Affinity Purification of Tag-Free *Shewanella oneidensis* Cystathionine Beta-Lyase (MetC) Using the Profinity eXact™ Fusion-Tag and Profinia™ Protein Purification Systems

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Introduction

Shewanella oneidensis MR-1 is a non-pathogenic gram-negative bacterium used as a model organism due to its versatile metabolic and respiratory capabilities, which include a number of novel mechanisms for electron transfer to insoluble metals. *S. oneidensis* can grow both aerobically and anaerobically and uses a variety of compounds such as solid metals and radionuclides as terminal electron acceptors (DiChristina et al. 2005). The molecular mechanisms of electron transfer to insoluble metal oxides however, remain poorly understood. Such mechanistic studies have been hampered by the difficulty of generating milligram quantities of pure, native proteins for crystallography and in vitro enzymatic studies.

Cystathionine β-lyase (MetC, CBL), which cleaves cystathionine to generate L-homocysteine, is a crucial enzyme involved in methionine biosynthesis (Dwivedi et al. 1982). L-homocysteine is further methylated to form L-methionine (Martel et al. 1987). MetC can also act as a carbon-sulfur (C-S) lyase for other substrates containing C-S linkages (e.g., L-cysteine, L-cystine). MetC may therefore play an important role in the central metabolism of *S. oneidensis* by regulating sulfur-containing compounds that can affect redox-balance. Although *S. oneidensis* MR-1 MetC displays an amino acid similarity to *E. coli* MetC, the structural and functional characteristics unique to the *S. oneidensis* MetC protein are not well understood.

Purification of soluble, cytoplasmic proteins is traditionally performed using affinity chromatography following overexpression of a recombinant fusion protein in *E. coli*. Most common fusion tags, such as glutathione-S-transferase (GST) or polyhistidine (HIS) sequences, allow the target protein to bind an immobilized ligand on an affinity column. However, fusion tags remain attached to the target protein following purification and may inhibit crystallization or lead to artificial results in functional assays. Thus, it is often necessary to introduce an enzymatic cleavage site between the target protein sequence and the fusion tag to facilitate purification of a tag-free protein. This is a laborious and time-consuming multistep process that includes removal of tags and proteases. The Profinity eXact fusion-tag system employs a novel one-step affinity purification and tag-removal process that produces native protein free of the fusion tag, often in less than one hour (Oganesyan and Strong 2007).

The Profinia protein purification system performs automated affinity chromatography of recombinant fusion-tagged proteins with a subsequent integrated desalting step. The Profinia system uses preprogrammed methods and prepacked affinity cartridges and is capable of generating milligram quantities of protein in as little as 30 minutes. The Profinity eXact fusion-tag purification system is ideally suited for use with the Profinia system, enabling one-step, automated affinity purifications with subsequent cleavage of the fusion-tag and exchange of the native, tag-free protein into the buffer of choice.

In this study, the Profinity eXact fusion-tag was used with the automated Profinia purification methods in order to obtain sufficient quantities of purified protein for activity assays of MetC. Expression vectors containing the *S. oneidensis* MR-1 MetC coding region were constructed with an N-terminal Profinity eXact fusion-tag. Purifications were performed and optimized on multiple scales using both chemical and mechanical lysis (sonication) methods. In addition, the impact of cleavage time was examined and protein yield, purity, and activity were monitored following each expression experiment. Following optimization at a small scale, a scale-up experiment was performed. In these scale-up experiments, protein yield increased proportionally, while purity remained comparable to that of the small-scale purifications. These studies thus demonstrate the effectiveness of using the Profinity eXact technology on an automated chromatography system with preprogrammed methods for the rapid generation of milligram quantities of pure, tag-free protein for downstream functional assays.

Methods

Cloning of MetC Gene Into Profinity eXact pPAL7 Supercoiled Expression Vector

The MetC coding region was PCR-amplified with iProof™ high-fidelity polymerase (Bio-Rad Laboratories, Inc.) using primers containing HindIII (5' end) and XhoI (3' end), as directed in the Profinity eXact system manual. Profinity eXact pPAL7 supercoiled expression vector (Bio-Rad) and MetC DNA were double digested with HindIII and XhoI and ligated to form pPAL-MC. A second clone (pPAL-MCL) was constructed in an analogous manner, except that the 5' primer contained an additional 6 nucleotides coding for a Thr-Ser linker (for optimal cleavage, as recommended). Ligation products were transformed into an *E. coli* host strain (NovaBlue) via electroporation using the MicroPulser™ electroporator (Bio-Rad). Clones were verified via restriction analysis and subsequently transformed into BL21(DE3)pLysS expression strains (for strict control of MetC expression).

