

Combining a PagP fusion protein system with nickel ion-catalyzed cleavage to produce intrinsically disordered proteins in *E. coli*

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ABSTRACT

Many proteins contain intrinsically disordered regions that are highly solvent-exposed and susceptible to post-translational modifications. Studying these protein segments is critical to understanding their physiologic regulation, but proteolytic degradation can make them difficult to express and purify. We have designed a new protein expression vector that fuses the target protein to the N-terminus of the integral membrane protein, PagP. The two proteins are connected by a short linker containing the sequence SRHW, previously shown to be optimal for nickel ion-catalyzed cleavage. The methodology is demonstrated for an intrinsically disordered segment of cardiac troponin I. cTnI[135-209]-SRHW-PagP-His₆ fusion protein was overexpressed in *E. coli*, accumulating in insoluble inclusion bodies. The protein was solubilized, purified using nickel affinity chromatography, and then cleaved with 0.5 mM NiSO₄ at pH 9.0 and 50°C, all in 6 M guanidine-HCl. Nickel ion-catalyzed peptide bond hydrolysis is an effective chemical cleavage technique under denaturing conditions that preclude the use of proteases. Moreover, nickel-catalyzed cleavage is more specific than the most commonly used agent, cyanogen bromide, which cleaves C-terminal to methionine residues. We were able to produce 15 mg of purified cTnI[135-209] from 1 L of M9 minimal media using this protocol. The methodology is more generally applicable to the production of intrinsically disordered protein segments.

Key words: intrinsically disordered regions; inclusion bodies; fusion protein expression; *Escherichia coli*; nickel ion-catalyzed peptide bond hydrolysis; chemical cleavage

Abbreviations: PagP, PhoPQ-activated gene P; KSI, ketosteroid isomerase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; NMR, nuclear magnetic resonance; IDR, intrinsically disordered region

Introduction

Intrinsically disordered regions (IDRs) are protein segments that lack a fixed three-dimensional structure under physiologic conditions. Genomic analyses indicate that >30% of eukaryotic proteins contain at least one disordered region spanning 50 residues or more [1]. IDRs are solvent-exposed and susceptible to post-translational modifications such as phosphorylation and proteolysis. Thus, they are highly important in the regulation of numerous biological processes and cellular signal transduction [2].

It is of great interest to determine how post-translational modifications of IDRs gives rise to changes in function, but the structural malleability of IDRs can make biophysical characterization difficult [3]. Producing large quantities of pure intrinsically disordered proteins can also be challenging due to their susceptibility to proteolytic digestion both during expression and purification stages [4]. One solution is to target IDRs to inclusion bodies, an old method that is used to mass-produce a number of clinically important peptides like insulin [5]. Inclusion bodies are dense particles of aggregated (and usually misfolded) protein, a form that is largely protected from proteolytic enzymes [6]. The strategy allows for easy purification, since inclusion bodies can be separated from other cellular components through centrifugation. The primary disadvantage of producing proteins from inclusion bodies is that they are misfolded and have to be reconstituted into an active folded form [7]. Of course, this is not an issue for IDRs, suggesting that it might be an ideal strategy for their production.

Many proteins have been used as fusion partners to direct the expression of target proteins into inclusion bodies [8]. We previously developed a new fusion system based on PagP for this purpose [9]. PagP is a β -barrel integral membrane protein found in the outer membranes of many pathogenic Gram-negative bacteria [10]. It is directed to the outer membrane by a signal sequence, but when the signal sequence is deleted using recombinant DNA techniques, the misdirected protein accumulates in cytoplasmic inclusion bodies [11]. We used our PagP fusion protein to produce large amounts of a fragment of human cardiac troponin I, cTnI[1-71], which contains a large intrinsically disordered region spanning residues 1-38 [12]. Our PagP fusion protein was more effective at targeting large constructs into inclusion bodies than the commercially available fusion protein system based on ketosteroid isomerase (KSI) as a fusion partner, making feasible isotopic enrichment and multinuclear multidimensional NMR studies of cTnI[1-71] [12].

One challenge of using fusion proteins for protein expression is that the fusion partner must be cleaved and removed from the target protein. This is especially challenging in the case of inclusion body-targeted expression, because the inclusion bodies are usually solubilized in harsh denaturants or detergents [13], so that enzymatic cleavage (for example, with TEV protease or thrombin) is not possible. Cyanogen bromide (CNBr) is the most commonly used chemical cleavage technique [9], given that it is specific and highly effective even at room temperature. Cyanogen bromide cleaves peptide bonds C-terminal to methionine residues [14], though with reduced efficiency when the methionine is preceded by serine or threonine. The main disadvantage of CNBr cleavage is that the target protein of interest cannot contain internal

methionine residues.

