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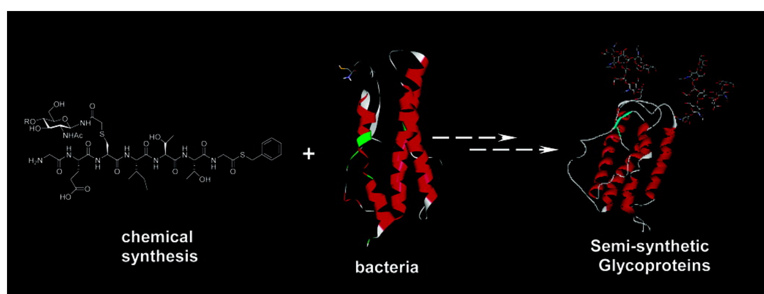

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Cyanogen Bromide Cleavage Generates Fragments Suitable for Expressed Protein and Glycoprotein Ligation

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Recently, the expressed protein ligation (EPL) methodology developed by Muir and co-workers has come to the fore as an extremely powerful technology for protein semisynthesis,[1] particularly for proteins bearing posttranslational modifications such as phosphorlyation[2a] and glycosylation[2b] and for the selective C-terminal modification of bacterially derived proteins with fluorescent probes,[2c] lipids,[2d] and biotin affinity tags used in the production of protein microarrays.[2e] Glycoproteins are particularly suited to EPL since short synthetically attainable glycopeptides can be fused with bacterially derived protein fragments of large molecular weight to give glycoproteins. Use of commercially available expression systems allows bacteria to be employed to express large quantities of C-terminal thioester components for EPL. Fragments bearing the N-terminal cysteine component are generally produced through enzymatic scission of pre-cysteine sequences from target fusion-proteins.[1b] Pre-cysteine sequences can be readily cleaved from *soluble* protein fragments using commercially available factor Xa[2b] or TEV proteases[3] after expression in *E. coli* and where additional flanking IEGR and ENLYFQ amino acid sequences have been encoded, respectively. One glycoprotein under study in our group, erythropoietin (EPO), is notoriously insoluble when expressed in bacteria, and five mutations have been incorporated into the protein sequence to confer sufficient solubility for structural investigations.[4] Consequently, these mutations have been shown not to affect the structure or activity of the folded protein and most were also incorporated into a recent pioneering total synthesis of this protein by native chemical ligation (NCL).[5] Our semisynthetic approach to glycosylated EPO has been targeted toward investigations of the potential to ligate synthetic glycopeptide thioesters to the bacterially derived fragments shown schematically in Figure 1. These fragments were chosen since **1** and **2** utilize native cysteine residues at cys29 and cys33 for ligation purposes where **3** introduces an additional cysteine residue (E55C) allowing for greater flexibility in glycopeptide synthesis.

On expression of fusion precursors to fragments **1**−**3** in bacteria (engineered to release N-terminal cysteine residues upon treatment with factor Xa protease), we quickly encountered problems (Table 1, constructs A and B) in that, although they were overexpressed in quantities of 5−10 mg L^{-1} of cell culture, they could not be solubilized in a buffer system compatible with factor Xa proteolysis at practical concentrations. Indeed rapid protein precipitation was observed in guanidine·HCl concentrations lower than 3 M. Use of the detergents *N*-laurylsarcosine and Triton X-100 to aid solubility was also investigated, but none of our desired cleavage products were observed upon treatment with protease. High detergent concentrations also complicated analysis by LC-MS. In an attempt to solubilize our protein fragments, glutathione-*S*-transferase (GST) fusion proteins were also prepared (construct B) since Muir and co-workers had successfully employed this strategy to solubilize an hydrophobic ion-channel protein fragment.[6] The GST fusion proteins were also efficiently produced but offered no advantage in terms of solubility in our case. Finally, we investigated cyanogen bromide (CNBr)[7] as cleavage reagent, as it has been widely used to cleave proteins at methionine residues[8] and most importantly is insensitive to the chaotropes required to solubilize our EPO fragments.

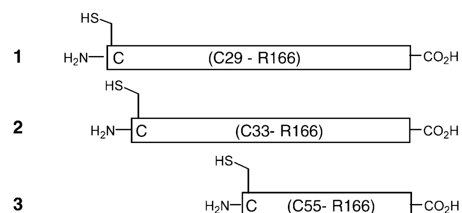

Figure 1. Designed protein fragments for EPL. **1**, **2** and **3** comprise 138, 134, and 112 amino acids, respectively.

Table 1. Designed Fusion Precursors to **1** for Use in Cleavage Studies

construct	fusion-protein-generated	cleavage method	cleavage (%)
A	His_{10}−IEGR−C29EPO[a]	factor Xa	0
B	GST−His_{10}−IEGR−C29EPO[b]	factor Xa	0
C	His_{10}−M−C29EPO[c]	CNBr	>95[d]

[a] Expressed from vector pET16b engineered to encode the amino acid presequence IEGR such that N-terminal cys results from factor Xa cleavage. [b] Gene excised with His_{10} tag (*Nco*I/*Bam*HI) from pET 16b and subcloned into pET 41a. [c] Expressed directly from nonengineered pET 16b. [d] No uncleaved material detected by LC-MS.

Table 2. Ligation Reactions with CNBr Cleavage Fragments **1**−**3**.[10]

bacterial fragment	thioester[a]	calcd mass for ligation product (Da)	obsd mass of ligation product (Da)
1	GEC(GlcNAc) ITTG (4)[b]	16 229.7	16 229
2	GC(Acm)AEH (5)	15 421.7	15 422
3	AWKRL (6)	12 887.1	−[c]

[a] SBn thioesters prepared according to Shin et al.[11] [b] Incorporates GlcNAc at the native position (N24C). [c] Only trace product observed after 48 h.