Protein Expression and Extraction

BL21(DE3)pLysS strains carrying pPAL-MC (BL-MC) and pPAL-MCL (BL-MCL) were cultured in the presence of ampicillin (100 µg/ml) and chloramphenicol (25 µg/ml). Overnight seed cultures were grown at 37˚C (200 rpm) and used to inoculate expression cultures. Expression cultures were grown at 30˚C (175 rpm) to OD_{600} ~1.0. IPTG was added to a final concentration of 1 mM and cultures were induced for 10 hr. Following expression, cell cultures were harvested via centrifugation (10,000 x g, 4˚C, 10 min). For chemical lysis, cells were resuspended in B-PER (Pierce Biotechnology, Inc.) (10 ml per gram wet cell weight). Cell suspensions were incubated with benzonase (Novagen, 25 U ml^{-1}, 25˚C, 10 min) to reduce viscosity. Lysates were cleared via centrifugation (20,000 x g, 4˚C, 20 min) and the resulting supernatants were filtered (0.45 µm polyethersulfone [PES] filters). For mechanical lysis, cells were resuspended in the recommended Profinity eXact 1X bind/wash buffer (100 mM Na_2HPO_4, 10 ml per gram wet cell weight). Samples were sonicated on ice at 100% intensity in 30 second pulses (60W) (total processing time 10 min) using an S4000 sonicator (Misonix, Inc.). Lysates were centrifuged and cleared as described above.

Affinity Purification

Cleared lysates were purified using the Profinia purification system utilizing the preprogrammed Profinity eXact purification methods. These methods consisted of using either the 1 ml and 10 ml or 5 ml and 50 ml Bio-Scale™ Mini Profinity eXact and Bio-Scale Mini P-6 desalting cartridges, respectively (both from Bio-Rad). Buffers were prepared at the following concentrations according to the Profinity eXact system manual: bind/wash buffer (100 mM NaH_2PO_4, pH 7.2); elution buffer (100 mM NaH_2PO_4, 100 mM NaF, pH 7.2); desalting buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 8.1 mM KH_2PO_4, pH 7.4). Samples were applied to Profinity eXact affinity cartridges using the standard flow rate (1.0 ml/min). Fractions for the column flowthrough, washes, and elution were collected via automatic peak detection. Purified protein was collected in a total of 4 ml for 1 ml Profinity eXact cartridge purifications and in a total of 10 ml for 5 ml Profinity eXact cartridge purifications.

SDS-PAGE and Experion™ Pro260 Analysis

SDS-PAGE analysis was performed using Criterion™ Tris-HCl 4–20% linear gradient gels (Bio-Rad). Samples were prepared with a 6-fold dilution into Laemmli sample buffer and 30 µL of sample or 10 µL of Precision Plus Protein™ Kaleidoscope™ standard (Bio-Rad) was loaded per lane. Gels were stained with Bio-Safe™ Coomassie Blue G-250 stain (Bio-Rad) following standard protocols and imaged with a Molecular Imager® Gel Doc™ XR system (Bio-Rad) using Quantity One® 1-D analysis software (Bio-Rad). Protein quantity and purity was determined using the Experion automated electrophoresis system with the Pro260 analysis kit (Bio-Rad) according to the standard protocol. Protein concentrations were independently confirmed using the Bio-Rad *RC DC*™ protein assay kit according to the instructions provided with the kit.

Native PAGE and Cystathionine β-lyase (CBL) Activity Assay

Native PAGE analysis was performed using Criterion Tris-HCl 8–16% linear gradient gels (Bio-Rad). Samples were diluted 2-fold into Native sample buffer and 45 µl of sample or 10 µl of Precision Plus Protein Kaleidoscope standard was loaded per lane. Gels were stained with Bio-Safe Coomassie Blue G-250 stain following standard protocols (in order to detect the total protein in each lane) or with CBL activity stain (100 mM Tris-HCl, 10 mM L-cysteine, 500 µM $Pb(NO_3)_2$, pH 8.2). For detection with the CBL activity stain, gels were washed three times in dH_2O (15 min, with shaking) then visualized (30 min, 25˚C, no shaking). Gels were imaged with a Molecular Imager Gel Doc XR system using Quantity One 1-D analysis software. Positive CBL activity results in an insoluble lead sulfide precipitate in the gel (which appears black in gel images).

