	
Two Alanines		
CO		174,2
α-CH	4,31 – 4,39	50,4
	4,31 – 4,39	50,6
β-CH_2	1,33	17,9
	1,36	18,6
NH	8,60	
Tyrosine		
CO		173,2
α-CH	4,48 – 4,50	56,3
β-CH_2	2,85 – 2,94	37,7
γ-CH_2	3,05 – 3,08	128,7
δ-CH_2	6,68	114,6
ω-CH_2	7,07	129,7
NH	8,10	
Leucine		
CO		170,5
α-CH	3,87	52,8
β-CH	1,64 – 1,72	41,6
γ-CH_2	1,64 – 1,72	25,4
δ-CH_3	0,96 - 0,99	21,9
δ'-CH_3	0,96 - 0,99	23,1
NH		

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