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Facile Synthetic Route to Ascorbic Acid-Dipeptide Conjugate *via* *N*-Terminal Activation of Peptide on Resin Support

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A solid-phase synthetic approach is reported for the synthesis of an ascorbic acid (ASA)-dipeptide conjugate that exhibited enhanced antioxidant activity. The *N*-terminal amino group of dipeptide (Ala-Ala) on a resin support was first activated by 1,1'-carbonyldiimidazole (CDI), and then reacted with an ASA derivative. The addition of a base, triethylamine (TEA), promoted nucleophilic acylation of ASA derivative and yielded a desired product (ASA-Ala-Ala) with enhanced purity, when cleaved from the resin. Compared to the approach where a C3 hydroxyl group of ASA was first activated with CDI and then reacted with the amino group of dipeptide on the resin, this new approach allowed a significant reduction of a total reaction time from 120 h to 8 h at 25 °C. As-prepared ASA-dipeptide conjugate (ASA-Ala-Ala) showed improved antioxidant activity compared to ASA.

Key Words : Carbamate formation, Ascorbic acid, Peptide conjugate, Antioxidant, Solid-phase synthesis

Introduction

Ascorbic acid (ASA), also known as vitamin C, has beneficial functions in various biological processes such as collagen synthesis,[1] antioxidative,[2] antiviral,[3] and antibacterial activities.[4] In particular, the strong antioxidant activity of ASA has drawn significant interests from the pharmaceutical and cosmetic industries as a potent anti-aging agent. However, ASA is highly unstable and degraded easily by exposure to moisture, light, and oxidative or high-temperature conditions, which results in the loss of its inherent, valuable biological activities. Furthermore, due to its super-hydrophilic property, ASA is difficult to deliver through the lipophilic skin barrier or cell membranes.[5]

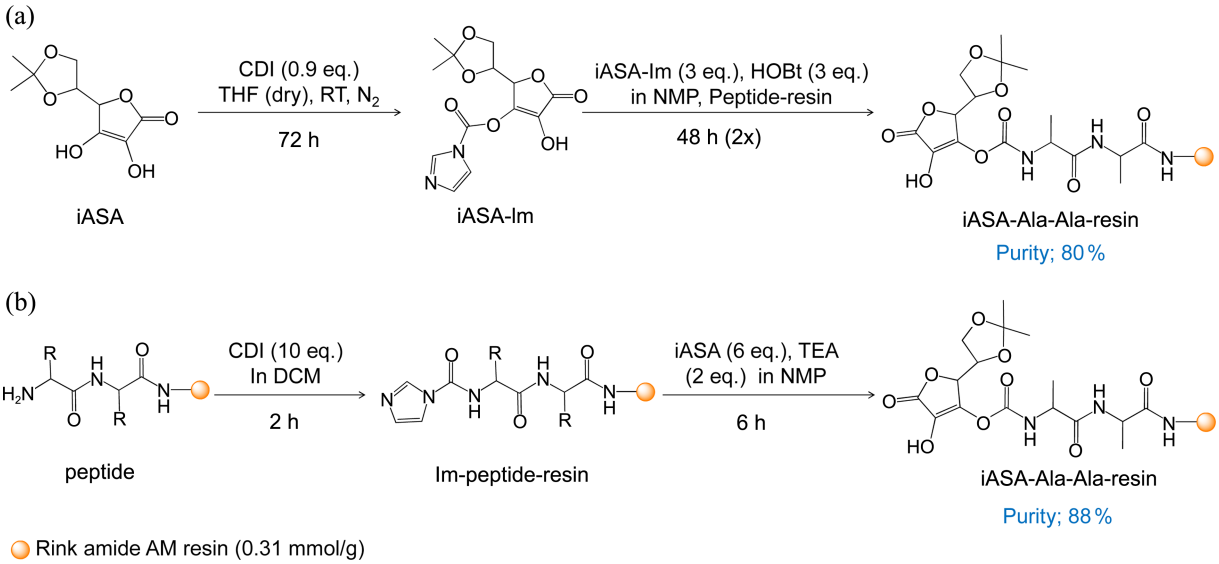
There have been numerous studies to improve the chemical and physical stability and to maintain or enhance biological activities of ASA, as well as its lipophilicity.[6-12] Among various approaches, peptide conjugation appears to be a promising strategy as it allows ASA various chemical properties of peptides with reduced safety concerns.[13] The peptide conjugation to ASA can also give rise to an enzymatic degradation by peptidase and subsequent on-site release of ASA as a pro-drug. For example, ASA-pentapeptide conjugate, which is derived from type I procollagen, has been reported to stimulate collagen biosynthesis.[14,15] In order to synthesize ASA-peptide conjugates with enhanced biological activities, multiple steps must be applied to introduce the peptide to a desired hydroxyl group of ASA, which involves several orthogonal protection and deprotection procedures.[14] It is often exacting to isolate the desired product from by-products and other reagents and, at the same time, to preserve the inherent biological activities of ASA. Therefore, it still remains as a challenge to develop efficient synthetic methodologies for ASA-peptide conjugates.