Protein Identification by MALDI-TOF MS/MS

Eluted proteins were confirmed by Matrix-Assisted Laser Desorption-Ionization–Time of Flight tandem mass spectrometry (MALDI-TOF MS). Proteins were extracted from polyacrylamide gels and reduced (tributylphosphine, 20 mM), alkylated (iodoacetamide, 40 mM), and digested in-gel with trypsin (Sigma-Aldrich). The trypsin-digested fragments were concentrated using C18 ZipTips (Millipore) and spotted onto a 192-well Applied Biosystems (ABI) MALDI plate for multi-stage MALDI-TOF analysis (Georgia Institute of Technology Bioanalytical Mass Spectrometry Facility). MALDI-TOF MS/MS analysis was carried out with the Voyager 4700 MALDI-TOF-TOF system (ABI) operated in reflector mode. Peptide ions were analyzed with the GPS Explorer software package using the MASCOT database.

Results and Discussion

Native MetC Is Efficiently and Rapidly Cleaved From the Profinity eXact Fusion Tag

Some proteins require a spacer or linker between the Profinity eXact affinity tag and the native protein sequence to promote efficient cleavage of the tag (see the Profinity eXact fusion-tag system manual). To determine if a linker was required, two clones were constructed with one containing only native MetC amino acid sequence (pPAL-MC), and another containing the recommended threonine-serine linker (pPAL-MCL). At the scales tested (250 ml culture volume), protein purification on the Profinia system using the 1 ml Profinity eXact plus 10 ml desalting method from each clone resulted in nearly identical yields and purity for the same incubation period (30 min) (data not shown). As native MetC is highly desirable for downstream applications, the clone without the linker region (pPAL-MC) was chosen for further analysis.

Chemical Versus Mechanical Cell Lysis Procedures

In order to determine the optimal method for isolation of total cell protein, both chemical and mechanical (sonication) lysis procedures were compared from two different expression culture volumes (250 ml culture, 10 ml total lysate; 1 L culture, 50 ml total lysate). The resulting chromatograms from the purifications on the Profinia system using the 1 ml Profinity eXact plus 10 ml desalting method (Figure 1) demonstrate that both chemical and sonication lysis methods were equally efficient for isolating soluble MetC protein from lysates prepared at both culture volume scales (250 ml and 1 L). Final protein yield and purity obtained from purification at both culture volumes tested and using chemical and sonication lysis methods are shown in Table 1.