In our previous work expressing PagP-cTnI[1-71]-His₆ fusion protein, we used CNBr as the peptide bond-cleaving agent. Achieving near-100% cleavage was challenging, but cleavage was more complete when steps were taken to ensure that methionine residues were fully reduced. Another issue was that CNBr predictably cleaved PagP at multiple methionine-containing sites, yielding multiple fragments. This did not create any issues for our construct, which had a C-terminal His-tag that facilitated purification of cTnI[1-71]-His₆ from fragments of PagP.

One research group has recently discovered that aqueous nickel ions can be used to selectively cleave the peptide bond N-terminal to the sequence S/T-X-H-Z, where “S/T” can be serine or threonine and “H” is histidine. The cleavage is most effective when “X” is a positively charged residue (R or K) and “Z” is I, K, L, R, or W [15]. The reaction is favoured by high temperatures, high Ni²⁺ concentrations, and alkaline conditions. It was demonstrated that incorporation of the sequence SRHWAPH₆ into the C-terminus of recombinant SPI2 protein yielded a His-tag that could be purified via immobilized metal affinity chromatography and then removed by Ni²⁺-catalyzed cleavage [16].

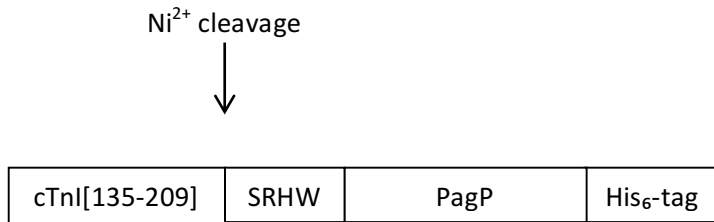
In the current study we combine our previous work with PagP as a fusion protein partner with nickel ion-catalyzed cleavage. Nickel ion-catalyzed cleavage is ideally suited to the PagP fusion protein system because it can be carried out under denaturing conditions. Moreover, the cleavage reaction is selective for sequences in the form S/T-X-H-Z, less common in protein sequences than the individual methionine residues targeted by CNBr. Thus, our system should be readily applicable to any intrinsically disordered protein segment, including those that include internal methionine residues. We demonstrate the utility of our new construct in producing an unstructured fragment of cardiac troponin I, cTnI[135-209], which contains three internal methionine residues.

Materials and Methods

Design of PagP fusion partner plasmid

A novel plasmid, pELECTRA-PagP-His₆, was developed with DNA 2.0 for expressing target proteins fused to PagP-His₆. In contrast to our previous system [9], the current plasmid fuses the C-terminus of the target protein to the N-terminus of PagP. Thus, when a nickel cleavage-sensitive SRHW site is added to the target protein C-terminus, nickel-mediated proteolysis will yield the target protein with its native C-terminus (see Figure 1A). The SRHW site was not included in pELECTRA-PagP-His₆, so that any cleavage site (whether for chemical or enzymatic cleavage) could be introduced onto the C-terminus of the target protein through recombinant DNA methods and inserted into the pELECTRA-PagP-His₆ plasmid. Because of this, all the internal methionine residues in PagP were also mutated to other residues (corresponding to other residues found in other Gram-negative bacterial homologues, see Figure 1B), so that a methionine residue could also be included in the linker sequence, allowing separation via cyanogen bromide cleavage without fragmentation of the PagP-His₆ fusion partner (although this strategy was not used in the current study).

(A)



(B)

MRGKFKRPTL	RRVRISADAM	MQALLGARAK	ESLDLRAHLK	QVKKEDTEKE	NREVGDWKRN
70	80	90	100	110	120
IDALSGMEGR	KKKFES SRHW	GNAD EW LT TF	RENIAQTWQQ	PEHYDLYIPA	ITWHARFAYD
130	140	150	160	170	180
KEKTDRYNER	PWGGGFGLSR	WDEKGNWHGL	YA I AFKDSWN	KWEPIAGYGW	ESTWRPLADE
190	200	210	220	230	240
NFHLGLGFTA	GVTARDNWN Y	IPLPVLLPLA	SVGYGPATFQ	A TYIPGTYN N	GNVYFAW I RF
250					
QFLE HHHHHH					

Fig. 1. (A) The cTnI[135-209]-SRHW-PagP-His₆ fusion protein. The SRHW sequence allows for nickel-catalyzed peptide bond cleavage N-terminal to the serine residue, allowing separation of the cTnI[135-209] target protein from SRHW-PagP-His₆.

