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Engineered Calcium-Precipitable Restriction Enzyme

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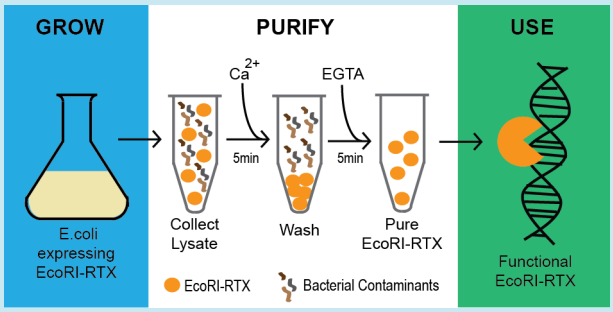
ABSTRACT: We have developed a simple system for tagging and purifying proteins. Recent experiments have demonstrated that RTX (Repeat in Toxin) motifs from the adenylate cyclase toxin gene (CyaA) of *B. pertussis* undergo a conformational change upon binding calcium, resulting in precipitation of fused proteins and making this method a viable alternative for bioseparation. We have designed an iGEM Biobrick comprised of an RTX tag that can be easily fused to any protein of interest. In this paper, we detail the process of creating an RTX tagged version of the restriction enzyme EcoRI and describe a method for expression and purification of the functional enzyme.



Commonly used methods for protein purification include high-performance liquid chromatography (HPLC) and other affinity based methods. While effective, these methods generate hazardous waste and require costly, limited-use materials. Recently developed methods for protein purification involve tagging the protein of interest and purifying with high heat or harsh chemical conditions, both of which can influence the activity of the protein.[1]

In the past few years, the RTX motif of *B. pertussis* has been investigated as a possible alternative for tagging and purifying proteins.[1] The RTX motif consists of a nine amino acid sequence that repeats up to 40 times.[2,3,1] In the presence of calcium, these motifs undergo a conformational change resulting in precipitation of the polypeptide.[4,2,3] These motifs can theoretically be appended to any protein to allow for its precipitation and purification. The precipitation reaction occurs rapidly at room temperature and requires a lower salt concentration than other stimulus-induced tags, protecting a tagged protein from potential degradation.

The purification of restriction enzymes is of particular interest to the scientific community as they allow for site-specific cleavage of DNA to facilitate cloning. While not overly expensive, restriction enzymes do present a significant financial burden when used in bulk; therefore, we chose to develop a method of purifying EcoRI that was cheap, easy and effective for synthetic biology use.

RESULTS

To demonstrate EcoRI-RTX precipitation in response to calcium, we added increasing amounts of $CaCl_2$ to whole cell lysate from plasmid harboring *E. coli* and monitored accumulation of pellets after centrifugation. Indeed, we did see pellets form specifically in the calcium containing samples, with the optimal calcium concentration of 50 mM. To verify that the pellet was in fact EcoRI-RTX, we performed SDS-PAGE analysis (Figure 1). A band is visible in the pellet at the predicted size.

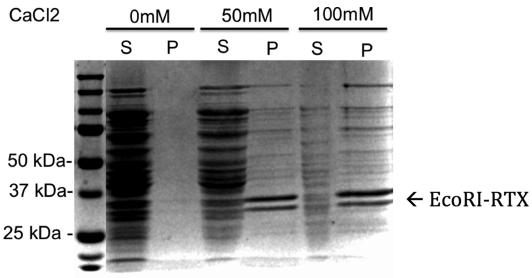


Figure 1. SDS-PAGE analysis of calcium precipitation of EcoRI-RTX. Calcium was added to EcoRI-RTX expressing whole cell lysate (supernatant S, pellet P) at increasing concentrations from 0 mM to 100 mM, run on a 10% SDS-PAGE gel and stained with coomassie blue.

