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Semisynthesis of Dimeric Proteins by Expressed Protein Ligation

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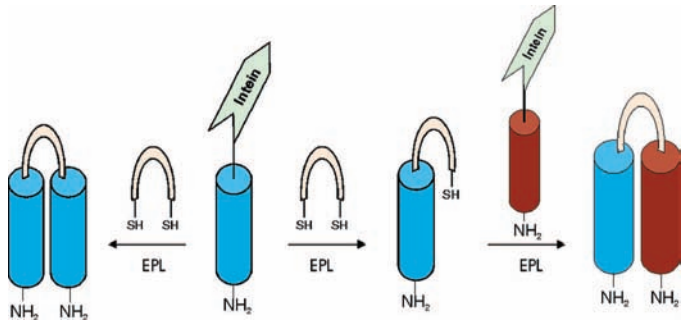
ABSTRACT



A one-pot synthesis of homodimeric proteins is described. The synthetic strategy is based on a double expressed protein ligation reaction between thioester peptides and a new bis-cysteinyl linker. The protocol was also applied to the synthesis of heterodimers.

Several proteins and peptides carry out their biological function in a dimeric form. For example, transcription factors regulate gene expression by binding DNA as dimers; antimicrobial peptides, such as Magainin 2, show increased biological activity in a dimeric form.[1] Investigating the sequence-specific DNA binding properties of dimeric proteins will allow for the development of new tools for the fine regulation of gene expression; on the other hand, understanding the functional role of dimerization of antimicrobial peptides will help comprehend their biological activity. In recent years, there have been significant efforts to obtain minimized versions of naturally occurring proteins such as dimeric DNA binding proteins which retain their biological function.[2−4] Dimeric peptides and proteins have been assembled both by noncovalent interactions and by covalent bonds.[5−7] The GCN4 basic region peptides were connected through a disulfide bond to give a dimer which specifically bound the AP1-DNA sequence.[8] Artificial dimerization domains, such as those derived from bulky metal complexes or enantiomeric bridged diphenyl derivatives, were employed for the dimerization of peptides possessing DNA binding specificity different from the native bZIP.[9] Host−guest inclusion complexes of β-cyclodextrin and adamantane were used for joining two monomers by non-

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covalent interactions.[10] Several scaffolds were employed for the synthesis of dendrimeric peptides/proteins.[11–13]

In this work, we propose a strategy for obtaining one pot protein homodimers covalently connected at the C-terminus by expressed protein ligation (EPL). The synthetic strategy was extended also to the synthesis of heterodimers. EPL is a protein engineering tool for the chemo- and regioselective modification of proteins based on the use of intein containing constructs.[14–16] Engineered inteins are used today for the production of C-terminus thioester and N-terminus cysteine proteins.[17,18] Reactive thioesters are used in ligation reactions with N-terminus cysteine peptides to give a new protein with all native peptide bonds. The ligation reaction proceeds through a trans thioesterification initiated by the cysteine thiol, followed by a spontaneous N−S acyl shift which affords the peptide bond between the two reacting moieties. EPL allows for the preparation of proteins containing natural and artificial modifications.[19–21]

In this paper dimers were obtained by reacting a new bifunctional linker with carboxyl-activated polypeptides. We synthesized a linker containing two cysteines in a N-terminal-like position, separated by an ethylendiamine spacer, and obtained thioester proteins by intein mediated splicing reactions. In order to demonstrate the feasibility of the synthetic strategy, we carried out the reactions on polypeptides obtained by expression in bacteria.

Thioesters were generated after splicing of proteins containing the MxeGyrA intein at the C-terminus of the amino acid sequences codified by the multicloning site of vectors such as a modified pTrcHisA (peptide A)[22] and pTXB1 (peptide B), containing as purification tag a hexa-histidine sequence and a chitin binding domain respectively.

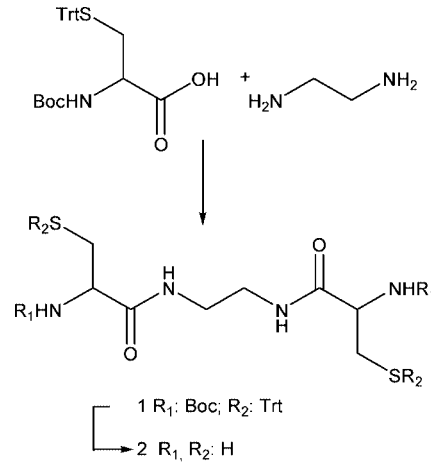
In these constructs, the MxeGyrA intein contains a single mutation, Asn-198-Ala, which prevents cleavage of the intein−C-extein peptide bond, without affecting the intein N-terminal splicing reaction (see the Supporting Information) After induction of splicing in the presence of thiols, these constructs generate a 42 and a 17 amino acid polypeptides respectively (peptide A and peptide B, respectively) as thioesters. The sequences of the peptides are as follows:

