Condensation of Cys(Acm)-Containing Peptide Segments with Silver Chloride as an Activator of Peptide Thioesters

Toru Kawakami and Saburo Aimoto*
Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565

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The undesirable removal of an acetamidomethyl (Acm) group in a Cys(Acm) residue is suppressed during the segment condensation of peptides, when silver chloride is used as an activator of the thioester moiety in a partially protected peptide thioester. The rate of segment condensation is sufficiently high enough for its application to practical peptide synthesis.

We have reported polypeptide syntheses using a thioester method, in which peptide thioesters are used as building blocks.[1] In the previous paper we described a method for the synthesis of a Cys-containing polypeptide using an acetamidomethyl (Acm) group for the protection of the mercapto group.[2] Since an Acm group is stable under anhydrous HF treatment conditions, a peptide thioester, containing Cys(Acm) residues, can be easily prepared by a Boc solid-phase method. If a peptide thioester containing Cys(Acm) residues could be used for segment condensation without loss of the Acm groups, the thioester method can be easily applied to the synthesis of Cys-containing polypeptides.

Adrenomedullin, which contains a cystine residue, was synthesized successfully.[2] A peptide thioester, containing a Cys(Acm) residue, was condensed in the presence of silver nitrate and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBt). The loss of the Acm groups during segment condensation was not observed in this synthesis. However, the removal of an Acm group was observed in the synthesis of reaper protein,[3] which contains 65 amino acid residues, including one Cys residue.

To find the conditions under which the Acm group is stable and the condensation reactions proceeded at an acceptable rate, the effect of silver salts was examined, using model peptide thioesters.

Fmoc-Phe-Thr-Ser-Cys(Acm)-His-Gly-SCH_2CH_2CO-β-Ala-NH_2 (**1**)
+
Leu-Arg-Glu-Ser-NH_2 (**2**)
↓ Ag salt, HOOBt, DIEA, DMSO
Fmoc-Phe-Thr-Ser-Cys(Acm)-His-Gly-Leu-Arg-Glu-Ser-NH_2 (**3**)

Figure 1. Model segment condensation.

Reaper protein contains the partial sequence Thr-Ser-Cys-His. Hence, we prepared a peptide thioester, Fmoc-Phe-Thr-Ser-Cys(Acm)-His-Gly-SCH_2CH_2CO-β-Ala-NH_2 (**1**), as a model compound (Figure 1). The segment condensations of peptide **1** and the tetrapeptide, Leu-Arg-Glu-Ser-NH_2 (**2**), were carried out using silver salts in the presence of HOOBt and *N*,*N*-diisopropylethylamine (DIEA) in DMSO.[4] The reactions were analyzed by reversed phase HPLC (RP-HPLC) and matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The RP-HPLC elution profiles of the reaction mixtures after 4 h reaction, are shown in Figure 2.

When silver nitrate was used, the coupling reaction reached completion within 1 h, and, at that time, 16% of the Acm group in the condensation product had been already removed, as evidenced by the area of the peaks. A desired product, Fmoc-Phe-Thr-Ser-Cys(Acm)-His-Gly-Leu-Arg-Glu-Ser-NH_2 (**3**), had a retention time of 31.8 min.[5] The des-Acm product, Fmoc-Phe-Thr-Ser-Cys-His-Gly-Leu-Arg-Glu-Ser-NH_2 (**4**), eluted at 32.1 min.[5] The mass numbers of the peaks, which eluted after the condensation products, corresponded to the derivatives of thioester **1** such as a hydrolyzed product. To estimate the stability of the Acm group under segment coupling conditions, the mixture was stirred for a further period of time. After 4 h, the percentage of peptide **4** in the condensation product increased to 43% (Figure 2 (A)).[6] After 24 h, the Acm group was completely removed.

When silver chloride was used for the activation of the thioester, the reaction proceeded more slowly. After 4 h, the reaction was complete, but peak **4** was not detected (Figure 2 (B)).[6] Stirring the reaction mixture for 24 h resulted in less than 10% loss of the Acm group.