Because EcoRI is an endonuclease, its expression in *E. coli* lacking EcoRI methylase is highly toxic.[5] We designed a Biobrick-compatible plasmid capable of expressing the EcoRI methylase and verified that the plasmid was protected from cleavage by EcoRI (Figure 2A). After isolating the plasmid, we digested with commercially available EcoRI and PstI, whose sites flanked EcoRI methylase. The presence of a single band after the digest in addition to robust growth of the cells clearly shows that the methylase is being expressed and is protecting the EcoRI site from cleavage *in vivo*.

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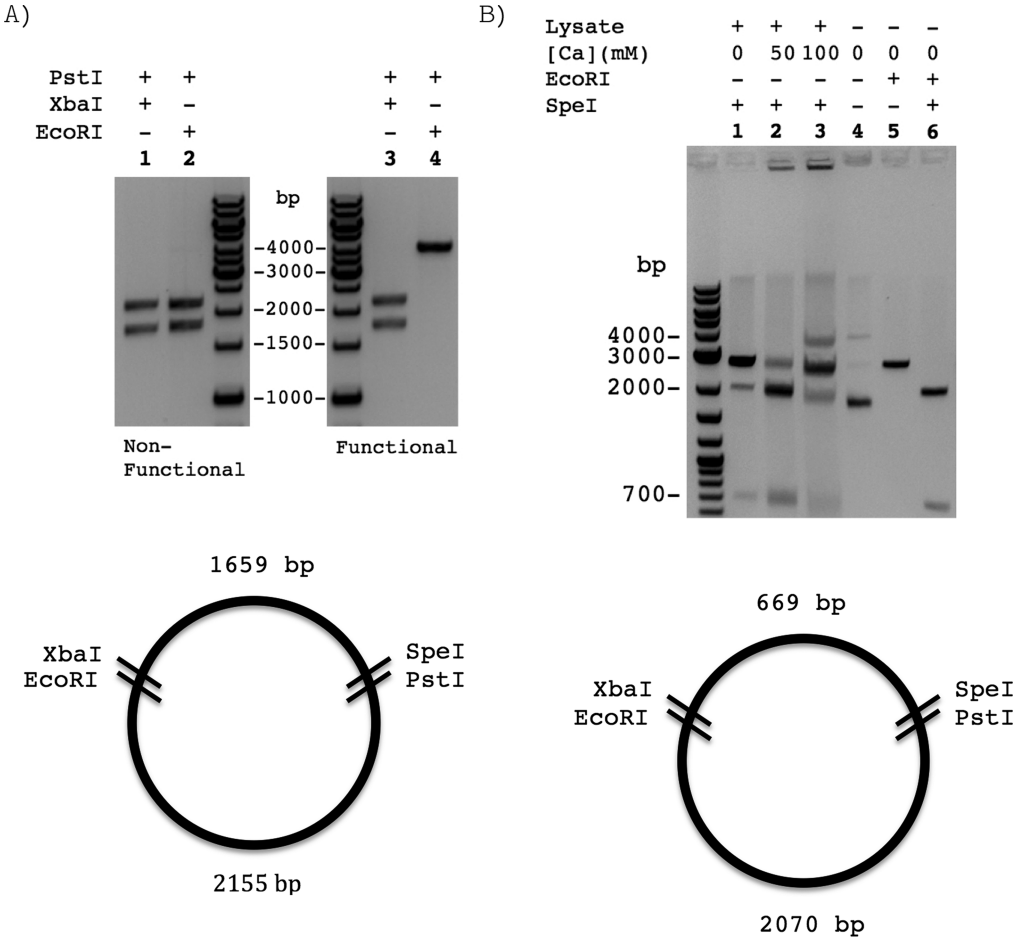

Figure 2. (A) Methylase prevents EcoRI cleavage. A plasmid containing the sequence of a nonfunctional EcoRI methylase (interrupted by a premature stop codon) was digested by PstI and XbaI (Lane 1), or PstI and EcoRI (Lane 2). Plasmid expressing a functional EcoRI methylase was digested by PstI and XbaI (Lane 3) or PstI and EcoRI (Lane 4). Diagram of plasmid with sizes shown below. (B) EcoRI-RTX is functional. An exogenous plasmid was run on a gel after digestion by lysate containing EcoRI-RTX (Lane 1), pellet after precipitation of EcoRI-RTX with 50 mM $CaCl_2$ (Lane 2), or 100 mM $CaCl_2$ (Lane 3). Exogenous plasmid prior to digestion (Lane 4), after digestion by commercially available EcoRI (Lane 5), or SpeI and EcoRI (Lane 6). Diagram of plasmid with sizes shown below.

