Project Information

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Deliverable Information

Deliverable Number	3.1
Deliverable Title	Engineered Boon2-BST and accompanying expression and purification SOP
Workpackage Number	WP3
WP Leader	UCAM
Authors	Dushanth Seevaratnam (UCAM)
Contributors	UCAM, DTU
Reviewers	Lisa Hall (UCAM)
Contractual Deadline	31 Jan 2021
Actual Delivery Date	28 Feb 2021

Delivery Type

R	Report	✓
DEM	Demonstrator, pilot, prototype, plan designs, new or revised health policies etc	
DEC	Websites, patents filing, press & media actions, etc	
OTHER	Other	

Dissemination Level

PU	Public*	✓
RE	Restricted to a group specified by the consortium.	

*Being prepared for open access publication

Document Log

Version	Date	Author	Description of Change
1.0	28/02/2021	Dushanth Seevaratnam	First iteration of the document
1.1	07/01/2021	Dushanth Seevaratnam	Focused deliverable report
1.2	07/01/2022	Dushanth Seevaratnam	Reported on additional variants
1.3	24/05/2022	Dushanth Seevaratnam	Additions for Final Report

Total number of pages:	10

List of content

Partner	Contribution to this deliverable
CAM	Engineered and Screened Boon-Enzymes. Produced accompanying SOPs in both text and video format.
DTU	Boon2-BST-PATHPOD compatibility test.

1 Status of the Deliverable

The deliverable has been completed. The silica-immobilising design of the Boon2-BST has been synthesised and characterised. This iteration of the enzyme has been delivered to DTU and the plasmid required for local expression of the protein has been delivered to KNUST along with the necessary SOPs and training videos.

2 Summary of the results (max. 1-2 pages)

Multiple silaffin-tags were tested to determine their adhesiveness to unmodified silica gel microparticles. Of the peptides tested, the 3 amino-acid truncated R5-tag (Δ3R5), the unmodified Car9 peptide and the Car9-[R4Q] peptide were observed to have the highest affinity to silica, as shown in Table 2.1. In all 6 cases, once the peptide was electrostatically bound to the silica gel particles, incubating the silica at temperatures of up to a maximum of 60°C did not dissociate the proteins off the silica.

Table 2.1: Fusion proteins were immobilised at a ratio of 250 µL of cell lysate (normalised via mCherry fluorescence) to 1.25 mg of 63µm silica gel.

Silaffin-Tag	Amino Acid Sequence	Immobilisation Percentage
R5	SSKKSGSYSGSKGSKRRIL	66 ± 5
Δ11R5	KGSKRRIL	85 ± 2
Δ3R5	KSGSYSGSKGSKRRIL	97 ± 5
Car9	DSARGFKKPGKR	95 ± 5
Car9-[R4Q]	DSAQGFKKPGKR	96 ± 8
Car9-[K8Q]	DSARGFKQPGKR	88 ± 7

The final designs of the Boon2-BST are comprised of 5 individual components synthetically linked together through protein engineering. As shown in Figure 2.1, the independent constituents are: an immobilisation-tag (silaffin/cellulose affinity), a photocleavable protein (PhoCl), a fluorescent protein (mCherry), a histidine (His)-loop, and a *Geobacillus stearothermophilus* (BST) DNA polymerase. This combination of proteins and tags would allow for low-cost purification (silaffin tag, cellulose tag, PhoCl), visual tracking (mCherry), DNA amplification for isothermal nucleic acid diagnostic assays (BST 2.0 DNA polymerase), and a secondary purification option for additional enzyme characterisation (His-loop).