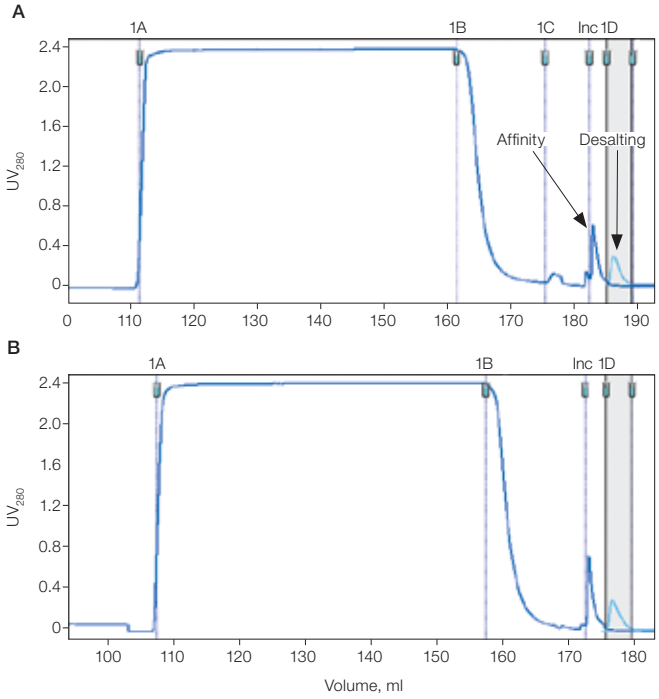

Fig.1. Comparison of chemical versus mechanical lysis methods using Profinity eXact affinity purification with integrated desalting. MetC from 10 ml of lysate (250 ml culture, data not shown) or 50 ml of lysate (1 L culture) was purified using a 1 ml Profinity eXact cartridge for the affinity step and a 10 ml Bio-Scale Mini Bio-Gel P-6 desalting cartridge for integrated desalting. **A**, chromatogram of lysate prepared by chemical lysis of a 1 L culture. (—) affinity purification (UV1); (—) desalting (UV2); **B**, chromatogram of lysate prepared by sonication of a 1 L culture. (—) affinity purification (UV1); (—) desalting (UV2). Collected fractions are indicated above the graphs: Inc, column incubation step; 1A, flowthrough fraction; 1B, column wash 1 fraction; 1C, column wash 2 fraction; 1D, elution fraction.

Table 1. Summary of MetC protein yield and purity from lysates prepared by mechanical versus chemical lysis methods*.

Culture Volume	Lysis Method	Lysis Volume, ml	Concentration, mg/ml	Purity, %	Total Protein Yield, mg
250 ml	Chemical	10	0.075	93	0.30
	Sonication	10	0.072	92	0.28
1 L	Chemical	50	0.6	91	2.40
	Sonication	50	0.72	87	2.88

* Protein concentration and purity were determined using the Experion Pro260 analysis kit.

Efficient Removal of Profinity eXact Tag From Native MetC Requires Only 12 Minutes

In an effort to further optimize the purification scheme, a series of on-cartridge incubation times were examined to determine the minimal time required for efficient cleavage of native MetC protein from the Profinity eXact fusion tag. 1 ml Profinity eXact cartridge methods were programmed with 12 min, 30 min, and 2 hr incubation times, followed by elution with integrated desalting on the Profinia instrument. A 1 L culture was grown as described above and prepared by sonication (50 ml total lysate). A total of 10 ml of lysate was loaded onto 3 separate 1 ml Bio-Scale Mini Profinity eXact cartridges, the cartridges were incubated at the three different incubation time periods, and each was subsequently eluted from the affinity column and loaded onto 10 ml desalting cartridges. Comparable yields and purity were obtained for each incubation time examined, indicating that the on-cartridge cleavage reaction proceeded to completion within 12 minutes (Table 2, Figure 2).

Table 2. Summary of protein yield and purity of MetC using three different incubation times with the Profinity eXact method*.

Incubation Time	12 min	30 min	2 hr
Concentration, mg/ml	0.09	0.08	0.12
Purity, %	86	87	85
Yield, mg	0.36	0.32	0.48

* Protein concentration and purity was determined using the Experion Pro260 analysis kit.

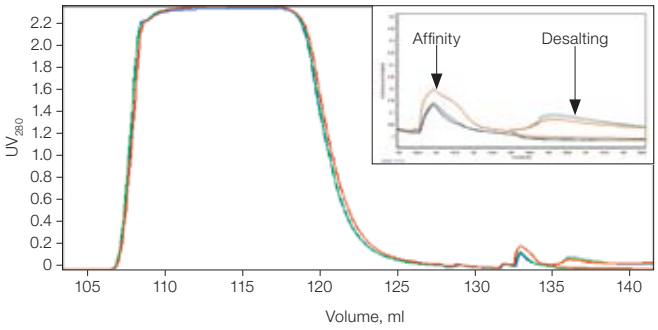

Fig. 2. Comparison of three different cartridge incubation times using Profinity eXact purification with integrated desalting. MetC was purified from 10 ml of lysate using a 1 ml Bio-Scale Mini Profinity eXact cartridge for the affinity step and a 10 ml Bio-Scale Mini Bio-Gel P-6 desalting cartridge for integrated desalting with recommended buffers. In each purification, a separate incubation time was used. An overlay of chromatograms from affinity purification and desalting are shown for 12 min, 30 min, and 2 hr incubations (—), (—), and (—) respectively. Inset shows the affinity and desalting peaks for the three incubations.