peptide A:
MGGSHHHHHHGMASMTGGQQMGRDLYDDDD
KDRWGSGHIE GR

peptide B: MASSRVDGGR EFLEGSS

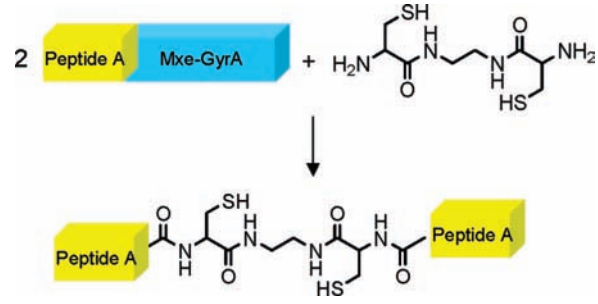
The linker was obtained by reacting ethylendiamine with a 2-fold excess of BocCys(Trt)OH. After acidic deprotection of the Boc and Trt groups and purification, the desired linker

Scheme 1. Synthesis of the Bis-cysteinyl Linker



was obtained (Scheme 1); its identity was confirmed by NMR and ESI-MS. This molecule is suitable for two chemical ligation reactions. Proteins were expressed in *Escherichia coli* BL21 (DE3) cells, transformed with the appropriate plasmid. The homodimer (A-linker-A) was obtained using the construct peptide A-MxeGyrA. The protein was purified by affinity chromatography using a Ni^{2+} NTA resin. The splicing and ligation reactions occurred simultaneously in phosphate buffer 20 mM, 0.18 mM Sodium 2-mercapto-ethanesulfonate (MESNA), 0.23 mM 1,2-ethanedithiol (EDT), pH 7 with 0.5 equiv of linker (Scheme 2).

Scheme 2. Synthesis of the Homodimer



The homodimer was obtained after an overnight incubation with a 50% yield. Several experimental conditions, using different pH and different protein concentrations, were

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explored (see the Supporting Information). We did not find a significant difference in reactions carried out at pH 7 and pH 8. pH 7 was preferred to suppress the competitive thioester hydrolysis. The yield of the reaction is strictly dependent on the concentration: when the peptide concentration is 50 μM or lower, only a single peptide A unit connected at the C terminus with the linker (A-linker), was obtained. At a concentration of 130 μM (or higher), the yield of dimer increased up to 50%. When a 3-fold excess of linker is used at a peptide concentration higher than 130 μM the yield of the homodimer is always 50%; the unreacted peptide is converted into the A-linker derivative (Figure 1).

