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Optimization and Expansion of a Site-Selective *N*-Methylpyridinium-4-carboxaldehyde-Mediated Transamination for Bacterially Expressed Proteins

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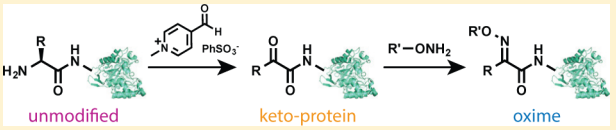
Supporting Information

ABSTRACT: Site-selective bioconjugation methods are valuable because of their ability to confer new properties to proteins by the chemical attachment of specific functional groups. Well-defined bioconjugates obtained through these methods have found utility for the study of protein function and the creation of protein-based materials. We have previously reported a protein modification strategy to modify the N-terminus of peptides and proteins using *N*-methylpyridinium-4-carboxaldehyde benzenesulfonate (Rapoport's salt, RS) as a transamination reagent, which oxidizes the N-terminal amino group to provide a uniquely reactive aldehyde or ketone. This functional handle can subsequently be modified with an alkoxyamine reagent of choice. Previous work had found glutamate terminal sequences to be highly reactive toward RS-mediated transamination. However, proteins of interest are often recombinantly expressed in *E. coli*, where the expression of a glutamate-terminal protein is rendered difficult because the N-terminal methionine derived from the start codon is not cleaved when Glu is in the second position. In this work, we describe a way to overcome this difficulty via the insertion of a Factor Xa proteolytic cleavage site to acquire the optimal glutamate residue at the N-terminus. Additionally, we present studies on alternative high-yielding sequences containing N-terminal residues that can be expressed directly. We have used site-directed mutagenesis to validate these findings on a model cellulase enzyme, an endoglucanase from the thermophilic *Pyrococcus horikoshii*. Activity assays performed with these mutants show that RS transamination and subsequent modification with alkoxyamines have no negative impact on cellulolytic ability.



INTRODUCTION

The attachment of synthetic molecules to proteins with desired structure and function allows for the formation of hybrid materials that can capitalize on the properties of both components. The utility of these bioconjugates has been realized in a variety of contexts, such as cellular imaging,[1] light harvesting,[2] drug delivery,[3] water remediation,[4] and tissue engineering.[5] Traditional methods used in protein modification target the side chains of native amino acid residues, such as lysines and cysteines.[6−8] However, depending on the relative abundance of these residues on the solvent-exposed protein surface, it can be difficult to target them in a controlled and site-specific manner while maintaining native protein function. As a result, many newer techniques for protein modification target uniquely reactive sites, such as C-terminal thioesters,[9,10] N-terminal groups,[11−14] artificial amino acids,[15] and specific recognition sequences for enzymatic ligations.[16−18] Each of these new methods has expanded the scope of bioconjugates that can be accessed, and the applications of well-defined bioconjugates are proceeding apace.

Recently, we reported a transamination reaction mediated by *N*-methylpyridinium-4-carboxaldehyde (Rapoport's salt, RS), which chemoselectively oxidizes the N-terminal amine of a polypeptide or protein substrate to afford a ketone or an aldehyde group.[19] This carbonyl functionality is unique within the protein structure and, therefore, can be used as a handle for appending a synthetic group of choice through oxime or hydrazone formation[20,21] (Figure 1). Relative to other techniques, N-terminal transamination strategies offer mild

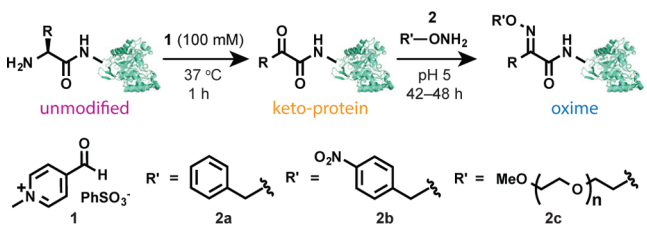


Figure 1. Using Rapoport's salt (RS, **1**), site-specific protein modification can be achieved at the N-terminus. RS converts the N-terminal amine into a ketone or an aldehyde group. This unique carbonyl functionality can subsequently form a stable oxime linkage with a synthetic alkoxyamine reagent. The alkoxyamines used in this study are benzylalkoxyamine (**2a**), nitrobenzylalkoxyamine (**2b**) and $PEG_{5\ kDa}$ alkoxyamine (**2c**).