Finally, to test the activity of our engineered EcoRI, we used our EcoRI-RTX harboring resuspensions in a restriction digest (Figure 2B). For this experiment, our substrate was a plasmid containing the sequence of AmilCP, flanked by a SpeI site and an EcoRI site. As indicated by the presence of two bands of the correct sizes in the gel, EcoRI-RTX was capable of digesting the plasmid, albeit with reduced efficiency compared to commercially available EcoRI.

We tested the activity of the endonuclease activity after precipitation with 0, 50, and 100 mM and found that 50 mM was ideal.

DISCUSSION

We demonstrated the effectiveness of a simple protein purification system by purifying the EcoRI restriction enzyme. We designed a plasmid containing an RTX tag sequence that can readily be fused to any protein of interest. We also verified that RTX-tagged EcoRI retained endonuclease activity after tagging and precipitation.

We note that there were specific aspects of the method where further optimization may be required. Unexpectedly, we observed reduced endonuclease activity in the sample precipitated in 100 mM Ca^{2+} relative to 50 mM Ca^{2+}. Because SpeI activity was also reduced in the 100 mM sample, it is likely that residual Ca^{2+} in the solution may have generally inhibited restriction enzyme activity, a phenomenon that has been described previously with high concentrations of Ca^{2+}.[6] Using lower concentrations of Ca^{2+} or more thorough washing of the pellet could help to alleviate this issue.

The RTX system is a useful protein purification tool that does not require harsh chemicals or extreme temperatures and significantly reduces the costs associated with traditional protein purification approaches. Many proteins require specific temperature conditions for transportation and storage. RTX tagged proteins can be produced quickly on site, eliminating the need for long-term cold storage. This may ultimately allow extremely rural laboratories, even those with no, or only intermittently available electricity, the ability to quickly purify proteins when they are needed. Overall, RTX precipitation offers a fast, inexpensive method for protein purification.

METHODS

Standard Biobrick assembly protocols were followed to generate a plasmid that simultaneously expresses the EcoRI-RTX construct and EcoRI methylase. DH10B cells were cultured in 250 mL flasks containing LB+100 μg/mL Ampicillin at 37 °C, shaking at 225 rpm until saturated. Cells were resuspended in 6 mL 50 mM Tris-HCl pH 7.5 and lysed

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using a French press. Lysates were centrifuged at high speed for 20 min and supernatants were collected. $CaCl_2$ was added at increasing concentrations ranging from 0 to 100 mM in a volume of 1 mL and incubated at room temperature for 2 min before centrifugation at 16 000*g* for 2 min. Supernatants were collected for SDS-PAGE analysis. Pellets were washed four times in 50 mM tris-HCl, pH 7.5. Finally, pellets were resuspended in 50 mM tris-HCl containing 50 mM EGTA. EcoRI-RTX containing pellets were solubilized and centrifuged for 10 min at 16 000*g*.

To monitor purification of EcoRI-RTX, we performed SDS-PAGE analysis. Samples were boiled in SDS buffer containing DTT for 10 min and loaded into a 10% SDS-PAGE gel. To test the functionality of EcoRI, we digested an exogenous plasmid with 10 units of SpeI and 7.5 μL of EcoRI-RTX. Digestions were compared to exogenous plasmid digested with commercial SpeI and EcoRI.

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Author Contributions

J.H. and J.F.L. cloned the biological parts, J.H. tested constructs, and P.K.J., W.H., E.L., and S.A.Z. assisted in idea development. T.R., J.R., M.B., and R.D.D. provided guidance and design assistance. T.R. wrote the initial manuscript and T.R., J.H., and R.D.D. revised and contributed to the final manuscript.

Notes

The authors declare no competing financial interest.

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