Presence and removal of a contaminating NADH oxidation activity in recombinant maltose-binding protein fusion proteins expressed in *Escherichia coli*

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We observed the presence of contaminating NADH oxidation activity in maltose binding protein (MBP) fusion proteins expressed in *Escherichia coli* and purified using conventional amylose resin-based affinity chromatography. This contaminating NADH oxidation activity was detectable with at least four different enzymes from *Cryptosporidium parvum* expressed as MBP-fusion proteins (i.e., an enoyl-reductase domain from a type I fatty acid synthase, a fatty acyl-CoA binding protein, the acyl-ligase domain from a polyketide synthase, and a putative thioesterase), regardless of their NADH dependence. However, contaminating NADH oxidation activity was not present when fusion proteins were engineered to contain a His-tag and were purified using a Ni-NTA resin-based protocol. Alternatively, for proteins containing only an MBP-tag, the contaminating activity could be eliminated through the addition of 0.1% Triton X-100 and 2% glycerol to the column buffer during homogenization of bacteria and first column wash, followed by an additional wash and elution with regular column and elution buffers. Removal of the artificial activity is very valuable in the study of enzymes using NADH as a cofactor, particularly when the native activity is low or the recombinant proteins are inactive.

Modern biological and biomedical research, as well as the biotechnology industry, relies heavily on the production of bioactive proteins. A large number of fusion systems are available for expressing recombinant proteins in prokaryotes or eukaryotes, including *Escherichia coli*, *Saccharomyces cerevisiae*, and baculovirus. Vectors based on T7 RNA polymerase (e.g., pET plasmids) and tac promoter (e.g., pMAL plasmids) are among the most popular expression systems used in *E. coli* (1). The majority of expression systems use certain tags to facilitate the expression and purification of recombinant proteins, including the His-tag, glutathione S-transferase (GST), maltose binding protein (MBP), N-utilization substance A (NusA), and thioredoxin (Trx). Among these, MBP fusion is a powerful system for expressing a large quantity of protein. It is able to enhance the solubility and proper folding of its fusion partners (2,3). The purification of MBP-fused proteins is achieved by a relatively easy and simple amylose resin-based chromatography.

Our laboratory has been using various systems including the MBP fusion system and derivatives to express a large number of *Cryptosporidium parvum* proteins for functional analysis (4–11). MBP fusion generally performs well, particularly in expressing very large proteins that are otherwise very difficult to express using many other fusion systems. *C. parvum*, a protozoan pathogen infecting both humans and animals, possesses a multifunctional type I fatty acid synthase and polyketide synthase (CpFAS1 and CpPKS1) (10,11). We have previously expressed these two megasynthases as MBP-fused protein with molecular weights up to 250 kDa. The expressed proteins contain multiple functional domains that are biochemically active as MBP-fusion proteins (5,8).

More recently, however, in our attempts to express individual enoyl reductase (ENR) domains from CpFAS1 and CpPKS1, we have observed that MBP-fused proteins expressed in *E. coli* and purified using conventional amylose resin-based affinity chromatography contained NADH oxidation activity that did not originate from the fusion proteins of interest. Further experiments confirmed that the NADH oxidation activity was in fact an artifact produced from a contaminating but undefined bacterial enzyme(s). More importantly, this unwanted artificial activity was present in most (if not all) MBP-fusion proteins purified by the conventional amylose resin-based protocol investigated so far in our laboratory, which had caused some confusion in our study of enzymes that used NADH as a cofactor and when the recombinant MBP-fusion proteins possessed very low or no enzyme activity. To overcome the problem, we have developed alternative protocols to eliminate this NADH oxidation activity.