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reaction conditions and compatibility with free cysteine residues. A particular strength of the RS method over our previously reported pyridoxal 5′-phosphate (PLP) based method[22,23] is the low cost of the *N*-methylpyridinium-4-carboxaldehyde reagent. In addition, it can be recrystallized and stored easily, preventing batch-to-batch variability. These aspects suggest that it could find use in large-scale bioconjugation applications, which are impractical with many other protein modification strategies.

In our initial report of this reaction,[19] a peptide library screening platform identified highly reactive N-terminal sequences, revealing that optimal yields (>80%) could be obtained with glutamic acid-terminal sequences (particularly Glu-Glu-Ser termini). This capability was demonstrated for the chain-specific modification of monoclonal antibodies. However, many proteins of interest are recombinantly expressed in bacterial cells that do not readily allow glutamate residues to be expressed at the N-termini. This is because the N-terminal methionines that arise from the start codons are not cleaved post-translationally when the second residue is large[24] (in contrast, the methionine residues are always removed in eukaryotic organisms). Keeping these expression parameters in mind, we sought to expand the RS-mediated transamination methodology for site selective protein modification of bacterially expressed proteins.

Herein, we show two different strategies for obtaining good yields of N-terminal bioconjugates through bacterial protein expression, followed by RS-mediated transamination. In the first strategy, we inserted a four-residue proteolytic site immediately before the desired N-terminal amino acid. Following expression of this construct in *E. coli*, the protein was cleaved with the protease Factor Xa to obtain the desired EES-terminal protein. Optimization of the RS transamination was then carried out using this model system. In the second strategy, we have turned to recently identified higher pH reaction conditions that increase the reactivity of nonglutamate residues. Taken together, the greater diversity of compatible N-termini and the expanded range of pH values at which this reaction can be performed make this RS-mediated transamination applicable to a significantly larger spectrum of proteins than was previously possible.

RESULTS AND DISCUSSION

Obtaining an EES Terminal Protein and Evaluating Its RS Reactivity. A cellulase was chosen as a protein of particular interest for these studies because of its relevance to the biofuels industry. This class of enzymes has the ability to hydrolyze the cellulose in biomass, releasing glucose for ultimate biofuel generation.[25] In previous studies,[26] we showed that a pyridoxal phosphate (PLP)-based N-terminal bioconjugation strategy could be used to install polymer chains with lower critical solution temperatures (LCSTs) on a thermostable endoglucanase from *Pyrococcus horikoshii* (EGPh).[27] The resulting enzyme could be recovered through modest increases in solution temperature and then reused. One hurdle to the practical use of this system, however, is the relatively high cost of the PLP reagent, limiting the scale on which the bioconjugate could be produced. In contrast, RS can be made from inexpensive precursors (pyridine carboxaldehyde and methylbenzenesulfonate) in high yields.[28] However, as this enzyme is most conveniently expressed in *E. coli*, it can not be generated with the N-terminal glutamate residue identified in the previous RS-transamination study.[19]

EGPh is a monomeric protein that is amenable to site-directed mutagenesis and can be heterologously expressed in high yield (>120 mg per liter of culture). Additionally, it has a solvent exposed N-terminus that is located on the opposite face of the enzyme active site, rendering it accessible for transamination and oxime formation.[29] To begin these studies, site-directed mutagenesis was carried out to obtain an IEGREES-terminal EGPh mutant. IE(or D)GR is the preferred cleavage site for the protease Factor Xa, a commercially available enzyme derived from the blood clotting cascade.[30,31] Factor Xa will not cleave a site followed by a proline or arginine, but remains unbiased toward all other residues if they follow the recognition site. During the expression and N-terminal proteolysis process, however, we found that the cellulase had an unexpected, yet inherent Factor Xa cleavage site of SVIR 20 amino acids away from the C-terminus. Because the His_6 tag that was inserted for ease of protein purification was also directly at the C-terminus, we were able to use Ni-NTA purification following proteolytic cleavage to separate EES-EGPh that was truncated at the C-terminus from EES-EGPh that still retained the His_6 tag (Figures S1 and S2, Supporting Information).

