Peptide Ligation Using a Building Block Having a Cysteinyl Prolyl Ester (CPE) Autoactivating Unit at the Carboxy Terminus

Toru Kawakami* and Saburo Aimoto*
Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871

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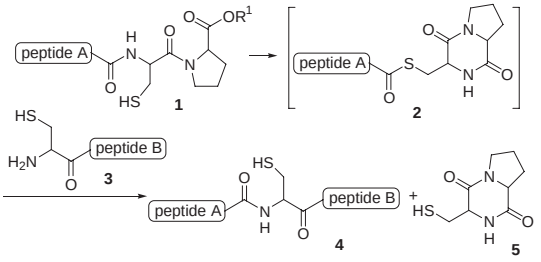
CPE peptide ligation using a building block containing a cysteinyl prolyl ester (CPE) autoactivating unit is described. The peptide building block can be prepared by a standard Fmoc chemistry and used in thiol-mediated ligation, such as native chemical ligation.

The chemical ligation of peptide segments is widely used in polypeptide synthesis.[1] Ligation chemistry has been developed based on the use of peptide thioesters, such as the thioester method,[2] native chemical ligation,[3] and extended chemical ligation[4] strategies. Here, we describe an alternative strategy, CPE peptide ligation, in which a building block contains no thioester moiety but a cysteinyl prolyl ester (CPE) autoactivating unit (Scheme 1).

A peptide, Fmoc-His-Pro-Ile-Arg-Gly-Cys-Pro-OCH_2-$CONH_2$ (**6a**) (0.57 μmol), was allowed to react with a cysteinyl peptide, H-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (**7**) (1.1 μmol), in a 0.1 M tricine buffer (pH 8.2) (0.13 mL) containing 20 mM tris-(hydroxylpropyl)phosphine (THP) and 6 M guanidine (Gdn). The reaction mixture was stirred at room temperature for 24 h. The elution profile of the reaction mixture by reversed phase (RP) HPLC after the addition of dithiothreitol (DTT) is shown in Figure 1. A new peak appeared at 24 min. MALDI-TOF-MS and an amino acid analysis indicated that it corresponded to the ligated product, Fmoc-His-Pro-Ile-Arg-Gly-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (**8a**) (MALDI-TOF-MS: found m/z 1414.9, calcd for $(M + H)^+$ 1414.7; Amino acid analysis: $Asp_{0.99}$-$Pro_{1.1}Gly_2Cys_{nd}Ile_{1.9}Leu_{2.0}His_{0.84}Arg_{0.92}$). A peptide having the same primary structure as peptide **8a** was prepared by all stepwise solid-phase peptide synthesis (SPPS). This peptide eluted at the same retention time as that of the ligated product **8a**. Peptide **8a**, thus ligated, was obtained in 60% yield after isolation by RP-HPLC. When a Gly residue in **6a** was replaced by Ala, Leu, or Val, respectively, the ligated products, **8b**–**8d**, were obtained in good yields, as shown in Table 1, although the ligation conditions were not optimized. The ligation reaction did not reach completion and a small amount of the hydrolysis product was observed under the conditions used. Epimerization during the ligation was checked in the reaction of peptide **6d**. The reaction mixture contained less than 2% of D-Val containing peptide **8d′** in the ligated product by the comparison with **8d′**, prepared by all stepwise SPPS, on RP-HPLC analysis.



Scheme 1. Peptide ligation using a cysteinyl prolyl ester (CPE) as an autoactivating unit.

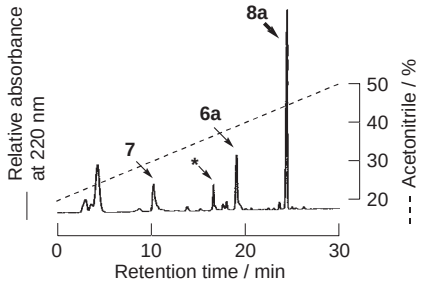


Figure 1. RP-HPLC of the reaction mixture of peptides **6a** and **7**. *: Fmoc-His-Pro-Ile-Arg-Gly-OH. Column: YMC-Pack ProC18 (4.6 × 150 mm), eluent: aq. acetonitrile containing 0.1% trifluoroacetic acid (TFA), flow rate: 1.0 mL/min.