(B) Amino acid sequence of cTnI[135-209]-SRHW-PagP-His₆. The nickel-sensitive SRHW sequence, His₆ tag, and former methionine residues in PagP mutated to other residues are highlighted in bold red.

The pELECTRA-PagP-His₆ plasmid contains a lacI repressor gene and a T5 promoter flanked with lacO sites, allowing for IPTG-inducible expression in any *E. coli* host (the T5 promoter utilizes native *E. coli* RNA polymerase). It also incorporates ampicillin selection, a high copy number origin of replication, and a strong ribosome binding site (Figure 2). The pELECTRA –PagP-His₆ plasmid is designed to allow insertion of any target sequence using the patented Electra cloning system of DNA 2.0 (see DNA 2.0 website: <https://www.dna20.com/products/expression-vectors/electra-system#2>). The double-stranded target sequence must have a 5'-ATG overhang at one end corresponding to the start codon (coding for methionine) and 5'-ACC overhang at the other end corresponding to the anti-sense codon of a C-terminal glycine residue (the last residue in the insert, which follows the SRHW

sequence in Figure 1). The target sequence (in this case, M-cTnI[135-209]-SRHWG) was synthesized by DNA 2.0 and inserted into the pELECTRA-PagP-His₆ plasmid, generating the sequence shown in Figure 1B and the cTnI[135-209]-SRHW-PagP-His₆ plasmid shown in Figure 2.

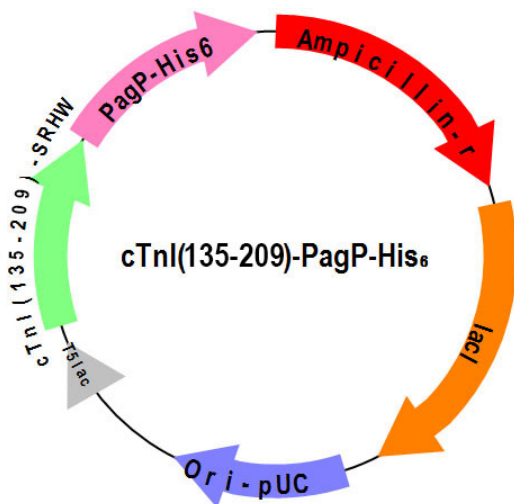


Fig. 2. A map of the cTnI[135-209]-SRHW-PagP-His₆ expression plasmid. The graphic was produced using the website: http://www.premierbiosoft.com/plasmid_maps/index.html, using the SimVector application

Protein expression and purification

The cTnI[135-209]-PagP-His₆ plasmid was transformed into calcium chloride-treated competent BL21(DE3) cells (though we did not need the T7 polymerase encoded by the BL21(DE3) strain). A few transformed colonies were inoculated into 10 mL LB media containing 100 mg/L ampicillin. After reaching an A₆₀₀ of about 1.0, the cells were diluted into one liter of M9 minimal media and allowed to grow overnight at 25°C until A₆₀₀ ~ 0.9. The cells were then induced with 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown for an additional 6 hours at 37°C. The cells were centrifuged at 4,500 x g for 10 minutes and harvested. The wet weight of the pellet was 5.3 g from a 1-L culture.

The cell pellet was resuspended in a total volume of 20 mL lysis buffer: 50 mM Tris, pH 8.0, 10 mM MgSO₄, and 10 μg/mL DNase I. Then, 200 mg deoxycholic acid and 20 mg lysozyme, each predissolved in 1 mL of distilled water, were added to the sample, which was then thoroughly mixed and incubated at room temperature for 20 minutes. The cell lysate was centrifuged at 27,000 x g for 10 minutes, and the pellet was resuspended with 20 mL of 1% Triton X-100 solution containing 5 mM EDTA, and vigorously stirred for an hour, followed by repeat centrifugation at 27,000 x g for 10 minutes. The pellet was washed with water and then dissolved in 20 mL denaturing nickel column binding buffer: 6 M guanidine-HCl, 20 mM Tris-HCl, 0.3 M NaCl, and 10 mM imidazole, pH 7.9. The sample was applied to a 30 mL Ni-NTA

column, which was further washed with binding buffer and then with wash buffer, which was identical to the binding buffer except that 33 mM imidazole was used. Finally, the fusion protein-target protein was eluted with the elution buffer, which contained 250 mM imidazole. Fractions were assessed by UV-vis spectrophotometry at 280 nm and SDS-PAGE.