Large Scale Purification of MetC

To obtain sufficient quantities of MetC for further downstream gel activity and mass spectrometry analyses, a large-scale purification of MetC from a 3 L culture was performed. Cells were grown as described above and lysed using sonication. Lysate (200 ml) was prepared as described and loaded onto a 5 ml Profinity eXact affinity column followed by a 50 ml desalting column using the 5 ml Profinity eXact plus 50 ml desalting method on the Profinia system. The column was incubated for 30 min to allow for sufficient cleavage, and the native MetC protein was eluted (10 ml) using PBS. The large-scale purification resulted in a yield of 8.71 mg total MetC protein (0.871 mg/ml), with measured purity comparable to that observed at the smaller scale (87%) (Figure 3).

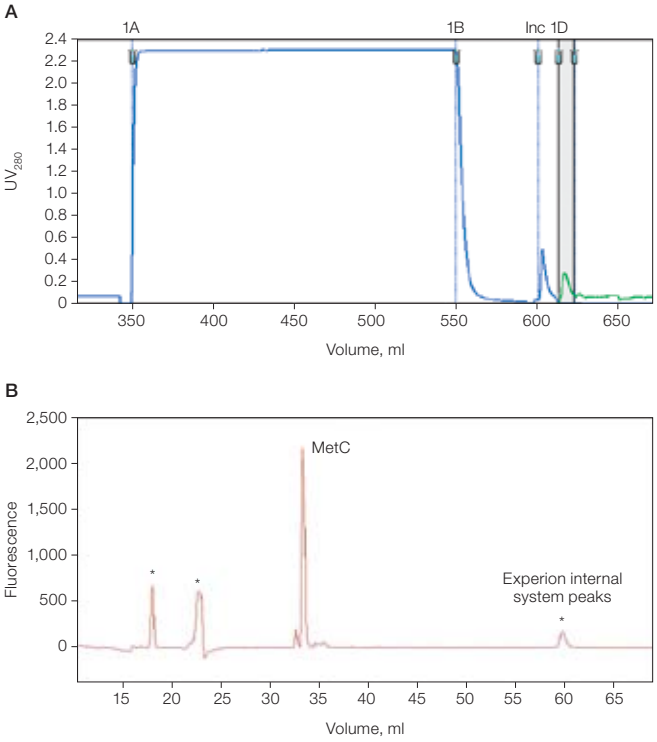

Fig. 3. Purification of MetC using the 5 ml Profinity eXact cartridge with integrated desalting method on the Profinia system. A, chromatogram of MetC purification from a 200 ml sonicated lysate using a 5 ml Profinity eXact cartridge for the affinity step and a 50 ml Bio-Scale Mini Bio-Gel P-6 desalting cartridge for integrated desalting with recommended buffers. (—), affinity purification (UV1); (—), desalting (UV2). Collected fractions are indicated above the graph: Inc, column incubation step; 1A, flowthrough fraction; 1B, column wash 1 fraction; 1D, elution fraction; **B**, representative Experion electropherogram of eluted MetC protein (87% purity at 0.871 mg/ml protein concentration).

Recombinant MetC Is Active in vitro

Protein fractions from the 5 ml Profinity eXact purification described in Figure 3 were loaded onto both native and denaturing polyacrylamide gels to determine activity, size, and purity, respectively. Activities of wild-type MetC (from *S. oneidensis* crude lysate) and purified, tag-free recombinant MetC recovered from the elution fraction were detected using an in-gel assay using native PAGE. The cleavage of thiol from L-cysteine, and subsequent reaction with lead nitrate to form an insoluble lead-sulfide resulted in black precipitates.

Other fractions (load, flowthrough, and wash) from the purification displayed C-S lyase activity at a position in the gel indicating the presence of a tag resulting from the fusion to the Profinity eXact tag (Figure 4A). SDS-PAGE gels were stained for total protein only, and purified MetC displayed a band at the expected size (~40 kD) (Figure 4B). The purity and identity of the *S. oneidensis* MetC band was confirmed by MALDI-TOF MS/MS (Figure 5). All detected peaks matched with the peptide sequence predicted for MetC. These results demonstrate that the Profinity eXact fusion-tag system could be used to generate sufficiently pure and active recombinant MetC suitable for downstream analysis, where native protein function is required.

