SNAC-tag for sequence-specific chemical protein cleavage

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Site-specific protein cleavage is essential for many protein-production protocols and typically requires proteases. We report the development of a chemical protein-cleavage method that is achieved through the use of a sequence-specific nickel-assisted cleavage (SNAC)-tag. We demonstrate that the SNAC-tag can be inserted before both water-soluble and membrane proteins to achieve fusion protein cleavage under biocompatible conditions with efficiency comparable to that of enzymes, and that the method works even when enzymatic cleavages fail.

Protein cleavage is an integral step of recombinant protein expression and purification processes. Different tags are often fused to the N terminus or C terminus of target proteins to improve expression yield, solubility, folding or purification 1,2. However, after the target protein is purified, removal of these tags is often desired. Currently, the only strategy to remove fusion tags under biocompatible conditions is enzymatic cleavage using proteases, including tobacco etch virus (TEV) protease and thrombin protease 3. Unfortunately, the enzymes added often need to be removed, which adds one more purification step. Enzymes can also be prohibitively expensive, especially when proteins are to be produced at large scale. Moreover, enzymatic cleavages often fail, and this is especially common for membrane proteins when the enzyme recognition site is proximal to the hydrophobic domain. Finally, detergents and denaturants, which are widely used to solubilize membrane proteins or proteins expressed in inclusion bodies, inactivate common proteolytic enzymes used to cleave tags 1.

One approach to circumvent enzymatic cleavage and its drawbacks is chemical cleavage. Cyanogen bromide has been used for chemical protein cleavages, but under harsh conditions with low sequence specificity (recognizing a single Met residue) 4. Metal ions including Pd 2+, Cu 2+ and Ni 2+ have also been explored to this end 5–8. The use of Pd 2+ and Cu 2+ has major obstacles, but Ni 2+ has shown more potential. It has been shown that Ni 2+ can cleave the sequence pattern –XXHZ– before the Ser residue 9. The best-optimized sequence was inserted in the mini-protein SPI-2 to show that cleavage yield under the same conditions. These data together designate the resulting sequence –GSHHW– as the sequence-specific pattern for cleavage using Ni 2+ ion (Fig. 1b).

We found that Gly at the P 1 position is critical for high cleavage efficiency. A Gly-to-Ala replacement leads to a dramatically reduced cleavage rate (Supplementary Fig. 2). This can be rationalized through the proposed cleavage mechanism (Supplementary Fig. 1), involving an Ni 2+–assisted N-to-O acyl shift of the P 1 carbonyl to the P 2 Ser side chain 10. The resulting ester intermediate is then cleaved during the rate-limiting ester hydrolysis, and hence is sensitive to steric effects that slow hydrolysis for residues other than Gly 10. We therefore introduced a Gly at position P1 of a previously reported cleavage sequence 7 to give –GSRHW–. This peptide performed slightly better than the best phage-selected sequence (–GSHHTDLP–) indeed performs better than Arg (–GSRHW–) when embedded in a short peptide construct (Fig. 1d,e). We then did a cleavage comparison without or with the critical Gly insertion on the mini-protein SPI-2 (ref. 7) (Supplementary Fig. 3). With the critical Gly insertion, the cleavage progressed to more than 90% yield (22 °C, 16 h). As expected, absence of the Gly gave only 15% cleavage yield under the same conditions. These data together designate the resulting sequence –GSHHW– as the sequence-specific nickel-assisted cleavage (SNAC)-tag, which was generated from the combined efforts of our phage selection, validation using synthetic peptides and previous reports.