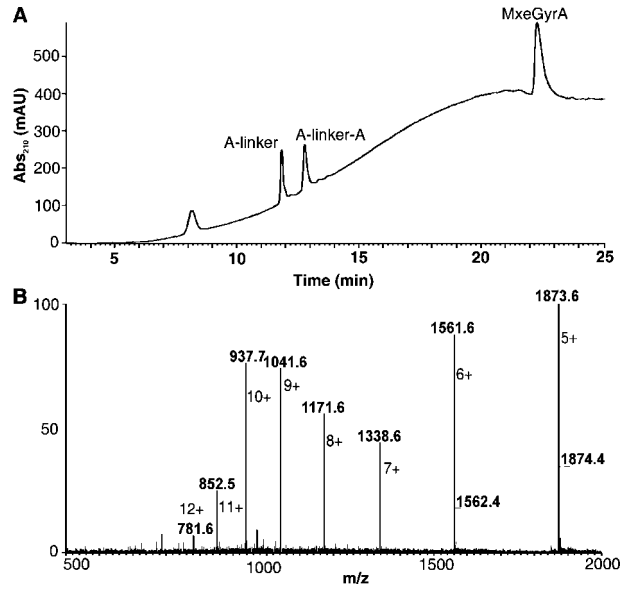


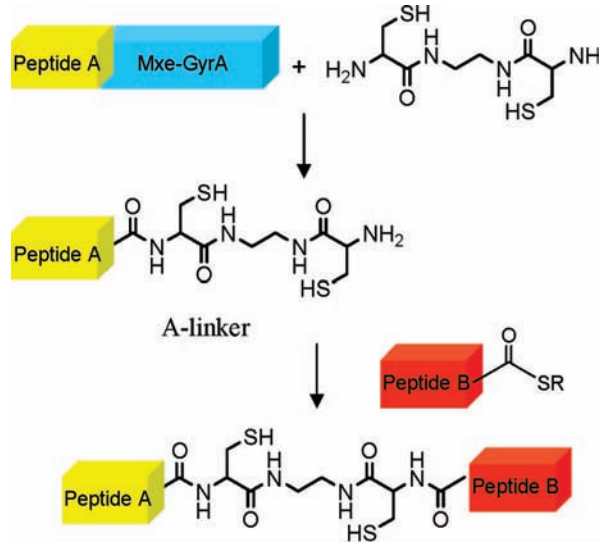
Figure 1. (A) RP-HPLC profile of the homodimer reaction formation; identified peaks are highlighted. (B) ESI-MS spectrum of "A-linker-A" peak.

Reaction did not occur without EDT, probably because the linker was easily oxidized. For the synthesis of the heterodimer (A-linker-B), we used the peptide A derivatized with the linker (A-linker) and reacted it with thioester peptide B (Scheme 3).

The A-linker derivative was obtained after the splicing of the peptide A-MxeGyrA fusion protein (20 μM) with a 3-fold excess of linker. The excess of linker was separated by dialysis, and the protein was identified by LCMS, purified by HPLC, and lyophilized. As the ligation between the thioester peptide A with the linker and the peptide A thioester hydrolysis reactions occurr simultaneously, a small amount of peptide A (C terminal carboxyl) which coelutes with the A-linker derivative was found.

The peptide B-MxeGyrA fusion protein was immobilized on the chitin resin; splicing was induced by incubating the resin in 20 mM phosphate buffer containing 500 mM NaCl, 50 mM MESNA, and 1 mM EDTA, pH 7. The thioester was identified by LCMS, purified by HPLC, and lyophilized. The purified A-linker (0.4 mM) derivative was reacted with

Scheme 3. Synthesis of the Heterodimer



2 equiv of thioester peptide B (0.4 mM). Reaction was carried out in 20 mM phosphate buffer pH 7, in the presence of MESNA and EDT overnight. A-linker was converted into the heterodimer A-linker-B, while the excess peptide B was converted into the EDT-thioester (Figure 2).

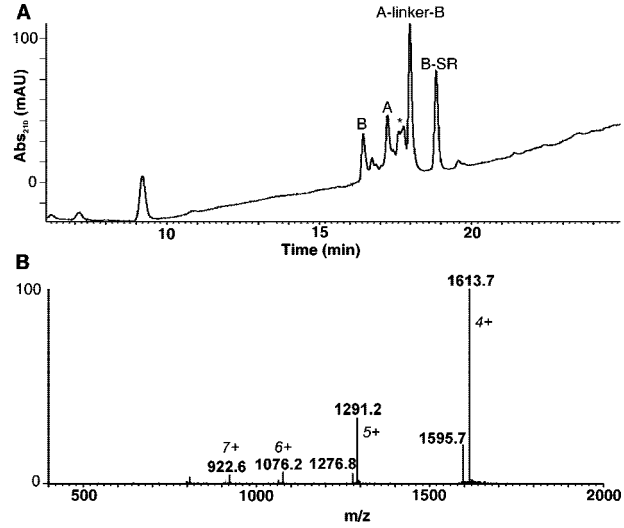


Figure 2. (A) RP-HPLC profile of the homodimer reaction formation, identified peaks are highlighted. $*$ indicates heterodimer oxidation byproducts, and B-SR designates the EDT thioester of protein B. (B) ESI-MS spectrum of "A-linker-B" peak.

We noticed that peptide B is very prone to degradation; in the mass spectra of the peptide B and the heterodimer we find oxidation products (M + 16) and products lacking the first two amino acids (M − 202).

In conclusion, a strategy for the preparation of peptide/proteins homo- and heterodimers covalently bound through a

linker at the C-terminus was developed. This strategy based on EPL reactions between thioester peptides and a new bifunctional linker affords chemically stable dimeric proteins. Homodimers can be obtained in a one-pot reaction. The linker can be easily modified as needed by changing the length and rigidity of the spacer between the cysteines. This strategy has potential in biochemical and bioorganic applications, for obtaining minimized and/or modified natural proteins and for joining two different proteins at the C-terminus position.

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Supporting Information Available: Synthesis and characterization of the linker and the proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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