Herein, a new solid phase-based synthetic approach was developed for the synthesis of ASA-dipeptide conjugate with high purity with a substantially shortened reaction time. This involved an initial activation of a *N*-terminal amino group of dipeptide on a resin support using 1,1'-carbonyldiimidazole (CDI), followed by the coupling of the activated dipeptide with the C3 hydroxyl group of ASA in the presence of triethylamine (TEA) to yield a desired product. Enhanced antioxidant activity of as-prepared ASA-peptide conjugate was also demonstrated.

Experimental

Chemicals. Fmoc-Rink amide linker coupled aminomethyl polystyrene (Rink amide AM) resin (0.33 mmol/g), 4-hydroxymethyl-2(5*H*)-furanone twenty-millilitre filtered reactors (Libra tube RT-20M), benzotriazole-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP), hydroxybenzotriazole (HOBt), and Fmoc-L-Ala-OH were obtained from BeadTech (Seoul, Korea). Diisopropylethylamine (DIPEA) was bought from Alfa Aesar (Ward Hill, MA). Ascorbic acid (ASA), ninhydrin, linoleic acid (~99%), ammonium thiocyanate (NH_4SCN), ferrous chloride ($FeCl_2$) and polyoxyethylenesorbitan monolaurate (Tween 20) were purchased from Sigma-Aldrich (St. Louis, MO). *N*-Methyl-2-pyrrolidone (NMP), piperidine, dichloromethane (DCM), tetrahydrofuran (THF), diethyl ether, ethanol and methanol were bought from Dae-Jung Chemicals (Siheung, Korea). 1,1'-carbonyldiimidazole (CDI) was bought from Tokyo Chemical Industry Co. (Tokyo, Japan). Triethylamine (TEA) was purchased from Junsei Chemical Co. (Tokyo, Japan). Trifluoroacetic acid (TFA) was bought from Acros Organics (Morris Plains, NJ).

Apparatus. ASA-Ala-Ala was analyzed by liquid chromato-



Scheme 1. Comparison of two methods to synthesize ASA-Ala-Ala. (a) Previous synthetic method of ASA-Ala-Ala conjugate, in which iASA was first activated by CDI, and then the activated iASA was coupled to peptide on solid resin supports. (b) New synthetic method of ASA-Ala-Ala conjugate, in which a peptide on solid resin supports was first activated, and then coupled with iASA in the presence of a basic additive.

graphy-mass spectrometry (LC/MS Thermo Finnigan surveyor HPLC system, Thermo Finnigan LCQ deca XP plus, Thermo-Fisher, Waltham, MA), using Phenomenex Polar embedded C18 column (80 Å, 4 μm, 100 × 3 mm; Phenomex, Torrance, CA). The colour reaction of linoleic acid peroxidation test was followed by UV/Visible spectrophotometry (Optizen 2120 UV, Mecasys Co. Ltd., Daejeon, Korea).