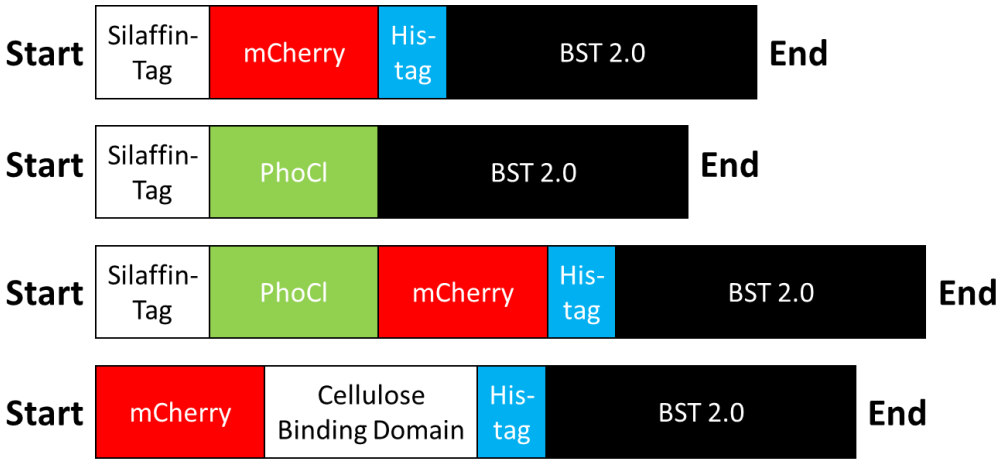

Figure 2.1: Schematic of proposed fusion protein.

The silaffin-tagged, mCherry iteration of Boon2-BST showed very good activity, even when compared to the commercial reagent, as shown in Figure 2.2. Due to the large quantity of protein produced, the untreated synthetic BST2.0 construct was able to react faster and produce a stronger fluorescent signal than its commercial counterpart.

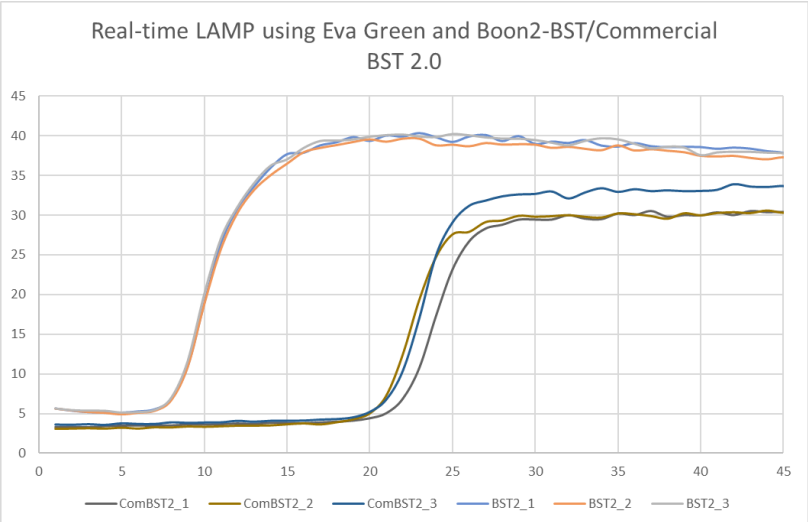

Figure 2.2: 1 µL of cell lysate was used for the synthetic BST2.0 DNA polymerase and a standard 8U of enzyme was used for the commercial assay. The rise in fluorescence signal (produced by Eva Green) is indicative of DNA amplification.

In addition, this synthetic enzyme was also shown to be active when immobilised to silica, as shown in Figure 2.3. Despite the strong activity, the recombinant protein was not compatible with the PATHPOD system (DTU) from WP2 due to the interference of the silica on the instrument's turbidimeter. For this reason, a photocleavable and a paper-based iteration of the enzyme were synthesised. Despite successful production and purification of the new variants, they were also not compatible with the PATHPOD (turbidity generation) system.

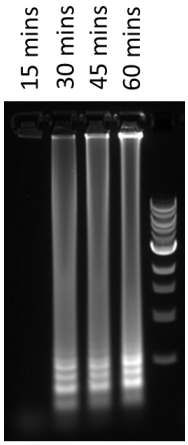

Figure 2.3: Gel electrophoresis of LAMP reactions using 0.2 mg of silica with immobilised BST2.0 at various time points. The illuminated smear represents a positive signal indicative of LAMP-based DNA amplification.

Compatible turbidity generation was finally achieved by eluting silica-immobilised BST2.0 DNA polymerase with L-arginine. The devised elution protocol resulted in improved enzyme purity and L-arginine was found to be compatible with the LAMP reaction.

3 Description of work performed and obtained results

As of this report we have synthesised and compared various iterations of silaffin-tags, and synthesised and characterised multiple fusion-constructs with BST2.0 DNA polymerase.