HPLC analysis suggested that the removal of the Acm group occurred mainly after the completion of the condensation reaction for the cases of both silver nitrate and silver chloride. Thus, silver ions have a higher affinity for the thioester moiety than for the mercapto group protected by an Acm group. This suggests that excess of silver ions in DMSO solution would be expected to accelerate the removal of the Acm group. It follows then, that the solubility of silver salt in DMSO is an important factor.[7] The removal of Acm groups proceeds slowly when silver chloride is used instead of silver nitrate. Therefore the reaction can be easily controlled by using silver chloride as the activator.

In order to examine the stability of the Acm group, as it is affected by the sequence around Cys(Acm), reactions of other two peptide thioesters were carried out. Initially, to examine the effect of hydroxyl groups of Thr and Ser in peptide **1**, a peptide thioester, Fmoc-Phe-Leu-Ala-Cys(Acm)-His-Gly-SCH_2CH_2CO-β-Ala-NH_2 (**5**), was prepared and condensed with peptide **2**. When silver nitrate was used as an activator, not only the desired product, Fmoc-Phe-Leu-Ala-Cys(Acm)-His-Gly-Leu-Arg-Glu-Ser-NH_2 (**6**),[8] but also a des-Acm product, Fmoc-Phe-Leu-Ala-Cys-His-Gly-Leu-Arg-Glu-Ser-NH_2 (**7**),[8] was observed. Sixty five percent of the Acm group in the condensation product was removed after 4 h stirring (Figure 2 (C)). In addition, the des-Acm product **7** was not observed after 4 h when silver chloride was used. These results suggest that the hydroxyl groups in the side chains are not responsible for destabilizing of Acm group during segment coupling.

Next, the His residue, next to Cys(Acm), was replaced by Phe, and the condensation of Fmoc-Phe-Thr-Ser-Cys(Acm)-Phe-Gly-SCH_2CH_2CO-Ala-NH_2 (**8**) with peptide **2** was carried out. The desired product, Fmoc-Phe-Thr-Ser-Cys(Acm)-Phe-Gly-Leu-