Nickel cleavage and final purification

Purified cTnI[135-209]-PagP-His₆ protein at 0.1 mM (3 mg/mL) was cleaved with 0.5 mM NiSO₄ in 6 M gdn-HCl, 50 mM HEPES buffer under different conditions of pH and temperature, with the pH corresponding to the 1 M stock solution of HEPES buffer used. No attempt was made to measure the final pH of solutions containing 6 M gdn-HCl, since the high concentration of gdn-HCl complicates pH measurement [17].

After cleavage, the protein sample was dialysed against water to get rid of guanidine-HCl, causing PagP to precipitate, while the target protein remained soluble. PagP was removed by centrifugation. Finally, the sample was lyophilized and then stored at 4°C.

Results

Protein expression and purification

cTnI[135-209]-PagP-His₆ was robustly produced in *E. coli* following induction with IPTG (Figure 3). The predicted molecular weight of the fusion protein is 29.4 kDa, and there is a corresponding band appearing on the SDS-PAGE gel post-IPTG induction (Figure 3, lane 2). Another band is seen at ~24 kDa, corresponding to folded PagP. PagP typically migrates as a double band in SDS-PAGE, with one band corresponding to a folded (β -barrel) form and the other corresponding to an unfolded form. This unusual behavior stems from the fact that PagP, unlike most other proteins, is an integral membrane protein that is capable of forming a folded structure even within an SDS micelle [18]. Depending on the composition of the SDS-PAGE gel, sometimes the folded form of PagP migrates slower and sometimes faster than the unfolded form.

Almost all of the cTnI[135-209]-PagP-His₆ fusion protein appeared in the insoluble fraction (Figure 3, lane 4) and not in the soluble fraction (lane 3), as expected for PagP protein on its own. However, the insoluble fraction was not as pure as when PagP is expressed on its own or with other PagP fusion constructs [9], suggesting that the cTnI[135-209] protein sequence may drag other protein contaminants into inclusion bodies. The cTnI[135-209]-PagP-His₆ fusion protein was purified to >95% purity by nickel affinity chromatography, as judged by SDS-PAGE.

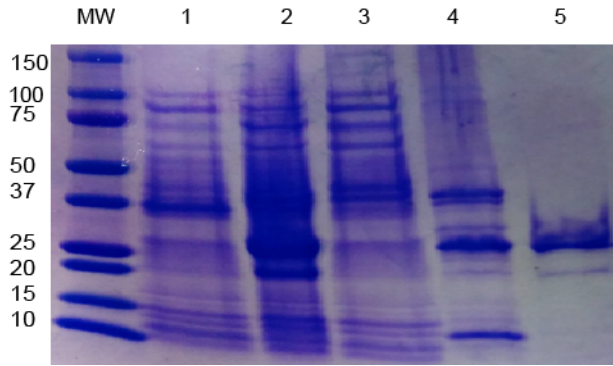


Fig. 3. SDS-PAGE gel stained with Coomassie Brilliant Blue, showing production and purification of cTnI[135-209]-PagP-His₆ protein. Lane 1: whole cells prior to induction. Lane 2: whole cells 6 hours post-induction with IPTG. Lane 3: cell lysate, soluble fraction. Lane 4: cell lysate, insoluble fraction. Lane 5: insoluble fraction dissolved in 6 M gdn-HCl and purified on a Ni-NTA column under denaturing conditions.

Nickel ion-catalyzed cleavage

Ni²⁺-catalyzed cleavage is predicted to generate two fragments, the target protein cTnI[135-209] with a molecular weight of 8.8 kDa, and the fusion partner, SRHWG-PagP-His₆ with a molecular weight of 20.5 kDa. The cleavage reaction produced two fragments, although on SDS-PAGE, SRHWG-PagP-His₆ ran as a doublet at about 20 kDa, corresponding to folded and unfolded forms, and cTnI[135-209] ran as a band at about 12 kDa (see Figure 4). It is possible that the positively charged nature of cTnI[135-209] caused it to migrate slower than expected, and its true mass was confirmed by MALDI-TOF (see below). No additional cleavage products were observed. The sequence most similar to SRHW in our fusion construct was noted to be ¹¹²TWHA (see Figure 1B), though a previous study of combinatorial peptide libraries predicted that 0% cleavage would occur at this site [15]. Indeed no additional fragments corresponding to this potential cleavage site were observed.