Fig. 4. Gel analysis of recombinant MetC activity. Gel electrophoresis analysis of *S. oneidensis* MR-1 crude lysate and different fractions collected from purification of 200 ml of lysate (Figure 3). **A**, Native PAGE; lane 1, Precision Plus Protein Kaleidoscope marker; 2, MR-1 crude lysate; 3, MetC loading fraction; 4, MetC flowthrough; 5, MetC wash; 6, MetC eluted protein; 7, Precision Plus Protein Kaleidoscope marker. Left panel, Bio-Safe Coomassie Blue G-250 stained gel; Right panel, cystathionine β-lyase activity gel. MR-1 lysate and eluted protein activity bands migrate faster due to absence of the Profinity eXact fusion-tag; **B**, SDS-PAGE, Bio-Safe Coomassie Blue G-250 stained gel of crude lysate and different fractions. Lanes are the same as in panel A.

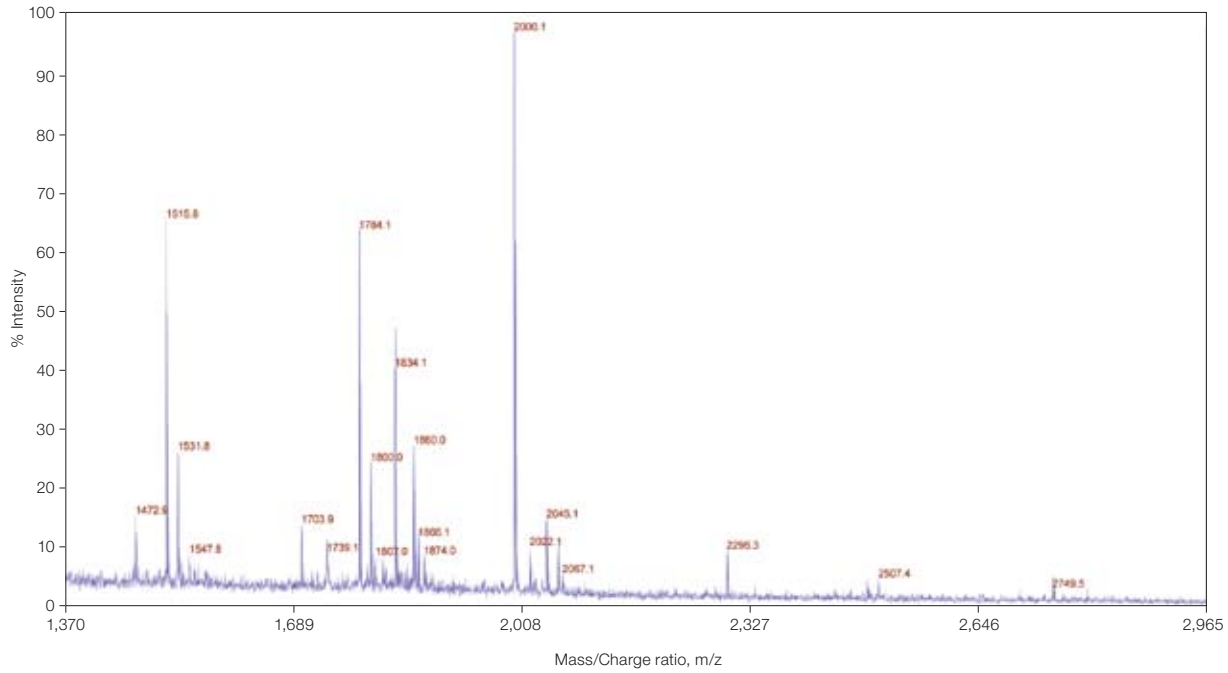

Fig. 5. Identification of purified MetC. MALDI-TOF MS/MS spectrum of eluted MetC protein. Spectrum and masses of peptides correspond to those predicted for MetC in the MASCOT database used to reference the sample.

Conclusions

Purification of tag-free native protein is an important step in elucidating steps in biochemical pathways. Here we report the purification of cystathionine β-lyase from a gram-negative bacterium, *Shewanella oneidensis* MR-1. The enzyme may play an important role in regulating the redox balance in an organism with a sophisticated respiratory chain that can utilize both soluble and insoluble terminal electron acceptors. The combination of the Profinity eXact fusion-tag and Profinia automated purification systems allowed recovery of milligram quantities of native, tag-free and functional MetC in a relatively short amount of time (<2 hours), with a purity suitable for use in sensitive downstream assays of protein activity and function.

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