3.1 Silaffin-Tag Assessment

A total of 2 native silaffin-tags and multiple unique mutated variants of said tags were tested for both their affinity and desorption properties. As was shown in Table 2.1, truncating the native R5 improved the peptide's affinity to silica, with the shorter truncation resulting in the greatest affinity. These results, in tandem with *ab initio* modelling, suggest that intramolecular binding is interfering with the native R5's ability to bind to silica. Truncating the N-terminal may disrupt these interactions, improving the peptides binding capabilities through the increased availability of the positively charge amino acids. As for the Car9 peptide, the R4 amino acid appears to have little impact on the tag's affinity to silica, despite its positive charge. On the other hand, the K8 amino acid does appear to play in some role with regards to silica affinity and could be a potential mutation location for the synthesis of a reversable silaffin-tag.

All 6 cases were tested to observe if heat could be a viable pathway for desorption of the immobilised protein. Going up to a maximum temperature of 60°C, there was no measurable desorption of the Boon2-BST. The proteins adhered to silica through silaffin tags were not reversible using quick low-cost methodologies.

Despite not being able to introduce a reversibility characteristic to the silaffin-tags, the Δ3R5-tag (that was generated in the process) was a drastic improvement over the native R5-tag. Thus, the tag was carried over to all the latter protein constructs synthesised.

3.2 Δ3R5-mCh-H10-BST2.0

The first engineered Boon2-BST was stable, active, and simple to produce. A standard expression protocol of 1 mM IPTG with a 4.5 hour incubation at 37 °C was enough to produce the protein. As shown in Figure 3.1, the confirmation of Boon2-BST expression was visualised through its pink appearance.

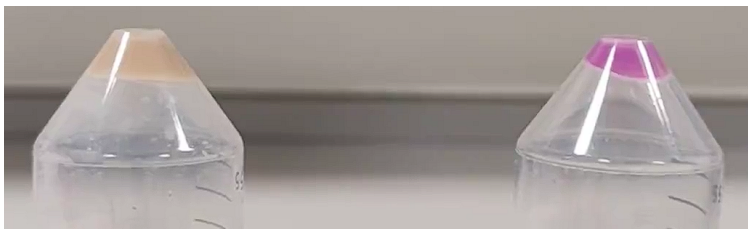

Figure 3.1: Cell pellet from a BL21 (DE3) culture without protein expression (left) and with protein expression (right).

As for silica immobilisation, the Δ3R5-tag does not appear to be hampered by the large size of the fusion protein. Incubating unmodified silica gel with the raw cell lysate revealed high affinity of the Boon2-BST, shown in Figure 3.2, and some degree of selectivity, as shown in Figure 3.3.

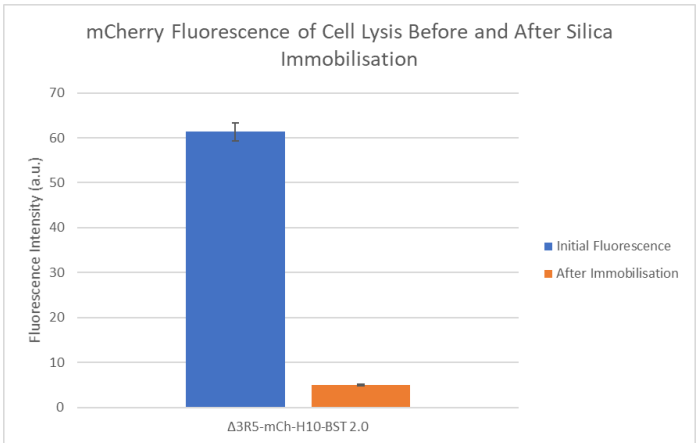

Figure 3.2: The mCherry fluorescence signal of the cell lysate drastically decreases after silica gel exposure. This indicated that the Boon2-BST is being removed from the lysate solution by the silica.