Once Factor Xa had been successfully used to obtain EES-terminal EGPh, the reactivity of the purified protein toward RS-mediated transamination was investigated. To assess the transamination yields, samples were subsequently reacted with nitrobenzylalkoxyamine (**2b**) at pH 4.5 for 42−48 h, followed by LC−MS analysis to quantify the oxime-modified protein, as seen in Figure 2. The long oxime-formation times were selected to ensure complete conversion for quantitative purposes. In some cases, shorter times may suffice, but in our experience, longer incubation times are required to reach high

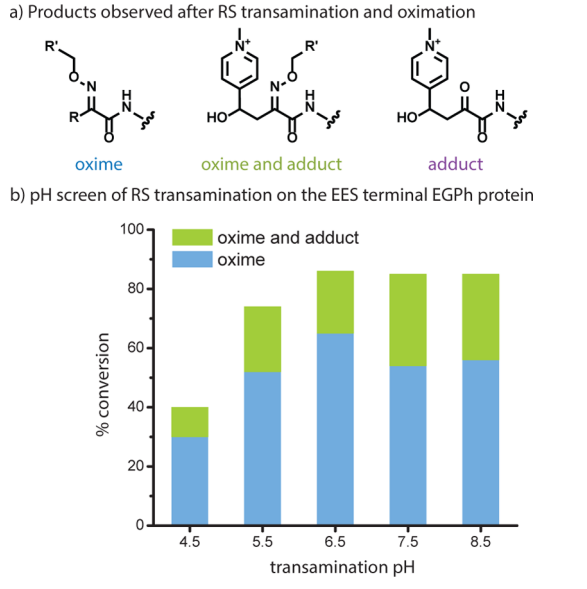

Figure 2. Testing the effect of pH on the EES terminal EGPh protein (after cleavage of the His_6 tag at the C-terminus). (a) Structures are shown for the three observed products. (b) EES N-terminal EGPh was subjected to RS transamination and subsequent oximation with **2b**. The pH during the transamination step was varied, but all other reaction conditions were kept the same. The levels of conversion were quantified using LC−MS. High levels of desired product were observed when the transamination step was performed at pH 6.5, 7.5, and 8.5.

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conversion with these ketone substrates. This screen validated previously published findings, showing that at pH 6.5 the EES-terminal protein underwent high conversion to the desired oxime product (blue). Covalent addition of RS to the N-terminus was also observed, through what is presumed to be an aldol-type addition. This resulted in some amount of product that contained both the oxime and an additional equivalent of RS (green). Additionally, it was observed that the reaction was tolerant to a wider-than-expected range of pH values without sacrificing high yield, a finding that facilitates the modification of proteins that have isoelectric points (pI values) near 6.5.

Studying Reactivity of Alternate N-Terminal Sequences with RS-Mediated Transamination Using Purified Peptides. While it was found that Factor Xa could be used to obtain bacterially expressed EES N-terminal proteins, it would clearly be preferable to identify termini that could be expressed directly in *E. coli* and then modified without the need for an additional protease step. In order to do this, the reactivity properties of other N-termini were also examined using different reaction conditions. More specifically, the transamination efficiency of RS was evaluated with N-terminal sequences beginning with residues that could be directly expressed bacterially. The residues for which the start codon Met will be post-translationally cleaved have been reported to be Gly, Ala, Pro, Ser, and Thr.[24] Therefore, peptides of the form XEEWSNAG were synthesized, where X represented a member of this set. In these model systems, Glu residues were incorporated in the second and third positions in order to retain the overall negative charge. Additionally, to represent those cases in which the Met residue is not cleaved off, a peptide of the sequence MEEWSNAG was included in these studies. Upon synthesis and purification, pH screens were carried out using each peptide. The EESWSNAG peptide was also included so that yield comparisons to the previously identified optimal sequence could be made. Figure 3 shows the pH screen of some of these peptides with Rapoport's salt, followed by oxime formation with benzylalkoxyamine (**2a**). LC−MS was used to quantify the yields. EESWSNAG reached quantitative conversion from pH 6.5 through 8.5, which matched the protein data closely (Figure 3a). The slightly higher yields are likely due to the fact that the N-termini of the peptides are more accessible than the termini of large proteins folded into higher order structures.