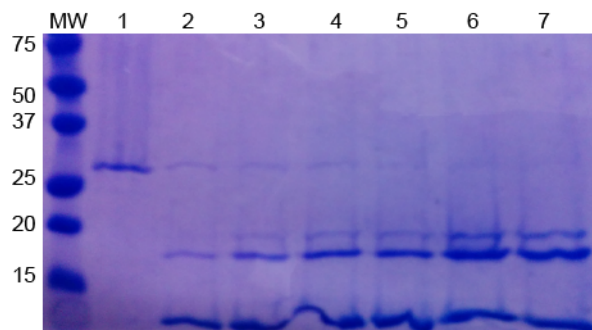


Fig. 4. Nickel cleavage time course. Lane 1: Purified fusion protein before cleavage. Lane 2: After 30 minutes. Lane 3: 60 minutes. Lane 4: 90 minutes. Lane 5: 2 hours. Lane 6: 3 hours. Lane 7: 4 hours.

Previous work had demonstrated that nickel-catalyzed cleavage required high temperatures and high pH. We found near-complete cleavage after 4 hours at 45°C and pH 9.0. Figure 5 shows partial cleavage under milder temperatures and pH conditions. At pH 9.0 and above, nickel hydroxide precipitate was apparent by the end of the incubation.

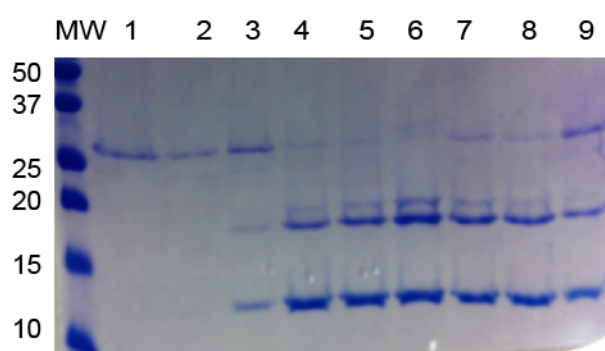


Fig. 5. Different conditions for nickel cleavage of cTnI[135-209]-PagP-His₆, all taken after 4 hours. Lane 1: Purified protein before cleavage. Lane 2: pH 7.0 at 45°C. Lane 3: pH 7.5 at 45°C. Lane 4: pH 8.0 at 45°C. Lane 5: pH 8.5, at 45°C. Lane 6: pH 9.0, at 45°C. Lane 7: pH 9.0 at 37°C. Lane 8: pH 9.0 at 30°C. Lane 9: pH 9.0 at 25°C.

Isolation and characterization of final product

After nickel-catalyzed cleavage, an excess of 15 mM EDTA and 55 mM sodium acetate, pH 4.0 was added, and the reaction mixture was incubated at 50°C for an additional 2 hours. The prolonged incubation under acidic conditions and elevated temperature was necessary to chelate Ni²⁺ that had precipitated out as nickel hydroxide. Following this, the sample was extensively dialyzed against water (for a minimum of 3 days and at least five 4 L changes). This caused PagP and any uncleaved fusion protein to precipitate out of solution, and this was removed by centrifugation. The remaining supernatant consisted mainly of cTnI[135-209] then was syringe filtered through a 0.45 µm filter. Thus, cTnI[135-209] retained its solubility in the presence of insoluble PagP. The purification protocol had also been performed without the prolonged incubation with EDTA under acidic conditions at 50°C. This step was not always needed for the current construct, though other constructs for expressing other fusion partners required this step, since separation from PagP required repeat nickel affinity chromatography (results not shown).

The final product, cTnI[135-209] was lyophilized and stored long-term at 4°C. Amino acid quantitation showed the expected amino acid composition and a final yield of 15 mg from 1 L M9 minimal media. MALDI-TOF mass spectrometry showed that the molecular weight of the final product was 8850, close to the expected molecular weight of 8842. An additional minor peak of unknown significance at 8948 was also observed, though this could be a sulfate ion (added to the cleavage reaction in the form of nickel sulfate).