We also examined the effect of buffers and exogenous nucleophiles on the rate of the cleavage reaction. We found that CHES and HEPES buffers performed the best (Supplementary Fig. 4), whereas the weak nucleophile acetone oxime could accelerate cleavage slightly without inducing nonspecific cleavages (Supplementary Fig. 4). Encouraged by the activity of the SNAC-tag, in peptides and a small protein, we sought to examine its utility as a cleavable tag in a number of difficult cases where fusion-tag enzymatic cleavage is very inefficient. We first examined its utility in conjunction with a His-tag (6×His) fused to the N terminus of a water-soluble α-helical

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bundle protein, HB2225. Between the His-tag and HB2225, a cleavage site was included: either TEV (–ENLYFQS–; Fig. 2a) or a SNAC-tag (–GSHHW–; Fig. 2b). Cleavage by TEV protease under standard conditions was mostly incomplete (Fig. 2a). However, cleavage of the SNAC-tag-containing construct yielded approximately 80% cleavage into the desired product (Fig. 2b), with good reagent compatibility (Fig. 2c). This result demonstrates that the SNAC-tag can indeed achieve biocompatible cleavage in full-sized globular proteins when enzymatic cleavage fails.

We next explored the utility of SNAC-tag for membrane-protein cleavage. In particular, we sought to cleave a construct consisting of a designed membrane protein 3hbtmV2 fused to T4 lysozyme via a TEV-cleavable linker. After screening a number of constructs, we found only a very low level of cleavage with TEV (less than 20%) (Fig. 2d). By contrast, replacing the TEV substrate sequence with the SNAC-tag (–GSHHW–) at the same site resulted in more than 90% cleavage (Fig. 2e). This demonstrates that SNAC-tag is perfectly compatible with cleavage of membrane proteins and detergent micelles. The SNAC-tag cleavage was further tested in two more water-soluble proteins and one more membrane protein; all cleavages achieved more than 80% completion at pH 8.2, 22°C, 18 h (Supplementary Fig. 7). In another T4L fusion membrane protein (T4L–PL5) in which thrombin cleavage was met with difficulty (Supplementary Fig. 8), we inserted SNAC-tag to replace the thrombin cleavage sequence, and found that SNAC-tag could be effectively cleaved (Supplementary Fig. 8).

We investigated the effect of different buffer and detergent conditions on the SNAC-tag cleavages (Fig. 2c–f,g). Cleavage was fully compatible with common mild detergents, although the reaction was partially inhibited by sodium dodecyl sulfate (SDS) (Fig. 2g). We found that strong denaturing conditions including 6 M guanidinium chloride or 8 M urea also were compatible with cleavage, though with decreased efficiency. Lower concentrations of up to 2 M guanidinium chloride or urea did not obviously affect cleavage efficiency (Supplementary Fig. 9). The SNAC-tag was most efficiently cleaved at pH 8.6, with a small decrease in yield at lower (8.2) and higher (9.0) pH. Removal of salt had little effect on T4L–3hbtmV2 cleavage, and only a modest effect for His-tag–HB2225 cleavage. We also found that HEPES and CHES buffers performed comparably, whereas the weakly Ni2+-chelating Tris buffer slowed the cleavage rate. The Ni2+ concentration at 1 mM seemed to be necessary for efficient cleavage. Low levels of reducing agent (1 mM TCEP, 0.5 mM DTT) were compatible, and in fact cleavage of a Cys-containing peptide showed negligible oxidation (Supplementary Fig. 10). For proteins that require low-temperature handling, cleavage at 4°C was approximately four times slower than that at room temperature.

We studied the potential for cleaving SNAC-tag from proteins bound to Ni2+-nitrilotriacetic acid (NTA) resins, via a His-tag. One milligram of His-tag–T4L–GSHHW–3hbtmV2 was loaded onto a bed volume of 0.5 ml of Ni-NTA resin. After exchange into cleavage buffer, 1 mM NiCl2 was added, and the reaction was incubated overnight at 22°C (Supplementary Fig. 11). On-resin cleavages proceeded nearly to completion, with efficiency similar to that observed in solution. No His-tag-containing protein was eluted off the beads during cleavage, nor did we observe protein cleavage while SNAC-tag-containing proteins were purified with Ni-NTA resins. Thus, SNAC-tag cleavage is compatible with Ni-NTA purifications and on-resin cleavages18. However, significant scale-up of protein loading for on-resin cleavage (50 mg protein per ml bed volume of Ni-NTA resin) resulted in visible protein precipitation as the cleavage proceeded, which suggests that loading is an important variable to consider for on-resin applications.