The C-terminal CPE structure is important. When the thiol group of the Cys residue was protected with a methylthio group, the ligated product **8a** was not observed in the reaction of **6e** in the absence of reducing reagents such as phosphines (Table 1, Entry 5). The proline residue could be replaced by a sarcosine (*N*-methylglycine) residue, but not by a glycine residue (Entries 6 and 7). Neither the C-terminal acid nor the amide of peptidyl cysteinyl proline gave the ligated product under the same reaction conditions (Entries 8 and 9).

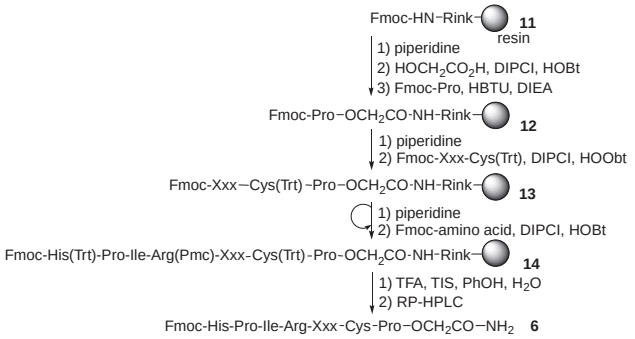
Zanotti et al. reported that a diketopiperazine (DKP) thioester, *cyclo*(-Cys($COCH_2$Ph)-Pro-) (**9**) was produced when a *p*-nitrophenyl (Np) ester, $PhCH_2CO$-Cys(S^{t}Bu)-Pro-ONp (**10**), was treated with tributylphosphine.[5] Our ligation reaction appears to proceed through a similar pathway (Scheme 1): The

Table 1. Ligation reaction of peptides **6** and **7**

Fmoc-His-Pro-Ile-Arg-**Aaa**-**Bbb**-**Ccc**-**Ddd** (**6**) + H-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (**7**)
↓ 0.1 M tricine, 6 M Gdn, 0.02 M THP, pH 8.2, 24 h
Fmoc-His-Pro-Ile-Arg-**Aaa**-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (**8**)

Entry	6	Aaa	Bbb	Ccc	Ddd	8	Yield/%[a]
1	**6a**	Gly	Cys	Pro	$-OCH_2CONH_2$	**8a**	60
2	**6b**	Ala	Cys	Pro	$-OCH_2CONH_2$	**8b**	56
3	**6c**	Leu	Cys	Pro	$-OCH_2CONH_2$	**8c**	54
4	**6d**	Val	Cys	Pro	$-OCH_2CONH_2$	**8d**	49
5	**6e**	Gly	Cys(SCH_3)	Pro	$-OCH_2CONH_2$	**8a**	0[b]
6	**6f**	Gly	Cys	Sar	$-OCH_2CONH_2$	**8a**	28
7	**6g**	Gly	Cys	Gly	$-OCH_2CONH_2$	**8a**	0
8	**6h**	Gly	Cys	Pro	–OH	**8a**	0
9	**6i**	Gly	Cys	Pro	$-NH_2$	**8a**	0

[a]Isolated yield by RP-HPLC. [b]In the absence of THP.