Fragments **1** and **2** both contained a single internal methionine residue that was first mutated to leucine (M54L). Polyhistidine-tagged precursors (Table 1, construct C) were purified in a single Ni^{2+} affinity chromatographic step. His_{10}-tagged **1**−**3** were then separated from buffer salts prior to CNBr cleavage by precipitation, which occurred during dialysis against water at 4 °C, followed by centrifugation. Initially, we were wary of potential damage to protein samples resulting from CNBr treatment and conducted

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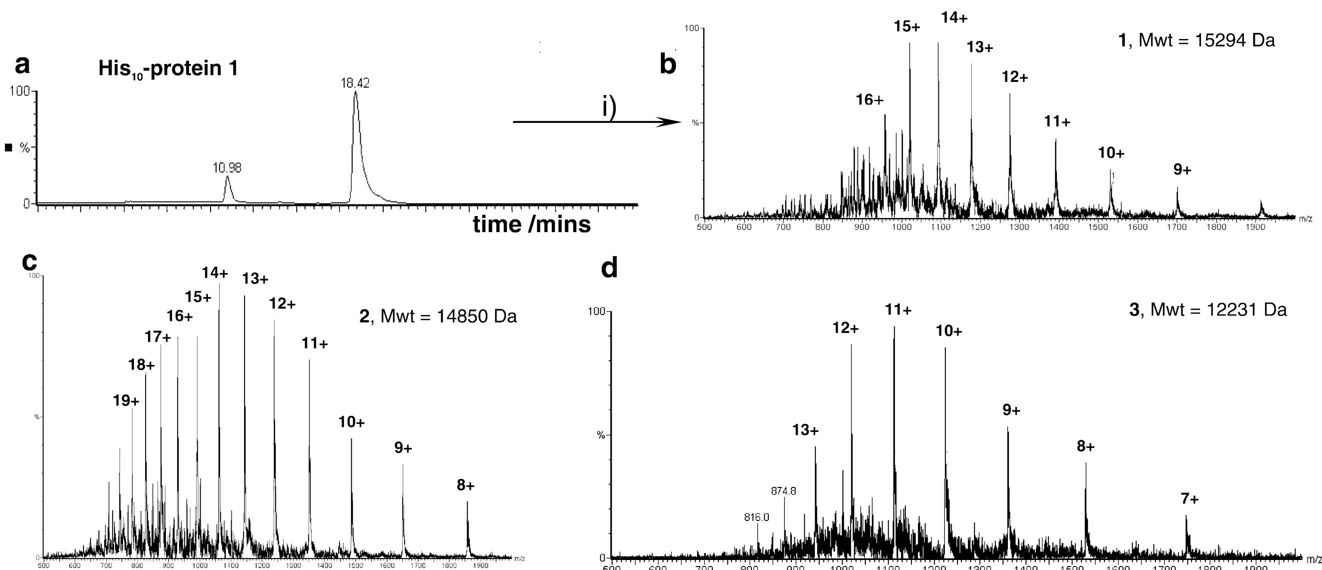

Figure 2. (a) LC trace of Ni^{2+} affinity purified His_{10}−**1**, retention time = 18.4 min. (b) CNBr cleavage of His_{10}−**1**. Reagents/conditions: (i) excess CNBr, 80% HCO_2H, 16 h, rt. Calcd mass = 15 292.7 Da. (c) **2**, calcd mass = 14 852.2 Da. (d) **3**, calcd mass = 12 232.2 Da.

preliminary reactions in parallel where fusion precursors had, and had not, been subjected to an oxidative sulfitolysis protocol that protects cysteine residues prior to CNBr treatment.[9] Surprisingly, we found that clean and efficient cleavage yielding N-terminal cys-containing fragments had occurred (Figure 2) in two solvent systems tested,[10] but no cleavage was observed in the case of the protected protein samples. Cleaved protein was again collected by simple dialysis against water at 4 °C and centrifugation.

With protein fragments **1**−**3** in hand, model thioesters **4**−**6**, which includes glycopeptide mimetic **4** (EPO residues 22−28), were prepared (Table 2) using standard solid-phase methodology[12] to test the viability of each potential protein ligation site. Finally, to conduct EPL the precipitated protein fragments **1**−**3** were each redissolved in 6 M guanidine hydrochloride containing 300 mM sodium phosphate buffer; pH 8.0, 2% w/v 2-mercaptoethanesulfonic acid (MESNA), and 20 mM tris-carboxyethylphosphine (TCEP). Ligation reactions proceeded smoothly under reducing conditions and (with the exception of that between **3** and thioester **6**) were virtually complete within 48 h.

To summarize, cyanogen bromide has been employed to efficiently release protein fragments containing N-terminal Cys residues from insoluble polyhistidine-fused precursors, though minor adjustments to the method described here should render it applicable to soluble proteins. Uncleaved protein can potentially be removed by Ni^{2+} affinity chromatography. This advance constitutes a useful addition to the tools available for the semisynthesis of proteins, glycoproteins, and glycoprotein mimetics by EPL since methionine is encountered relatively infrequently in protein sequences. Furthermore, the use of CNBr for the production of protein fragments for EPL has not been previously reported. Recently, a complex-type undecasaccharide was appended to the cysteine residue of a short synthetic peptide using α-haloacetamide sugars.[13] Hopefully, the fusion of EPL with such oligosaccharide coupling strategies will expedite the production of semisynthetic glycoproteins and mimetics.

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Supporting Information Available: Synthetic procedures, mass spectra, and LC traces for **1**−**6** and ligation products (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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