Knowing that Gly at the P1 position plays a key role in the rate of hydrolysis, we generated a second targeted phage library (–X1X2X3GSX4HX5X6X7–) to further explore the influences of nearby residues on cleavage efficiency. We carried out four more rounds of selection and sequenced the final output library. We ranked each particular sequence observed in the output library (normalized to the control library: the same selection protocol in the absence of Ni2+) shown in Supplementary Table 2. Guided by this analysis, we then picked 11 distinct sequences from this selection and the top two sequences from the initial phage selection to replace the TEV substrate site in His-tag–HB2225 and in His-tag–T4L–3hbtmV2 for Ni2+ cleavage. We confirmed that several of these SNAC-tag variants yielded more than 80% cleavage under our standard conditions (Fig. 2h). For the watersoluble His-tag–HB2225, efficiency was slightly lower (Fig. 2i). This result indicates that a number of diverse sequence combinations can be used to achieve high cleavage efficiency. All the high-ranking cleavage sequences are listed in Supplementary Table 2. To achieve high cleavage efficiency, Gly at P1, Ser at P1′, and His P3′ seem to be mandatory, whereas hydrophobic residues at P2′ are not favored. For the sequences listed in Fig. 2h,i and Supplementary Table 2, the specific sequences seem to be important to achieve high cleavage efficiency, though we did not investigate further to find the minimal sequence for each individual sequence. Furthermore, cleavage requires correct geometry of the Ni2+ complex (Supplementary Fig. 1), and is likely to be achieved only in poorly structured protein regions19,17.
We also systematically evaluated the effect of the surrounding residues by screening hundreds of peptides at five nearby positions (Supplementary Tables 3 and 4). Peptide sequences with faster cleavage than –GSHHW– were identified. However, they did not perform better when inserted in proteins (Supplementary Fig. 12).

In summary, we have developed the SNAC-tag as a new chemical protein-cleavage strategy. The SNAC-tag can be inserted into both water-soluble and membrane proteins to achieve biocompatible protein cleavage in a sequence-specific manner with efficiency comparable to that of enzymes. The method is particularly attractive because it leaves only a small Gly residue at the C terminus of the released protein when it is used as a C-terminal tag. Additionally, this approach circumvents the demands of subsequent enzyme removal. Critically, we show cases where the SNAC-tag can succeed when enzymatic cleavage fails. We suggest using –GSHHW– as a general SNAC-tag, although – GSRHW– is also efficiently cleaved. Cleavage conditions are as follows: protein concentration approximately 1 mg ml\(^{-1}\), 1 mM NiCl\(_2\), 0.1 M CHES buffer, pH 8.2, incubation at room temperature, 0.1–0.5 M NaCl. For specific sequence requirements and additional cleavage details, consult the Methods.

We expect the SNAC-tag to greatly reduce the cost and labor of recombinant-protein production at both laboratory and industrial scale and to find broad applications throughout medicine, basic biological research, and biotechnology.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability, and associated accession codes are available at https://doi.org/10.1038/s41592-019-0357-3.

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Author contributions
B.D. and W.F.D. designed the project. B.D. carried out most of the experiments. M.M. helped with data analysis and some protein expression. H.H. helped with data analysis. N.S. helped with one protein expression. B.M. helped with phage library construction and selection of experiment design.

Competing interests
The authors declare no competing interests.

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Methods

A step-by-step protocol for SNAC-tag cleavage is available at Protocol Exchange.18

Reagents. All reagents were used without further treatment. Fmoc-protected amino acids were purchased from GL Biochem. 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylxylidinium hexafluorophosphate (HCTU), trifluoroacetic acid (TFA), and hydroxybenzotriazole hydrate were purchased from Chem-Impex International. 4-Methylpiperidine was purchased from Acros Organics. Rink Amide-ChemMatrix resin (0.5 mmol g−1 loading) was purchased from Biotage. All other reagents, including 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylxylidinium hydrochloride (TCPH), DL-dithiothreitol (DTT), N,N-dimethyleedodecylamine N-oxide, N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (C12 Betaine), and Triton X-100 were purchased from Sigma-Aldrich. Fos-choline-12 (DPC) and decyl-N-dimethyl-3-ammonio-1-propanesulfonate (C12 Betaine) was purchased from Genesee Scientific Corporation.