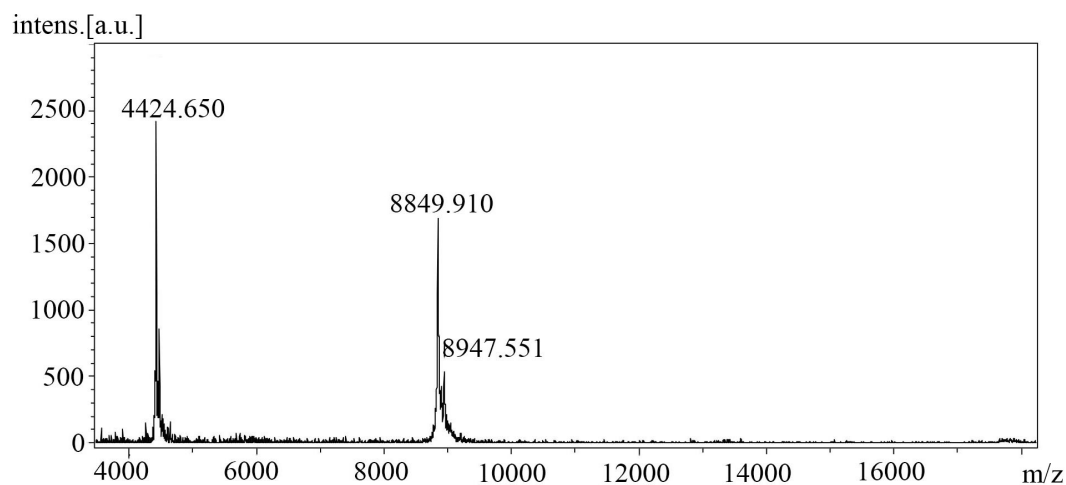


Fig 6. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry of final purified cTnI[135-209] product.

Protein production procedures were also repeated using M9 minimal media supplemented with $^{15}\text{NH}_4\text{Cl}$ and uniformly ^{13}C -labeled glucose to produce double ^{13}C , ^{15}N -labeled cTnI[135-209]. We were able to assign almost all ^1H , ^{13}C , and ^{15}N atom NMR resonances for the sample in aqueous conditions, suggesting that there were no chemical modifications for any of the amino acid residues. Moreover, there were only single NMR resonances for every atom and no multiple peaks suggestive of heterogeneity. As a side note, NMR studies revealed that EDTA was still present in the final product despite extensive dialysis. It is well known but not well documented that EDTA is not readily removed by dialysis for unknown reasons. However, the tenacity of EDTA is likely due to charged interactions with the protein, which could be overcome by including some salts in the dialysate buffer.

Discussion

We have previously demonstrated the utility of PagP as an N-terminal fusion partner for directing target proteins into inclusion bodies [9]. In the current study, we demonstrate that PagP is also effective at targeting proteins to inclusion bodies as a C-terminal fusion partner, so it does not seem to matter that the target protein is translated by the ribosome before PagP. The PagP protein sequence used in the current study also has a C-terminal histidine tag with all internal

methionine residues mutated out, and these changes do not appear to disrupt the ability of PagP to direct fusion partners into inclusion bodies. Use of PagP as a C-terminal fusion partner is advantageous when combined with an SRHW sequence-containing linker region, because Ni²⁺-catalyzed cleavage N-terminal to the serine residue leaves the SRHW sequence attached to PagP, leaving the native C-terminus of the target protein intact.

A previous study identified SRHW as the optimal sequence for Ni²⁺-catalyzed cleavage [15]. However, the influence of residues N-terminal to this sequence has not been studied, and it is possible that certain amino acid types could inhibit or facilitate the reaction. Solvent exposure of the target sequence is another important factor affecting cleavage efficiency. This is more of a concern under physiologic conditions than in the harsh denaturant used in the current study. However, solubility could be an issue even in 6 M gdn-HCl. We have found that some fusion partners are difficult to solubilize, and this may be a function of metal ion binding sites. Our fusion protein construct contains at least two metal ion binding sites, SRHW and HHHHHH. Additional Cys, His, Ser, Thr, Gln/Glu, or Asn/Asp residues could potentially contribute additional weak metal ion binding sites leading to metal-ion-mediated aggregation and poor cleavage efficiencies. We have found that nickel-catalyzed cleavage efficiency varies with the nature of the fusion partner. The construct used in the current study was unusual in that it required only 4 hours to achieve near-complete cleavage. Other constructs we have studied required overnight or even 48-hour incubations (data not shown).