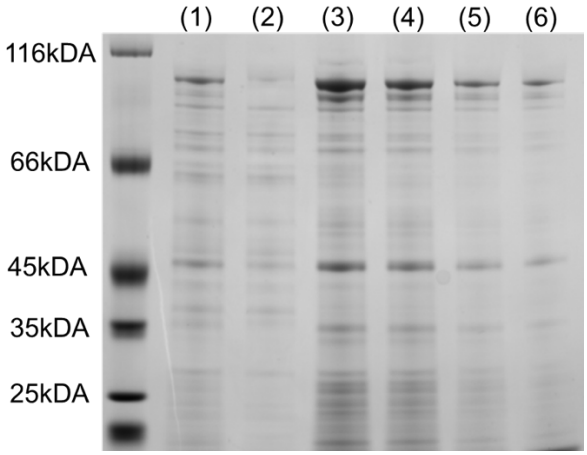

Figure 3.3: SDS-Page gel highlighting silica absorption and selectivity. The band for the Boon2-BST (98 kDa) is visible in the cell lysate (lane 1), but significantly less visible after silica absorption (lane 2). Lanes 3 to 6 are the absorbed proteins from 0.2 mg, 0.1 mg, 0.04 mg, and 0.02 mg of silica, respectively. The strong presence of the Boon2-BST band in the silica sample further emphasises the strong affinity of the silaffin-tag.

Lastly, the quantity of immobilised protein necessary for nucleic acid amplification (within a 1 hour timeframe) was tested by varying the quantity of protein absorbed silica per reaction sample. As shown in Figure 3.4, if at least 0.1 mg of silica was used, a positive reaction can be visualised via gel electrophoresis at the 45-minute mark.

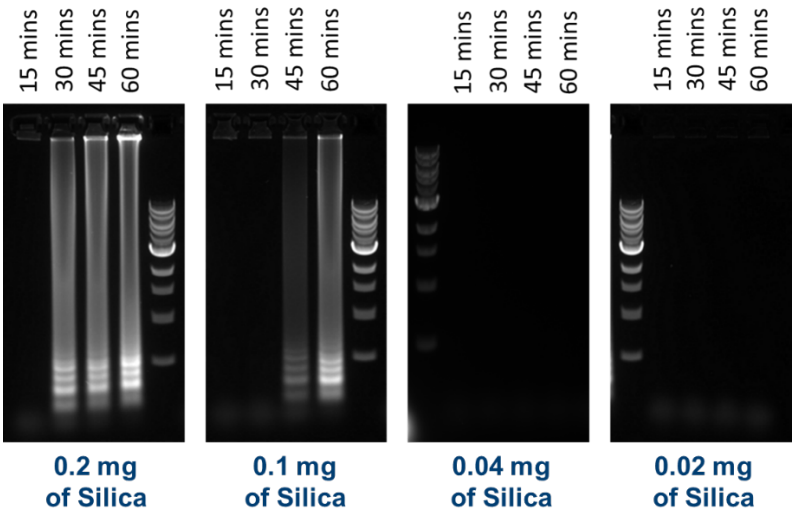

Figure 3.4: 2% agarose gels highlighting the activity of the immobilised protein. As expected, increasing the quantity of enzyme improves the reactivity of the assay. At lower silica quantities, there is a decrease in likelihood for the formation of a polymerase, primer, DNA triplex.

Though the enzyme is active and stable on silica, it was eluted off to provide the PATHPOD (WP2) system with turbidity compatible enzyme. Despite the compatibility of LAMP with native *E. coli* proteins, they were found to prevent the generation of magnesium pyrophosphate, which in turn inhibited the turbidity from increasing. Using controlled levels of L-arginine to wash and elute the Boon2-BST, turbid compatible enzyme was isolated, as shown in Figure 3.5.

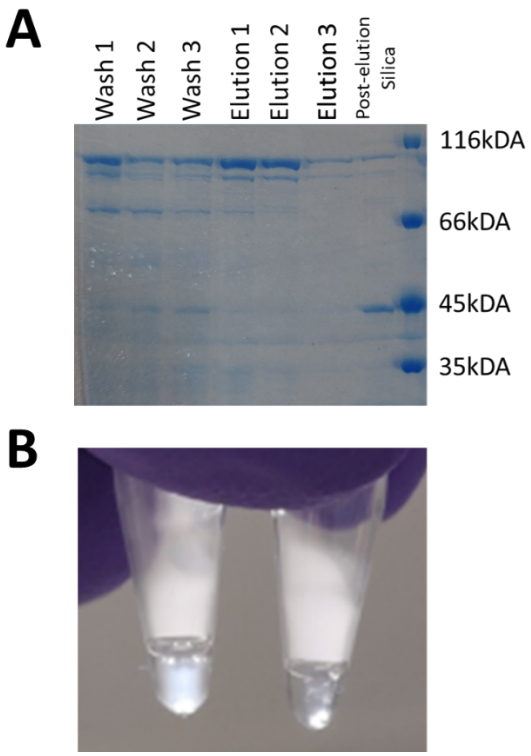

*Figure 3.5: **A** SDS-PAGE displaying the proteins washed and eluted off silica using L-arginine. **B** Image of PCR tubes displaying a positive (turbid) and negative (clear) LAMP reaction using the L-arginine eluted Boon2-BST.*