Materials and methods

Cloning and expression of MBP-fusion proteins
Several *C. parvum* genes including *CpFAS-ENR1*, *CpFAS-CBP1*, *CpPKS-AL1*, and *CpTE1* were cloned into the pMAL-c2x or its derivative vector as described below.
CpFAS1-ENR1 is one of the 21 functional domains within a unique type I fatty acid synthase (CpFAS1) elongation module-1 (8,11). All CpFAS1 modules containing multiple enzymatic domains have been previously cloned and expressed, in which all enzyme activities of individual domains in the recombinant proteins were functionally active (8). In this study, our original intent was to express the CpFAS-ENR1 domain to study whether this single domain was functional when expressed alone rather than expressed as part of a multidomain fusion protein. A gene fragment encoding the CpFAS-ENR1 domain was amplified from a large synthetic gene containing the entire module 1 with codons optimized for expression in E. coli using primers CpCFAS-ENR1-F1 (5′-aga gaattcGGCAGTATCAGCAATCTGA GCTTG-3′) (lower case represent artificially added EcoRI linker) and CpCFAS-ENR1-R1 (5′-caggctacCTCTCAGGA ATGCTATTGTCGCC-3′) (lower case represent artificially added SalI linker). PCR amplicons were digested with EcoRI and SalI, and the released CpFAS-ENR1 insert was purified from a 1% agarose gel, ligated into the pMAL-c2x vector (New England Biolabs, Ipswich, MA, USA), and transformed into One Shot TOP10 competent E. coli cells (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was isolated from positive clones and sequenced to confirm its identity. Additionally, CpFAS-ENR1 was also similarly cloned into a pMAL-c2E-derived vector (pMAL-c2E-TEV-His) between the EcoRI and SalI sites, which produces an MBP-fused protein containing a TEV cleavage site between MBP and fused partner and a His-tag fused at the C-terminus of recombinant protein.

The cloning and expression of C. parvum fatty acyl-CoA binding protein (CpACBP1) and the N-terminal acyl ligase within a type I polyketide synthase (CpPKSI-ALI) have been previously reported (5,7). Their fusion proteins were expressed from those constructs and used in this study. A thioesterase homolog (CpTE1; GenBank accession no. XM_628403) was also cloned into pMAL-c2E-TEV-His vector, expressed as an MBP-CpTE1-His fusion protein, and used as an additional negative control. Details on the cloning and protein expression of MBP-CpTE1 will be reported along with its biological and biochemical data elsewhere.

The expression of all fusion proteins was carried out in the E. coli Rosetta 2 strain (EMD Biosciences, Madison, WI, USA), which contains an extra set of tRNA genes to enhance the expression of foreign proteins from species with different codon usages. The BL21 strain was also used to express fusion proteins in some control experiments. Briefly, transformed Rosetta 2 or BL21 cells were plated onto solid Lysogeny Broth (LB-Miller) agar medium containing ampicillin (50 µg/mL), chloramphenicol (25 µg/mL), and glucose (2 mg/mL). After incubation overnight at 37°C, a single colony of transformed bacteria was first inoculated into 50 mL LB media containing the same amounts of antibiotics and glucose and grown overnight at 30°C in a shaking incubator. The overnight cultures were diluted 1:20 with fresh medium and allowed to grow for approximately 2 h at 30°C until their OD600 reached to ~0.5. At this time, isopropyl-1-thio-β-D galactopyranoside (IPTG) was added to a final concentration of 0.3 mM to induce protein expression, and cells were grown for ~16 h at 16°C before being collected by centrifugation.

Bacteria were resuspended in 25 mL column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA) containing a protease inhibitor cocktail for bacteria (Sigma-Aldrich, St. Louis, MO, USA) and frozen at -20°C for overnight or for a few days. All samples were then placed in an ice bath and subjected to about 2-min sonication in short pulses with 15-s intervals. Lysates were centrifuged at 8000×g at 4°C for 30 min to remove insoluble debris.
All expressed proteins were first purified by an amylose resin-based affinity chromatography following manufacturer’s protocol (New England Biolabs). Triton X-100 and glycerol at specified concentrations were included in the column and elution buffers in an alternative purification protocol to increase the stringency of the purification. For CpFAS-ENR1 expressed in pMALTc2xTEV-His vector, proteins were also purified using a nickel nitrilotriacetic acid (Ni-NTA) resin-based affinity chromatography (Qiagen, Valencia, CA, USA). Eluted fusion proteins were desalted using PD-10 Desalting Columns (GE Healthcare Biosciences, Piscataway, NJ, USA) in 20 mM Tris-HCl, pH 7.4. Protein quality (sizes and purity) was assessed with 10% SDS-PAGE, and concentrations were determined by a Bradford protein assay using BSA as a standard.