For the glycine-, serine-, and threonine-terminal peptides, complex product mixtures were observed due to competing β-elimination and retro-Mannich pathways.[32,33] We therefore do not recommend using these terminal residues with this technique. These data are represented in Figure S5 (Supporting Information). Additionally, the proline terminal peptide showed little-to-no reactivity, and is a useful sequence if one requires reactivity to be turned "off" (Figure 3b). For the methionine terminal peptide, moderate yields (<70%) of desired product were still observed (Figure 3c). The highest yielding sequences were those that retained an alanine residue at the N-terminus. For the alanine-terminal peptide, AEEWSNAG, high oxime yields were observed at both pH 7.5 and pH 8.5, with conversion surpassing 80% in the latter case (Figure 3d). Additionally, no side reactivity was observed other than the aldol addition pathway. With this finding, we concluded that an AEE terminus is an ideal alternative to EES, as long as transamination is carried out at the optimal higher pH values.

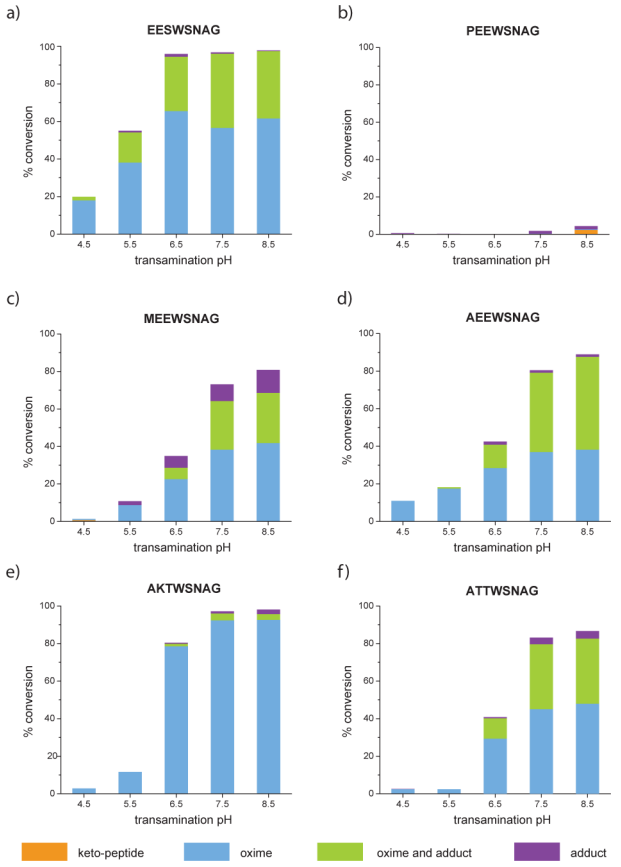

Figure 3. Testing the effect of pH on RS-mediated transamination with multiple N-terminal sequences. The peptides studied were of the sequence XXXWSNAG, in which the first three residues were varied as denoted on each graph. Each peptide was subjected to RS transamination at pH values ranging from 4.5−8.5, followed by oxime formation with **2a** at pH 5.0 for 40−48 h. These results indicated that the previously identified requirement for negative charges holds true at pH 6.5 but can be circumvented if the transmination reaction is performed at higher pH values. The levels of conversion were quantified using LC−MS, and the data shown are an average of three replicate experiments (standard deviation is tabulated in Figure S4, Supporting Information).

From these peptide data, we noticed a trend that regardless of the absolute level of transamination, Rapoport's salt reactivity was improved when the pH was increased from 6.5 to 8.5. EES was reactive at all pH values between 6.5 and 8.5, while the other sequences had significantly higher yields at higher pH values. Since the original library screen for reactive sequences was performed at pH 6.5, this explained why EES was identified. At the higher end of pH values tested herein, the reaction appeared to be less dependent on the specific sequence at the N-terminus. To test this hypothesis, we also screened AKT and ATT terminal peptides. The sequences were chosen because they substituted the presumed beneficial negatively charged penultimate residue with a positive charge and no charge, respectively. The pH screen results of these peptides after RS transamination and oxime formation are illustrated in Figure 3e,f. AKTWSNAG showed high levels of modification from pH 6.5 to 8.5, and ATTWSNAG exhibited a high yield at pH 8.5. These trends validate that at a higher pH, RS is less selective toward the specific sequence at or close to the N-terminus.