Synthetic Procedure for ASA-Ala-Ala Conjugate.

Route A. Previously Reported Method: Firstly, 5,6-O-isopropylidene-L-ascorbic acid (iASA) was synthesized by Jung's method.[16] To activate C3 hydroxyl group of iASA, iASA was added to CDI (0.9 eq.) in THF solution and reacted for 72 h under N_2 environment. Ala-Ala was manually synthesized on Rink Amide AM resin (0.33 mmol/g) by solid-phase peptide synthesis method. The Fmoc group was removed with 20% piperidine/NMP for 30 min, and Fmoc-Ala-OH (2 eq.) was coupled to the resin with BOP (2 eq.), HOBt (2eq.), and DIPEA (4 eq.) for 1.5 h at room temperature. Fmoc deprotection, and Fmoc-Ala-OH coupling were repeated and each steps were monitored by Kaiser's ninhydrin color test. Next, iASA carbonylimidazolide (3 eq.) was coupled twice to Ala-Ala on resin support with HOBt (3 eq.) in NMP for 48 h. The product was separated from the resin by treating 50% TFA/DCM (v/v) for 1 h. The resin was filtered, the filtrate was concentrated in high vacuum, and precipitated with cold diethyl ether. The resulting ASA-Ala-Ala was confirmed by LC/MS using 0.1% formic acid/methanol as an eluent at a flow rate of 250 μL/min over 30 min and monitored at 230 nm.

Route B. New Synthetic Method: Ala-Ala on Rink Amide AM resin was synthesized as decribed in route A. For activation, CDI (10 eq.) was treated to the Ala-Ala anchored resin for 2 h. Activation of *N*-terminal amine of Ala-Ala, after cleavage from the resin, was confirmed by RP-HPLC using the following conditions: A to B (A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile; from 0% to 60% B over 30 min, at a flow rate of 1.0 mL/min); detection, UV 230 nm. Next, iASA was introduced to the activated Ala-Ala anchored resin with or without additives depending on the reaction condition. The final product was separated from the resin support and characterized as described in *route A*.

Determination of Antioxidant Activity.[17] Linoleic acid emulsion (50 mM) was prepared by mixing 0.284 g of linoleic acid, 0.284 g of Tween 20 and 50 mL of 0.1 M sodium phosphate buffer (pH 7.0). For the oxidation test, reaction mixture containing 0.5 mL of water, 2.5 mL of the linoleic acid emulsion, 2.0 mL of 0.1 M sodium phosphate buffer (pH 7.0) and 0.5 mL of methanolic test samples were prepared in a glass vial (10 mL-volume). The total reaction volume was fixed to 5.5 mL, and the final concentration of antioxidant was 450 μM. The glass vials containing reaction

Table 1. Effect of Reaction Time and Additives on the Purity of ASA-Ala-Ala

iASA (eq.)	Additives	Rxn Time (h)	Purity (%)[a]
10	None	6	45
		12	47
		24	42
	Acetic acid (0.1 eq.)	6	41
	HOBt (2 eq)		37
	TEA (3 eq.)		88
6	TEA (2 eq.)	6	87
		12	75
3	TEA (1 eq.)	6	53
		12	56

[a]These results were obtained by LC/MS analysis.