Biochemical assays
Our original intent was to determine whether a single CpFAS-ENR1 domain expressed as MBP-fused protein was bioactive and able to reduce enoyl-CoA (e.g., crotonoyl-CoA) (11–14). A typical assay was performed in a final volume of 200 μL reaction buffer (0.1 M Tris-HCl, pH 7.5) containing 10–20 μg recombinant CpFAS-ENR1 and 200 μM NADH (or NADPH) with or without 100 μM crotonoyl-CoA. MBP-tag alone was used in all experimental groups as a control. The oxidation of NAD(P)H was monitored by the reduction of absorption at 340 nm (OD₃₄₀) with a Multiskan Spectrum spectrophotometer (Thermo Scientific, West Palm Beach, FL, USA). The concentrations of NAD(P)H were calculated according to the extinction coefficient (ε₃₄₀) at 6.22 mM⁻¹ cm⁻¹ (15). All assays were carried out in at least duplicate, and at least two independent assays were performed for each experiment.

After having observed that recombinant MBP-CpFAS-ENR1 purified by amylose resin affinity chromatography was capable of oxidizing NADH without reducing crotonoyl-CoA, we further tested whether this type of NADH oxidation activity originated from CpFAS-ENR1 by using proteins (i.e., MBP-CpFAS-ENR1-His) purified by Ni-NTA resin-based chromatography and by a modified amylose resin-based chromatography. To determine whether the artifactual activity was a common problem associated with the conventional amylose-resin purification protocol, the same assay was also used to test several other MBP-fused proteins that were known to be unable to use NADH as cofactor (i.e., CpACBP1, CpPKS-AL1, and CpTE1). The effect of the alternative purification protocol on the native enzyme activity was evaluated using MBP-fused CpTE1, in which the thioesterase activity was not NADPH (even in the absence of the substrate crotonoyl-CoA), and 50 μM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The formation of chromophore 5-thio-2-nitrobenzoate was then spectrophotometrically monitored at 412 nm (ε = 13,600 M⁻¹ cm⁻¹) (16).

Results and discussion
We initially observed that recombinant MBP-CpFAS-ENR1 purified by conventional amylose-resin chromatography was able to specifically oxidize NADH (but not NADPH) even in the absence of the substrate crotonoyl-CoA (Figure 1A). This activity was not observed in the MBP-tag control groups, indicating that the activity was not a result of self-oxidation of NADH in the solution. The presence of crotonoyl-CoA at varied concentrations had no effect on the activity either, suggesting that the electrons released from NADH were not...
transferred to the substrate (Figure 1B). These preliminary observations indicated that the CpFAS-ENRI domain expressed alone as a single protein was unable to use crotonoyl-CoA as a substrate (or the activity was extremely low), although it was functional when expressed together with other enzymatic domains in the modules (5). The unexpected NADH oxidation activity could originate from either an unreported biochemical feature of the recombinant CpFAS-ENRI protein or from some contaminating activity. To test these two possibilities, we first examined whether other MBP-fused proteins purified by conventional amylose resin-based purification protocols also possessed this NADH oxidation activity. These proteins (CpACBP, CpPKS-AL1, and CpTE1) were selected because they were known not to use NADH as a cofactor and could serve as authentic negative controls. To our surprise, all three recombinant proteins, together with MBP-CpFAS-ENRI, possessed the NADH oxidation activity at similar levels (Figure 2A). The artifactual activity was not only detected from fusion proteins expressed in Rosetta 2 cells, but also in BL21 cells (Figure 2B), suggesting that this unwanted NADH oxidation activity is an artifact associated with some conventionally purified MBP-fusion proteins expressed in E. coli.