Another factor limiting nickel-catalyzed cleavage efficiency is the inherent instability of the reaction mixture. The solubility product, K_{sp} , of Ni(OH)₂ at 25°C is only 5.48×10^{-16} [19], so that the cleavage reaction could not be substantially enhanced by addition of more Ni²⁺ or base. Under the reaction conditions employed in this study, Ni(OH)₂ precipitate is invariably present at the end of the reaction. Care must be taken to ensure that Ni(OH)₂ does not precipitate at the beginning of the reaction, as can occur if concentrated Ni(OH)₂ stock solution is added to a pH 9.0 solution (thus, NiSO₄ should be diluted before alkaline buffer is added). We note that the pK_a of HEPES buffer (used in the current study as well as in the original nickel cleavage study [16]) is only 7.5, so one would expect the pH to drop significantly as Ni(OH)₂ precipitates out of solution. A pH above 8.0 is beyond the optimal buffering capacity of HEPES. The drop in pH would be expected to directly slow the rate of nickel-catalyzed hydrolysis, but it would also help to keep more Ni²⁺ in solution. A buffer with pK_a > 9.0 would be expected to better maintain the pH of the reaction mixture, and use of CHES buffer (pK_a 9.3) was found to yield slightly better peptide hydrolysis rates. Finally, certain buffers with Ni²⁺-coordinating activity do prevent the formation of Ni(OH)₂ precipitate (like imidazole and Tris), but these also interfered with the peptide hydrolysis reaction (inhibition by imidazole >> Tris).

The primary concern about Ni²⁺-catalyzed peptide bond hydrolysis is the possible effects of high temperature and pH on amino acid sidechains [20] [21] [22]. There was no evidence of oxidation from MALDI-TOF mass spectrometry or multinuclear NMR spectroscopy. In particular, there was no evidence of modification of Phe, Trp, His, or Met, the amino acid residues in cTnI[135-209] that are most sensitive to oxidation. Moreover, NMR analysis found no evidence for hydrolysis of Asn or Gln residues to Asp/Glu, modifications that would not be

detectable by MALDI-TOF. There are no Tyr or Cys residues present in cTnI[135-209]. Cys residues would be inevitably oxidized in the presence of Ni²⁺ and alkaline pH. Thus, they would either need to be protected before the cleavage reaction or reduced afterwards.

Our study further confirms the utility of nickel ion-catalyzed cleavage as a method for cleaving specifically designed linkers for fusion protein expression. We suggest that the technique is most useful in the setting of protein expression from inclusion bodies, where the high concentrations of denaturant employed precludes the use of proteases. The technique is generally applicable, but further experience with a wide range of systems is needed to better define its limitations. Further refinements are needed to allow for less harsh conditions in terms of pH and temperature. Interestingly, other group 10 element ions besides nickel, namely palladium and platinum, have been found to be useful for the cleavage of peptide bonds [23] [24]. A complete exploration of these agents, their mechanism of action, amino acid sequence specificity, and effect of co-ordinating small molecules can be expected to further improve upon the current strategy.

Acknowledgements

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References

- [1] A.K. Dunker, J.D. Lawson, C.J. Brown, R.M. Williams, P. Romero, J.S. Oh, Oldfield CJ, Campen AM, Ratliff CM, Hipps KW, Ausio J, Nissen MS, Reeves R, Kang C, Kissinger CR, Bailey RW, Griswold MD, Chiu W, Garner EC, Obradovic Z., Intrinsically disordered protein, *J. Mol. Graph. Model.* 19 (2001) 26–59.
- [2] P. Tompa, P. Csermely, The role of structural disorder in the function of RNA and protein chaperones, *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* 18 (2004) 1169–1175.
- [3] V.N. Uversky, A decade and a half of protein intrinsic disorder: Biology still waits for physics, *Protein Sci.* 22 (2013) 693–724.
- [4] L.M. Iakoucheva, P. Radivojac, C.J. Brown, T.R. O'Connor, J.G. Sikes, Z. Obradovic, Dunker AK., The importance of intrinsic disorder for protein phosphorylation, *Nucleic Acids Res.* 32 (2004) 1037–1049.
- [5] R.E. Chance, B.H. Frank, Research, development, production, and safety of biosynthetic human insulin, *Diabetes Care.* 16 Suppl 3 (1993) 133–142.
- [6] H. Lilie, E. Schwarz, R. Rudolph, Advances in refolding of proteins produced in *E. coli*, *Curr. Opin. Biotechnol.* 9 (1998) 497–501.
- [7] H. Yamaguchi, M. Miyazaki, Refolding techniques for recovering biologically active recombinant proteins from inclusion bodies, *Biomolecules.* 4 (2014) 235–251.
- [8] P.M. Hwang, J.S. Pan, B.D. Sykes, Targeted expression, purification, and cleavage of fusion proteins from inclusion bodies in *Escherichia coli*, *FEBS Lett.* 588 (2014) 247–252.
- [9] P.M. Hwang, J.S. Pan, B.D. Sykes, A PagP fusion protein system for the expression of intrinsically disordered proteins in *Escherichia coli*, *Protein Expr. Purif.* 85 (2012) 148–151.
- [10] P.M. Hwang, W.-Y. Choy, E.I. Lo, L. Chen, J.D. Forman-Kay, C.R.H. Raetz, Privé GG,