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Applying New Findings to a Model Protein System: EGPh. From these peptide studies, we found that alanine at the N-terminus undergoes high levels of conversion with RS. To confirm these results on a protein substrate, AEE- and AKT-terminal EGPh mutants were made through site-directed mutagenesis and expressed. Since the start codon methionine is cleaved when alanine is in the neighboring position, the Factor Xa proteolytic cleavage site was not necessary to obtain alanine-terminal proteins after expression. The mutants were reacted with RS over a range of pH values, followed by oxime formation at pH 4.5. Yields were quantified by LC–MS. Both the AEE-EGPh and AKT-EGPh mutants resulted in high yields of modification at pH 7.5 and pH 8.5 (Figure 4). It should be noted that some of the lack of reactivity at pH 5.5 and 6.5 for these mutants can be attributed to their theoretical isoelectric points being in that range (AEE-EGPh pI 5.77: AKT-EGPh pI: 6.01), making them less soluble during the reaction.

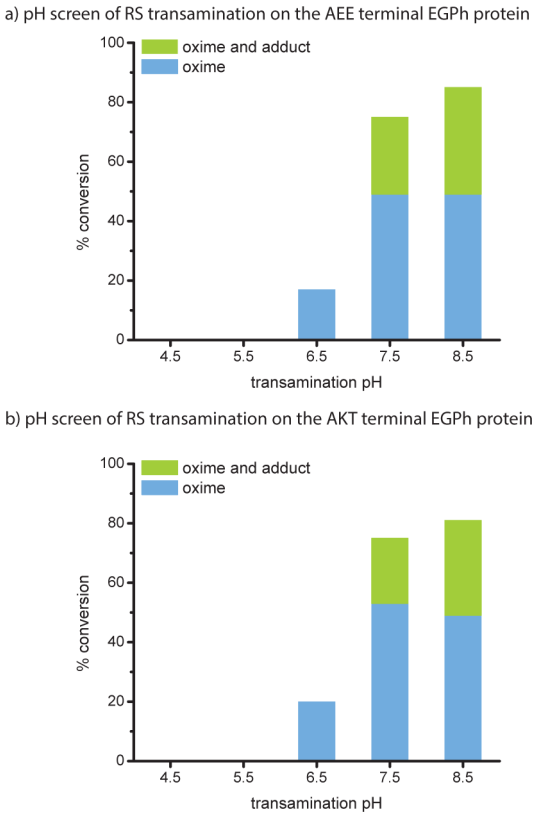

Figure 4. Verifying successful alanine terminal peptide trends on a protein. The proteins were subjected to RS transamination and subsequent oximation with **2b**. The pH values during the transamination steps were varied, but all other reaction conditions were kept the same. At pH 8.5, both (a) AEE-EGPh and (b) AKT-EGPh exhibited over 80% conversion to desired oxime products. The levels of conversion were quantified using LC–MS.

Evaluating the Activity of Modified EGPh. The activities of the modified EGPh proteins are compared in Figure 5. For each mutant type, a small molecule alkoxyamine (**2b**, green) and a $PEG_{5\ kDa}$ alkoxyamine (**2c**, blue) modified sample were assayed. Additionally, an unmodified, nontransaminated control of each mutant was evaluated (pink). In order to evaluate the activity of the EES-terminal mutant systematically, a second control was included with purified protein prior to proteolytic cleavage using Factor Xa. This unmodified IEGREES-terminal