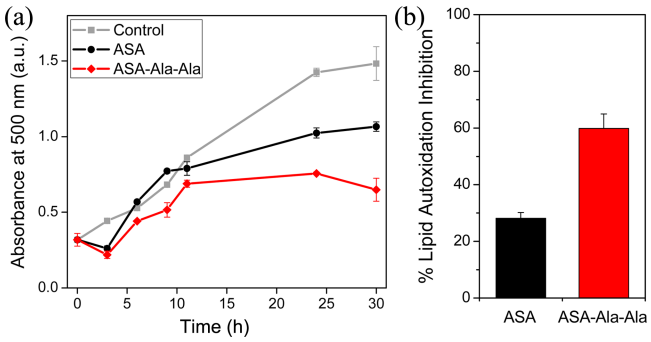


Figure 1. (a) Lipid peroxidation inhibitiory activity of ASA-Ala-Ala as a function of time and (b) Quantitative comparison of the activities between ASA and ASA-Ala-Ala after incubation for 30 h. The absorbance was determined at 500 nm by a ferric thiocyanate method. Conditions: lipid peroxidation proceeded in an emulsified system. The final concentration of antioxidant was 450 μM; the reaction was performed at 50 °C in a dark condition. As a negative control, methanol was added instead of antioxidant. Each experiment was performed in triplicate and repeated three times.

mixture were capped with rubber septum, and then kept at 50 °C under dark condition for 30 h. As a negative control, methanol was added instead of antioxidant. To evaluate antioxidant activity, we carried out a modified ferric thiocyanate method as follows. After a part of the reaction mixture was withdrawn at specific time intervals, the reaction mixture (25 μL) was mixed with 1.175 mL of 75% ethanol, 25 μL of 20 mM ferrous chloride in 3.5% HCl and 25 μL of 30% ammonium thiocyanate in an Eppendorf tube (1.5 mL-volume). After 3 min, the absorbance was measured at 500 nm, when the color developed by ferric ion and thiocyanate reached maximum. Each assay was carried out in triplicate and repeated three times. The absorbance of the control was repeated five times.

Results and Discussion

Synthesis of ASA-Ala-Ala based on Route A. We chose L-alanine as a model amino acid on the basis of its antioxidative activity in lipophilic condition,[18,19] structural simplicity, and easy coupling. From previous attempts, we found that a single amino acid conjugation to ASA was not able to enhance the stability of ASA and failed to preserve its practical biological activity. Consequently, dipeptide (Ala-Ala) was tried as a simple model peptide to improve both stability and activity of ASA. First, we coupled 1,1'-carbonyldiimidazole (CDI) activated ASA to Ala-Ala on a resin support to obtain ASA-Ala-Ala by the previously reported method, which has been well studied for the synthesis of kojic acid-peptide conjugates.[20-22] As shown in Scheme 1-a, ascorbic acid-carbonylimidazolide (iASA-Im), a C3 hydroxyl group activated form of ASA, was prepared initially with CDI and then reacted with Ala-Ala on the resin support prepared by a solid phase peptide synthesis method in the presence of 1-hydroxybenzotriazole (HOBt) to catalyze the coupling reaction. The first step for the synthesis of iASA-Im required a long reaction time (72 h, 25 °C) to obtain a moderate yield of 68%. The iASA-Im revealed not only poor stability during storage and the coupling reaction, but also showed low solubility in common reaction media of a solid-phase reaction such as DCM, NMP, and THF. Therefore, the second step for the coupling of iASA-Im to Ala-Ala on the resin support should be carried out at least twice to obtain the appropriate yield, which required a long reaction time (2 × 48 h) even with an addition of HOBt as a catalyst. ASA-Ala-Ala was obtained as yellowish powder by diethyl ether precipitation with 80% purity and characterized by LC/MS; calculated exact mass = 361.11 for $C_{13}H_{19}N_3O_9$ (ASA-Ala-Ala) $[M+H]^+$, found 362.3.