- Bishop RE, Kay LE, Solution structure and dynamics of the outer membrane enzyme PagP by NMR, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 13560–13565.
- [11] P.J. Booth, A.R. Curran, Membrane protein folding, Curr. Opin. Struct. Biol. 9 (1999) 115–121.
- [12] P.M. Hwang, F. Cai, S.E. Pineda-Sanabria, D.C. Corson, B.D. Sykes, The cardiac-specific N-terminal region of troponin I positions the regulatory domain of troponin C, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 14412–14417.
- [13] S. Misawa, I. Kumagai, Refolding of therapeutic proteins produced in *Escherichia coli* as inclusion bodies, Biopolymers. 51 (1999) 297–307.
- [14] E. Gross, B. Witkop, Nonenzymatic cleavage of peptide bonds: the methionine residues in bovine pancreatic ribonuclease, J. Biol. Chem. 237 (1962) 1856–1860.
- [15] A. Krezel, E. Kopera, A.M. Protas, J. Poznański, A. Wyślouch-Cieszyńska, W. Bal, Sequence-specific Ni(II)-dependent peptide bond hydrolysis for protein engineering. Combinatorial library determination of optimal sequences, J. Am. Chem. Soc. 132 (2010) 3355–3366.
- [16] E. Kopera, A. Belczyk-Ciesielska, W. Bal, Application of Ni(II)-assisted peptide bond hydrolysis to non-enzymatic affinity tag removal, PLoS ONE. 7 (2012).
- [17] M.M. Garcia-Mira, J.M. Sanchez-Ruiz, pH corrections and protein ionization in water/guanidinium chloride., Biophys. J. 81 (2001) 3489–3502.
- [18] C. Michaux, N.C. Pomroy, G.G. Privé, Refolding SDS-denatured proteins by the addition of amphipathic cosolvents, J. Mol. Biol. 375 (2008) 1477–1488.
- [19] W.M. Haynes, CRC handbook of chemistry and physics : a ready-reference book of chemical and physical data, Boca Raton, Florida : CRC Press, [2014], 2014.
- [20] W. Vogt, Oxidation of methionyl residues in proteins: tools, targets, and reversal, Free Radic. Biol. Med. 18 (1995) 93–105.
- [21] A. Fontana, D. Dalzoppo, C. Grandi, M. Zambonin, Chemical cleavage of tryptophanyl and tyrosyl peptide bonds via oxidative halogenation mediated by o-iodosobenzoic acid, Biochemistry (Mosc.). 20 (1981) 6997–7004.
- [22] S.W. Griffiths, J. King, C.L. Cooney, The reactivity and oxidation pathway of cysteine 232 in recombinant human α 1-antitrypsin, J. Biol. Chem. 277 (2002) 25486–25492.
- [23] N.V. Kaminskaia, T.W. Johnson, N.M. Kostić, Regioselective hydrolysis of tryptophan-containing peptides promoted by palladium(II) complexes, J. Am. Chem. Soc. 121 (1999) 8663–8664.
- [24] J. Hong, Y. Jiao, W. He, Z. Guo, Z. Yu, J. Zhang, Zhu L., His-Oriented Peptide Hydrolysis Promoted by cis-[Pt(en)(H₂O)₂]²⁺: a New Specific Peptide Cleavage Site, Inorg. Chem. 49 (2010) 8148–8154.