To further test whether the artifactual NADH oxidation activity was specifically associated with the conventional amylose resin-based purification protocol, we reengineered the CpFAS-ENRI protein to contain both an N-terminal MBP and a C-terminal His-tag (MBP-CpFAS-ENRI-His). This new fusion, together with CpTE1 that had already contained a C-terminal His-tag, could be purified by both amylose resin-based and Ni-NTA resin-based protocols. Subsequent biochemical assay clearly showed that NADH oxidation activity was undetectable (or at most at near background levels) in both proteins purified by Ni-NTA resin protocol, but detectable again in the same proteins purified by conventional amylose resin protocol (Figure 3A).

These observations confirmed that the artifactual NADH oxidation activity among MBP-fusion proteins originated from a yet undefined bacterial contaminant(s) copurified using the conventional amylose resin-based purification protocol. The problem was likely associated with foreign gene expression in E. coli, because the MBP-tag alone (which is of bacterial origin) that was expressed and purified under the same conditions displayed no such activity (Figures 1–3). The presence of bacterial contaminants in fusion proteins is not uncommon. Some contaminants are simply nonspecific proteins, while others may be specific proteins associated with the processing of fusion proteins in bacteria during expression, such as DnaK and flagellin (17,18).

The conventional amylose resin-based purification protocol used relatively mild wash and elution conditions, which might not be strong enough to remove some minor but highly bioactive contaminants. We thus tested whether increasing the stringency in the amylose resin purification protocol could improve the removal of the contaminating activity. After testing several different combinations of reagents, we observed that the addition of 0.1% Triton X-100 and 2% glycerol in the column and elution buffers could actually eliminate the contaminating NADH oxidation activity (Figure 3B). The NADH oxidase activity appeared to be related to a very minor protein contaminant(s) that could not be easily identified by standard SDS-PAGE analysis, as the protein band patterns were virtually indistinguishable between modified and conventional protocols (Figure 3B, inset). We also tested whether the addition of Triton X-100 in the column and elution buffers could affect the native enzyme activity using MBP-CpTE1 as an example. The result showed that although Triton X-100 at either 0.01%, 0.05%, or 0.1% concentrations could eliminate the contaminating NADH oxidation activity (Figure 4A), it also reduces CpTE1 activity by 15.3%, 23.8%, and 74.8%, respectively. When Triton X-100 is undesirable in the final preparations, the detergent could be removed by adding a second wash step with regular column buffer, followed by elution of fusion proteins with regular elution buffer (Figure 5). The fact that NADH oxidation activity was undetectable in the final preparation lacking detergent indicates that the contaminants were truly removed, rather than being suppressed by Triton X-100 (Figure 5, compare group 3 with group 4).

In summary, we have observed contaminating NADH oxidation activity present in some MBP fusion proteins expressed in E. coli and purified by conventional amylose resin-based affinity chromatography. The contamination could be removed by either addition of 0.1% Triton X-100 and 2% glycerol in the homogenization and wash steps to the conventional purification system for MBP-fusion protein, or by Ni-NTA resin-based purification protocols if a His-tag is
also engineered into the fusion proteins. It is possible that some MBP-fusion proteins may not bind well to anylose in the presence of this much detergent, in which case adding a His-tag to facilitate Ni-NTA resin-based purification protocol may be a better choice, or different concentrations of detergent may be tested to identify the best protein conditions. Because MBP fusion systems are commonly used to express foreign proteins in E. coli, and a large number of enzymes use NADH as a cofactor, it is necessary for the research community, particularly for those who study enzymes that use NADH as a cofactor, to be aware of this problem and the alternative protocols to eliminate any contamination.

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Competing interests

The authors declare no competing interests.

References


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