Synthesis of ASA-Ala-Ala based on Route B. As the poor stability and solubility of iASA-Im led to an extremely long reaction time, a small change was made in the reaction procedures for the synthesis of ASA-Ala-Ala on resin supports. As shown in Scheme 1-b, the *N*-terminal of Ala-Ala on resin supports was activated by CDI to give resin bound Ala-Ala-carbonylimidazolide. As shown in Scheme S1, this step was completed within 2 h, since the *N*-terminal amino group of peptide was more nucleophilic than the C3 hydroxyl group of ASA. The reaction time (72 h) was dramatically reduced compared to the activation time (2 h) of the C3 hydroxyl group of iASA by CDI in the previous method. 5,6-*O*-Isopropylidene ascorbic acid (iASA)[16] (10 eq.) was then added into the activated Ala-Ala on the resins to obtain desired ASA-Ala-Ala on the resin *via* nucleophilic acylation of the C3 hydroxyl group of ASA. The effect of the reaction time and several additives on the purity of the product (ASA-Ala-Ala) in the coupling step was investigated. As shown in Table 1, 6 h reaction without any additives gave 45% product purity. Longer reaction times (12 or 24 h) did not further improve the purity. Therefore, the reaction time was fixed as 6 h, and the effect of acidic and basic additives on the product purity was examined. Simple organic acids and bases were selected that could influence either the nucleophilicity of iASA or the activation of carbonylimidazole through protonation. The addition of acetic acid in the second step caused a slight decrease in the product purity (41%), compared to without additives. Initially, we expected that the acidic conditions could improve the purity of the product by increasing reactivity of carbonylimidazole through protonation. However, the nucleophilicity of the C3 hydroxyl group in iASA could be decreased in the acidic conditions, and thus, gave rise to a decrease in purity. As the acidic additive HOBt was added into the reaction media, the purity of the desired product also decreased. These results indicate that the increase in nucleophilicity of iASA is more crucial to improve the reaction yield and purity than the activation of carbonylimidazole. Hence, the second reaction step (iASA coupling) was carried out in the presence of a basic additive, triethylamine (TEA). As shown in Table 1, the purity of the product significantly increased up to 88% after the addition of TEA (6 eq.). The reaction was completed within 6 h at 25 °C, which is a dramatic result compared to the reactions from the route A. For further optimization of reaction condition, the amount of TEA and iASA was reduced. However,

the product purity fell to 53%, when the amount of TEA and iASA was decreased to 1 eq. and 3 eq. respectively. Based on these results, the optimal condition for iASA coupling to Ala-Ala carbonylimidazolide on resin supports was found to be 2 eq. of TEA, 6 eq. of iASA, and 6 h at 25 °C.

This new approach has made a huge progression in the reaction efficiency with the same step procedure of CDI activation and iASA coupling, when compare to the previous method. In particular, the approach where Ala-Ala on the resin was first activated by CDI and then coupled with iASA, significantly reduced the reaction times of the first and the second steps, and thus, gave high yield and purity of the final product. In terms of the overall reaction time, the efficiency of this approach for the synthesis of ASA-Ala-Ala has been improved by a factor of twenty compared to the previous method.

Antioxidant Activity of ASA-Ala-Ala. Finally, the antioxidant activity of the as-prepared ASA-Ala-Ala was evaluated by a lipid peroxidation inhibition test. Since the peroxidation of lipid or lipoprotein is associated with various diseases,[23] it is important to examine the antioxidant activity in lipophilic environments. As shown in Figure 1, ASA-Ala-Ala showed enhanced inhibition activity for lipid peroxidation compared to ASA alone. After incubation for 30 h at 50 °C, the lipid peroxidation inhibitory activity of ASA-Ala-Ala remained about twice as high as that of ASA. This can be attributed to an increase in the stability of ASA *via* conjugation with Ala-Ala. In addition, ASA-Ala-Ala have higher ClogP value (−3.2) compared to ascorbate anion (−6.5), predominant form of ASA in biological environment, which suggest that increased lipophilicity of ASA-Ala-Ala could give rise to improvement of antioxidant activity in lipophilic condition.[24] This result clearly demonstrates that the physical and chemical stability and biological activity of ASA can be effectively modulated by peptide conjugation.

Conclusion

An efficient solid-phase synthetic approach has been demonstrated with the synthesis of ASA-Ala-Ala. The initial activation of the *N*-terminal amino group of Ala-Ala by CDI on the resin supports enabled the significant reduction in overall reaction time and the increase in the level of purity. The as-prepared ASA-Ala-Ala showed enhanced antioxidant activity compared to ASA in lipophilic conditions even at high temperature. This synthetic approach can be applied to the conjugation of various unstable bioactive compounds with peptide.

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