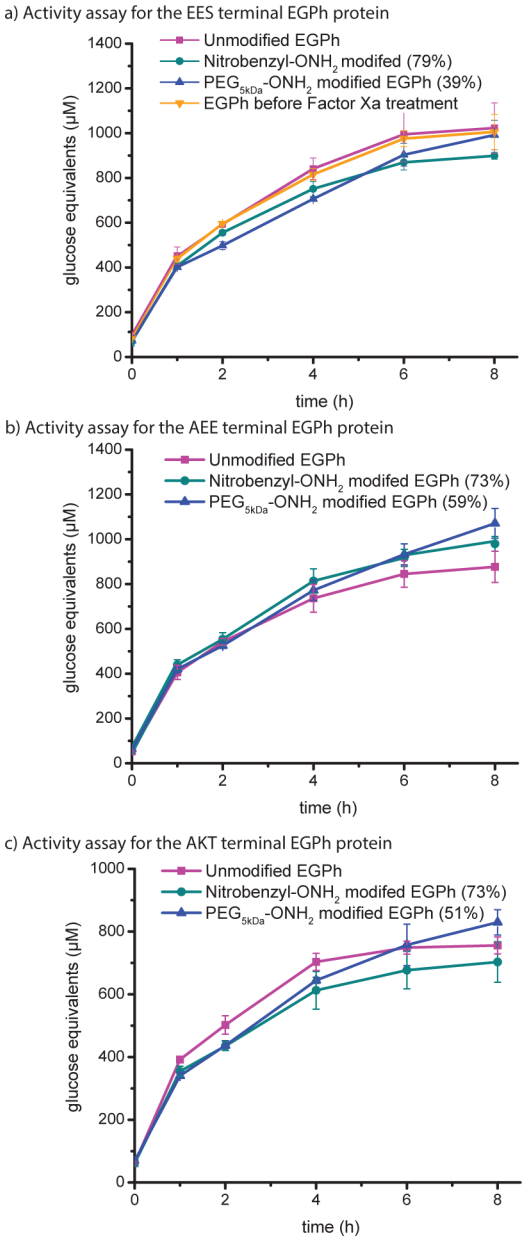

Figure 5. Quantification of cellulolytic activity following RS transamination and subsequent oxime formation with either a small molecule alkoxyamine or a 5 kDa PEG alkoxyamine. Samples were analyzed in triplicate, and error bars indicate standard deviation. Three samples of each mutant were studied: unmodified protein that was not exposed to any modification steps (pink), RS transaminated proteins coupled to nitrobenzyl-ONH_2 (**2b**, green), and $PEG_{5\ kDa}$-ONH_2 (**2c**, blue). Additionally, the IEGREES terminal EGPh protein was used without exposure to Factor Xa. RS transamination was carried out at pH 6.5 for (a) the EES terminal mutant and at pH 8.5 for both (b) the AEE and (c) the AKT terminal mutants. The modification levels within the samples used are denoted in the legend. All modified samples exhibited activity comparable to their unmodified controls over the span of 8 h, indicating that RS transamination, subsequent oximation, and Factor Xa proteolysis all led to unaltered protein activity.

mutant of EGPh was studied in order to ascertain how the truncation at the C-terminus of EES-EGPh affected its activity (orange). As compared to the controls, we observed that both the small molecule modified protein and the $PEG_{5\ kDa}$ modified

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protein exhibited similar activity levels, indicating that the tertiary structure and active site of the enzyme were not affected by the N-terminal modifications made through RS transamination. To be sure the observed activity was not merely due to remaining unmodified protein, an additional control assay was carried out using 50% less EGPh (Figure S11, Supporting Information). As expected, a significant reduction in activity was observed, indicating that the modified enzyme must be responsible for a substantial portion of the activity in Figure 5.

CONCLUSIONS

In the proposed mechanism for transamination, the first step is the formation of a Schiff base at the N-terminus. This is followed by tautomerization due to proton abstraction at the α carbon. With PLP, slightly acidic conditions are required for the subsequent protonation of the nitrogen on the pyridine ring. However, RS is methylated at the analogous nitrogen, and thus protonation is not required. This factor contributes to higher levels of RS reactivity at higher pH values.

We have previously only studied transamination with PLP and RS on proteins at pH 6.5. It was observed through our pH screens herein that RS reactivity for termini that did not contain a glutamate residue was enhanced at higher pH values. At low pH, the requirement for a glutamate residue in the first position could be due to its role as an internal base for the proton abstraction step. Replacing that residue at the first position with a residue that could not provide this type of anchimeric assistance results in the necessity for the reaction to be carried out at higher pH values with the buffer acting as an exogenous base in that step.

These studies have validated and expanded the utility of Rapoport's salt as a protein modification strategy for recombinant proteins. We have confirmed that an EES terminus is a high yielding sequence for pH values ranging from pH 6.5−8.5, and we have also observed that at the upper end of that range, transamination is less restricted to N-terminal sequences that possess negative charges and can be applied to different alanine terminal sequences with high yields (Figure 6). This provides flexibility in the residues inserted at the N-terminus and lowers mutagenesis requirements. This is important because alanine residues at the N-terminus of a recombinantly expressed protein do not prevent cleavage of the starting methionine residue during post-translational processes, thus alleviating the need to insert a proteolytic cleavage site at the N-terminus of the protein.