Protein gels and DNA gels. Precast NuPAGE 4–12% Bis-Tris polyacrylamide protein gels were purchased from Invitrogen. Protein gels were run at 200 V for 35 min at 50 °E/M buffer. Gels were then washed nine times with warm water and stained with Coomassie blue for 4 h. They were then destained with tap water for 16 h before we imaged them on a Bio-Rad ChemiDoc MP imaging system. All protein bands were analyzed and quantified using ImageJ.

Each peptide was purified and analyzed by reverse-phase HPLC and mass spectrometry. The peptides were purified on a C18 column (1.5 μm C18, 50 × 2.1 mm column). Chromatographic separations were obtained using a linear gradient of 1–61% acetonitrile (with 0.08% TFA) in water (with 0.1% TFA) over 40 min with the column at room temperature. Reverse-phase HPLC and mass spectrometry. A standard double coupling was done for 8 min at room temperature or 5 min at 37 °C before cell collection. Cell pellets were resuspended in buffer containing 0.125 M amino acids. A shallow gradient of acetonitrile (with 0.08% TFA) versus water (with 0.1% TFA) was designed for UV absorption at 220 nm, and mass spectrometry data were obtained using a reverse-phase HPLC and mass spectrometry.

Table 5) were then introduced into the pET-28a vector containing the HB2225 and 3hbtmV2 genes after Gibson assembly protocol (New England Biolabs).

Protein expression, purification, and cleavage. Initial HB2225 and 3hbtmV2 genes were introduced into a PET-28a vector (Novagen) by Gibson assembly; their sequences were confirmed (Genewiz San Francisco), and they were then transformed and expressed in One Shot BL21(DE3) chemically competent E. coli (Thermo Fisher Scientific). Different cleavage peptide sequences (Supplementary Table 5) were then introduced into the pET-28a vector containing the HB2225 and 3hbtmV2 genes after Gibson assembly protocol.
buffer containing 50 mM Tris, pH 8.0, 300 mM NaCl, 2% glycerol, 5 mM DPC and lyzed by sonication, and cell debris was subsequently removed by centrifugation (18,000 r.p.m. and 4 °C for 30 min). The soluble cell lysate was purified with Ni-NTA affinity chromatography.

For cleavage of HB2225 constructs, proteins were first exchanged into buffer containing 0.1 M CHES, 0.1 M acetone oxime, 0.1 M NaCl at appropriate pH, and 1 mM NiCl2 was then added, after which the solution was mixed well and left at room temperature (approximately 22 °C) for cleavage to proceed without shaking or stirring. Imidazole from Ni-NTA elution needs to be removed completely before cleavage. For cleavage of 3hbtmV2 constructs, the procedure is essentially the same as for HB2225, except appropriate detergents need to be added to the cleavage solutions. Cleavage efficiency was quantified using ImageLab 5.2.1 on the Bio-Rad ChemiDoc MP imaging system. A time-course cleavage of His-tag–T4L–ENLYFQS–3hbtmV2 and His-tag–GSHHW–HB2225 is shown in Supplementary Fig. 13. TEV protease was purchased from Sigma-Aldrich. TEV cleavage was carried out under standard conditions: protein 1 mg ml\(^{-1}\), 50 mM Tris, pH 8.0, 0.5 mM EDTA, 1 mM DTT, 0.25 M NaCl, 22 °C, 5 mM DPC, 16 h, TEV 0.04 mg ml\(^{-1}\). We also tried TEV cleavage at 34 °C for His-tag–T4L–ENLYFQS–3hbtmV2 and His-tag–ENLYFQS–HB2225, and observed no obvious differences compared with 22 °C cleavage. We ran a positive control of TEV protease cleavage on a different protein that could be efficiently cleaved by TEV protease to show that TEV protease we obtained indeed was of good quality (Supplementary Fig. 14).