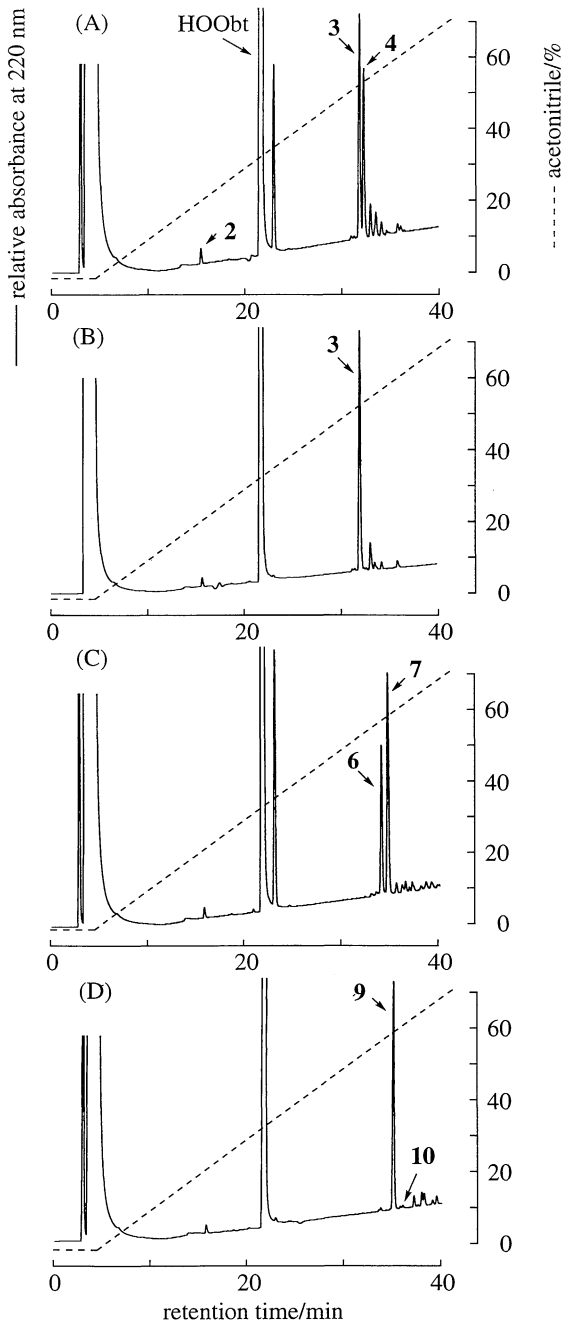

Figure 2. RP-HPLC elution profiles of the reaction mixtures after 4 h. Panels A and B show the reaction of peptide thioester **1** and peptide **2**. $AgNO_3$ was used in panel A, and AgCl was in panel B, respectively. Panel C shows the reaction of peptide thioester **5** and peptide **2** in which $AgNO_3$ was used. Panel D shows the reaction of peptide thioester **8** and peptide **2** in which $AgNO_3$ was used. Gradient is shown on panels using aqueous acetonitrile containing 0.1% trifluoroacetic acid at flow rate of 1.0 mL/min on Cosmosil $5C_{18}$ARII (4.6 x 250 mm).

Arg-Glu-Ser-NH_2 (**9**),[9] was obtained even when silver nitrate was used. After 4 h, the des-Acm product, Fmoc-Phe-Thr-Ser-Cys-Phe-Gly-Leu-Arg-Glu-Ser-NH_2 (**10**),[9] was observed in less than 1% (Figure 2 (D)). Even after 24 h, only 7% of the Acm group was removed.

These results strongly suggests that the His residue is involved in the coordination of the sulfur atom on Cys(Acm) with silver ion, which must activate the Acm group toward nucleophiles. The peak at 22.8 min on RP-HPLC (Figure 2) seemed to be a compound derived from an Acm group, and was produced when the Acm group was removed. We attempted to measure the mass number of the product by MALDI-TOF MS, but were not able to accomplish this.

We conclude that the stability of the Acm group in the presence of silver ions is dependent on the type of the amino acid residues in the vicinity of Cys(Acm). Although silver chloride is only sparingly soluble in DMSO, the condensation reaction proceeded at a reasonable rate with minimal removal of the Acm group. These data indicate that reaper protein could be synthesized using silver chloride as the activator with negligible loss of the Acm group.

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References and Notes

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4 Experimental procedures are as follows: A silver salt (1.5 μmol) was added to the solution of HOObt (2.5 mg, 15 μmol) and DIEA (1.7 μL, 10 μmol) in DMSO (20 μL). To this mixture were added the solutions of peptide **2** (55 mM, 10 μL, 0.55 μmol) and thioester **1** (57 mM, 10 μL, 0.57 μmol), and the mixture was stirred at room temperature in the dark. The reaction mixtures were analyzed by RP-HPLC and MALDI-TOF MS. RP-HPLC was performed on Cosmosil $5C_{18}$ARII (4.6 x 250 mm) (Nacalai tesque, Kyoto) using an increasing linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. Mass numbers were determined by a Voyager™ DE (PerSeptive Biosystems, Inc., Framingham, MA) using α-cyano-4-hydroxycinnamic acid as a matrix.
5 **3**: MS (MALDI-TOF) Found: *m/z* 1428.5. Calcd for $[M+H]^+$, 1428.6.
4: MS (MALDI-TOF) Found: *m/z* 1357.4. Calcd for $[M+H]^+$, 1357.6.
6 When silver nitrate was used, the desired product **4** was obtained in 28% yield, and the des-Acm product **5** was in 15% after isolation by RP-HPLC. When silver chloride was used, the desired product **4** was obtained in 56% yield after isolation by RP-HPLC.
7 Silver nitrate dissolves completely in DMSO at these condensations. The solubility of the silver chloride was measured in DMSO at room temperature and found to be less than 0.3 mg/mL.
8 **6**: MS (MALDI-TOF) Found: *m/z* 1424.5. Calcd for $[M+H]^+$, 1424.7.
7: MS (MALDI-TOF) Found: *m/z* 1353.3. Calcd for $[M+H]^+$, 1353.4.
9 **8**: MS (MALDI-TOF) Found: *m/z* 1438.6. Calcd for $[M+H]^+$, 1438.6.
9: MS (MALDI-TOF) Found: *m/z* 1367.8. Calcd for $[M+H]^+$, 1367.6.