We have used these findings to demonstrate site-specific modification of three mutants of a cellulase enzyme, EES-, AEE-, and AKT-EGPh in high yields. The enzymatic activity of the protein mutants remained intact following modification, thus confirming that RS-mediated transamination is a mild and simple method for the production of protein bioconjugates. The newly appreciated pH profile of RS-based transamination indicates that alanine terminal sequences give the highest yields of desired product among the residues screened. While combinatorial screening efforts could perhaps identify additional sequences, the work herein provides a clear design lead for expressing proteins in *E. coli* that are compatible with this chemistry.

EXPERIMENTAL SECTION

General Procedure for the RS-Mediated Transamination of Peptides. Peptide and RS stock solutions were prepared at twice the desired final concentrations and mixed in equal volumes in a 1.5 mL Eppendorf tube. The final volume of each reaction was 400 μL. The 2× peptide stock solutions were prepared at 50 μM in 25 mM phosphate buffer (with 0.02% NaN_3) at the desired pH. The 2× RS stock solution (200 mM) was freshly prepared before each reaction in 25 mM phosphate buffer (with 0.02% NaN_3) at the desired pH. The reaction mixture was briefly agitated to ensure mixing and was then incubated without further agitation at 37 °C for 1 h. Following the reaction, the excess aldehyde was removed using a Sep-Pak C18 1 mL vac cartridge (Waters, Milford, MA). Elution was carried out with 100% acetonitrile. The resulting keto-peptide solution was concentrated through evaporation and lyophilization. The white lyophilized powder was resuspended in 40 μL of 25 mM phosphate buffer (with 0.02% NaN_3), pH 5.0, and was then treated with the alkoxyamine stock solution in a 1.5 mL Eppendorf tube and incubated at room temperature for 48 h. The alkoxyamine stock solution used was a 250 mM benzyl-ONH_2 solution (in water, pH adjusted to pH 5.0). A 10 μL portion of the stock solution was added to give a final concentration of 50 mM benzyl-ONH_2. Controls were conducted following the same procedure, but without RS. After oxime formation, the modified peptide sample was analyzed using mass spectrometry.

N-terminus (XXXWSNAG)	Percent conversion at pH 6.5	Percent conversion at pH 8.5
AEE*	41†	88†
AKT*	80‡	96†
ATT*	40	83
GEE*	40	55
PEE*	0	0
SEE*	30	59
TEE*	6	28
MEE*	29	69
*Sequences that can be used to directly express proteins in bacteria when inserted at the N-terminus		
EES**	95†	98†
**Sequence that does not allow post-translational methionine cleavage in bacteria and requires the use of a proteolytic cleavage site for expression		
†Similar result observed for proteins		
‡Similar result not observed for proteins		

Figure 6. Summary of conversion results from the XXXWSNAG peptides studied with RS transamination at pH 6.5 and 8.5. At higher pH, peptides exhibited less dependence on nearby negative charges. Alanine terminal peptides proved most successful, as they led to high yields and do not prevent post-translational cleavage of methionine at the N-terminus in proteins expressed bacterially. The numbers are a sum of all products that retained the oxime linkage. For a detailed breakdown of the product distribution, see Figure 3 and Figures S4 and S6 (Supporting Information).

General Procedure for RS-Mediated Transamination of Proteins. Transamination of the EGPh N-terminus was performed using a previously reported method.[19] Protein and RS stock solutions were prepared at twice the desired final concentrations and mixed in equal volumes in a 1.5 mL Eppendorf tube. The final volume of each reaction was 200 μL. The 2× protein stock solutions were prepared at 50 μM in 25 mM phosphate buffer (with 0.02% NaN_3) at the desired pH. The 2× RS stock solution (200 mM) was freshly prepared before each reaction in 25 mM phosphate buffer (with 0.02% NaN_3) at the desired pH (using RS recrystallized from acetonitrile). The reaction mixture was briefly agitated to ensure mixing and then incubated without further agitation at 37 °C for 1 h. Following the reaction, the excess aldehyde was removed, and the resulting keto-protein solution was concentrated and buffer exchanged using 0.5 mL spin concentrators with a MWCO of 10 kDa (Millipore, Billerica, MA). The buffer exchange first involved the dilution of each sample to 500 μL with 25 mM phosphate buffer (pH 4.5). Each sample was then