Thrombin was purchased from EMD Millipore. Cleavage conditions were as follows: 50 mM Tris, pH 8.4, 150 mM NaCl, 0.5 mM CaCl\(_2\), 5 mM DPC, 22 °C, 16 h. One thrombin unit is defined as the amount of enzyme required to cleave 1 mg of a test protein when incubated in standard digest buffer at 20 °C for 16 h.

Pep tide cleavage screening. As an alternative approach to explore the influence of nearby amino acids on cleavage efficiency, we made approximately 100 peptides (Supplementary Table 3), varying the amino acid identity individually around the cleavage site in the peptide WLX\(_1\)X\(_2\)SX\(_3\)HX\(_4\)X\(_5\). Peptide cleavage was carried out under cleavage conditions described above in Fig. 1; cleavage yields were quantified using the Agilent HPLC online system by integration of peak area. The sum of the extinction coefficient for the two product peaks was assumed to be the same as that of the initial peptide. We found that position X\(_1\) had minimal impact on cleavage; position X\(_2\), His and Asn performed the best, followed by Lys and Arg; and position X\(_4\) preferred Pro. We then further picked approximately 30 sequence combinations (Supplementary Table 4) and tested the cleavage efficiency. We found two sequence combinations (–PGSHHW– and –HNSHHW–) that indeed gave better cleavage efficiency (Supplementary Fig. 12). But when we then inserted these two sequences individually into the globular protein and membrane protein construct we had tested previously, unfortunately, they did not perform better (Supplementary Fig. 12). Thus, for searching best protein cleavage sequences, peptide models did not seem to be the best system. Protein-based selections such as phage display are probably more reliable, as we did here. Another thing to point out is that the amino-acid preference at the X\(_4\) position did not fully hold up in a different peptide system (Supplementary Table 4), so we think Gly is still the best amino acid to use for best cleavage at the X\(_4\) position.

A few tips for running cleavage reactions. For peptide cleavage tests, lyophilized peptides were dissolved in cleavage buffer (0.1 M CHES, 0.1 M acetone oxime, pH 8.2 or otherwise noted in the text) at 0.2 mM concentration; 1 mM NiCl\(_2\) was then added for the reaction to proceed without stirring or shaking.

We tested the compatibility of single unpaired cysteine with cleavage conditions in a peptide sample (Supplementary Fig. 10) and observed no obvious Cys oxidation during the cleavage process. In cases where cysteine oxidation is observed, 1–2 mM TCEP could be added to keep cleavage under reducing conditions to minimize oxidation while keeping the cleavage rate essentially the same.

For the cleavage of short peptides, pH does make a big difference: cleavage rates increased about twofold from pH 8.2 to pH 8.6, as well as from pH 8.6 to pH 9.0 (Supplementary Fig. 15). However, this trend of rate increase did not hold for the two protein cleavage examples. We therefore recommend doing cleavage at pH 8.6, as it yielded slightly better performance in the two protein examples shown in Fig. 2.

For some proteins, adding 1 mM NiCl\(_2\) could cause protein precipitation. Under this situation, adding 0.5 M GuHCl to the protein solution before mixing protein with Ni\(^{2+}\) could dramatically reduce protein precipitation without affecting cleavage efficiency (Supplementary Fig. 9), as most of the proteins cannot be denatured with this diluted GuHCl concentration; we recommend using 0.5 M GuHCl when protein precipitation occurs. Alternatively, 1 mM TCEP could be added to the protein solution before the addition of NiCl\(_2\), as TCEP binds with Ni\(^{2+}\) weakly and could minimize nonspecific metal-mediated protein–protein interactions, thus preventing protein precipitation. Other weak Ni\(^{2+}\) ligands could potentially be explored to achieve the same result.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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