Scheme 2. Preparation of the CPE peptide **6** (Xxx = **a**: Gly, **b**: Ala, **c**: Leu, **d**: Val). Yields were calculated based on the proline content in the Fmoc-Pro-OCH_2CO-NH-resin **12**: **6a**, 25%; **6b**, 42%; **6c** (a 4-methoxybenzyl group was used for the protection of the cysteine residue, and the final deprotection was achieved by HF treatment), 18%; **6d**, 20%. DIPCI: diisopropylcarbodiimide; HBTU: 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate; HOBt: 1-hydroxybenzotriazole; HOObt: 3-hydroxy-1,2,3-bezotriazin-4(3*H*)-one; Pmc: 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TIS: triisopropylsilane; Trt: Trityl.

CPE peptide **1** initially forms the *S*-peptidyl *cyclo*(-Cys-Pro-) **2** as an analog to **9** through intramolecular *N* to *S* acyl shift followed by DKP formation. A reaction between the DKP peptide **2** and a cysteinyl peptide **3** gives the ligated peptide **4**, releasing *cyclo*(-Cys-Pro-) (**5**).[6,7] The mass number corresponding to **2**, derived from peptide **6d**, was detected in the reaction mixture (MALDI-TOF-MS: found m/z 1025.8, calcd for $(M + H)^+$ 1025.5). We failed to detect the DKP thioester in the reaction mixture of peptide **6a**, whereas the mass number corresponding to the DKP **5** was observed (ESI-MS: found m/z 201.3, calcd for $(M + H)^+$ 201.1).

We previously reported on the intramolecular *N* to *S* acyl shift reaction in a peptide bond under acidic conditions. When a peptide, attached by a 2-mercapto-4,5-dimethoxybenzyl group to the backbone, was treated with a TFA solution, an intramolecular *N–S* acyl shift occurred, giving a thioester intermediate.[8] A cysteine-containing peptide was also converted to the corresponding *S*-peptide (thioester).[8b] Although the thioester intermediate released the corresponding peptide thioester under the neutral conditions by the intermolecular thiol-exchange reaction,[8b,9] the thioester intermediate simultaneously produced the starting peptide by the *S* to *N* acyl shift reaction, that occurred even under weakly acidic aqueous conditions (aq. acetonitrile containing 0.1% TFA). In the ligation reaction using the CPE unit, the equilibrium between the thioester and the amide at the -Xxx-Cys- site shifts in favor of the thioester, because the amino group of the Cys residue, produced via the *N–S* acyl shift, is trapped by the formation of DKP with the Pro residue. Therefore the *S*-peptide is sufficiently stabilized to permit a ligated peptide to be produced through an intermolecular thiol-exchange reaction followed by an intramolecular *S–N* acyl shift reaction.

The thioester is quickly decomposed when treated with piperidine. Low nucleophilic bases permit a peptide thioester to be synthesized by Fmoc SPPS, however, the amino acid residue adjacent to the thioester is known to be racemized.[10] A peptide containing the CPE unit can be readily and safely prepared by the standard Fmoc SPPS because no thioester bond is present. A typical procedure is shown in Scheme 2. Glycolic acid was first attached to the Rink amide resin, and Fmoc-Pro-OH is then introduced. A dipeptide, Fmoc-Xxx-Cys(Trt)-OH, was introduced using DIPCI-HOObt. Here, HOObt was used to suppress the epimerization of the Cys residue, but note that the Cys residue is removed after the ligation. Therefore, the epimerization does not matter in the CPE peptide ligation. The peptide chain was then elongated according to standard Fmoc chemistry to give the protected peptide resin **14**. Finally, the desired peptide was obtained by a TFA treatment[11] followed by RP-HPLC.

In summary, peptides containing the CPE unit can be prepared by standard Fmoc SPPS and used as building blocks for ligation with a cysteinyl peptide in a manner similar to that used in native chemical ligation.

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11 The *N–S* acyl shift reaction rate of a Cys-containing peptide appears to be slow, and even if the *S*-peptide is produced, it reproduces the starting peptide rapidly even under the RP-HPLC conditions.[8b] Therefore, decomposition of the CPE peptide might be negligible during TFA or HF treatment in which the DKP is not formed under the acidic conditions.

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