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concentrated to 100 μL, and the process was repeated five times. The resulting keto-protein was then treated with an equal volume of the alkoxyamine stock solution of choice in a 1.5 mL Eppendorf tube and incubated at room temperature for 48 h. The alkoxyamine stock solution concentrations were the following: 62.5 μM nitrobenzyl-ONH_2 (in 25 mM phosphate buffer with the pH adjusted to 5.0), and 5 mM $PEG_{5\ kDa}$-ONH_2 solution (adjusted to pH 5.0 in water). After oxime formation, the protein concentration and buffer exchange steps were again repeated to remove the excess alkoxyamine. Controls were conducted following the same procedure but without RS. Analysis of the protein modification was carried out with mass spectrometry for the nitrobenzyl-ONH_2 modified samples, and with an SDS-PAGE gel for the $PEG_{5\ kDa}$-ONH_2 modified samples (Figures S8 and S9, Supporting Information).

Expression and Purification of AKT-EGPh, AEE-EGPh, and IEGREES-EGPh. Expression of AKT-EGPh was performed following a previously reported method.[26] The AKT-EGPh plasmid was used to obtain the AEE-EGPh plasmid using standard techniques. Additionally, further rounds of mutation were carried out to obtain the IEGREES-EGPh plasmid. The primers used to carry out the sequential mutations as well as the subsequent expression and purification are described in the Supporting Information.

Protein Quantification. EGPh concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific), with an extinction coefficient at 280 nm of 139,020 M^{-1} cm^{-1}. The molecular weights of the different mutants were as follows: IEGREES-EGPh, 49 524 Da; EES-EGPh, 46 742 Da; AEE-EGPh, 49 052 Da; AKT-EGPh, 49 023 Da.

Activity of Modified EGPh. All protein samples were assayed in triplicate in 1.5 mL Eppendorf tubes containing stir bars at 40 °C, using a 1% (w/v) suspension of Sigmacell cellulose powder (Sigma-Aldrich) in pH 4.5 buffer and with 0.2 μM protein. To measure the extent of the reactions, each tube was shaken vigorously to ensure even distribution of the substrate and protein, and a 100 μL aliquot was immediately removed and transferred to a clean, empty Eppendorf tube. This aliquot was centrifuged for 1 min at 13.2*k* rpm, and then the clarified supernatant was transferred to a 0.6 mL Eppendorf tube and immediately frozen in dry ice. The supernatant aliquots were stored at −20 °C until analysis for the amount of soluble reducing sugar.

Analysis of Soluble Reducing Sugar. This procedure was performed following a previously reported method[34] using a paired glucose oxidase−peroxidase assay with OxiRed as the substrate. Analysis was performed in clear-bottom plastic 96-well plates, with each sample analyzed in triplicate. Internal standards of 300, 200, 100, 50, 25, and 0 μM glucose, and 150, 100, 50, 25, and 12.5 μM cellobiose in pH 4.5 buffer were included in each plate. Frozen aliquots from the activity assays were thawed on ice and then diluted 0- to 20-fold with cold buffer, and then 8 μL of the solution was incubated with 8 μL of β-glucosidase (5 mg/mL in 10 mM NaOAc pH 4.6) for 60 min at 37 °C to convert all of the cellobiose to glucose. The amount of glucose present was then measured by adding 65 μL of glucose oxidase (1.25 U/mL), horseradish peroxidase (1.25 U/mL), and OxiRed (60 μM) in 125 mM phosphate buffer (pH 7.45) and incubating at room temperature for 10 min in the dark. The amount of resorufin formed was measured on an optical plate reader with excitation at 535 nm and emission detection at 590 nm. The amount of resorufin formed corresponded to the amount of glucose present. Linear standard curves were made from the internal standards in each plate (all $R^2 > 0.97$), which were then used to calculate the amount of glucose equivalents present in each activity assay sample. The triplicate measurements of each supernatant sample were averaged, and then the measurements of the triplicate activity assay samples were averaged to calculate each data point.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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