3.3 Δ3R5-PhoCl-mCh-H10-BST2.0

A few characterisation tests were performed with the photocleavable iteration of the Boon2-BST. Similar to the non-photocleavable version, protein expression would produce a pink pellet. However, unlike the previous iteration, the expression protocol required 0.5 mM IPTG at 18 °C for 16 hours.

In addition, the Δ3R5-tag continued to provide high affinity to silica with a good level of selectivity, as shown in Figure 3.6. The elution of the enzyme was unfortunately not successful. Once cleaved (after exposure to UV light) the mCherry-BST2.0 remain absorbed on the silica surface, rendering it incompatible with the turbidity based PATHPOD system.

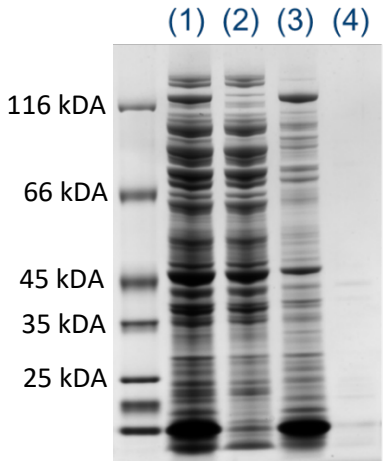

Figure 3.6: SDS-PAGE gel highlighting the presence and silica absorption of Δ3R5-PhoCl-mCh-H10-BST2.0 (126 kDa). The PhoCl-Boon2-BST band is present in the cell lysate (lane 1), not present in the lysate after silica exposure (lane 2) and is once again present on the silica itself (lane 3). Fortunately, exposing the immobilised protein to ambient indoor light is not enough for cleaving to occur, as there is no protein present the silica sample supernatant (lane 4). This also means extra safety procedures are not required to minimise pre-mature release.

Lastly, the presence of the photocleavable protein, aside from the general increase in the protein molecular weight, was determined though visualising the green fluorescence signal. As shown in Figure 3.7, whether it be in the *E. coli* or on the surface of the silica gel, the presence of green fluorescence indicated that the photocleavable protein folded correctly. In addition, the strong location overlap between the mCherry fluorescence (red) signal and the green signal suggest that the proteins are attached to one another.

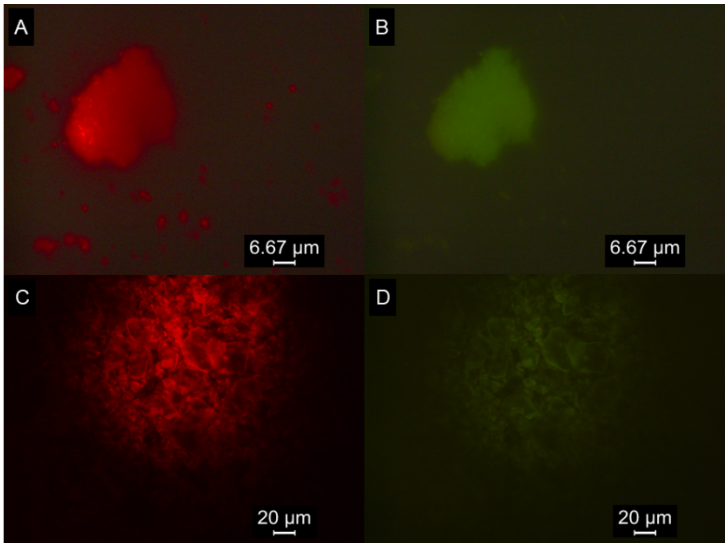

Figure 3.7: Fluorescence microscopy of E. coli from cell lysate (A, B) and of silica gel after protein immobilisation (C, D).

3.4 mCh-CipA-BST2.0

Similar to the photocleavable variant, the cellulose binding (CipA) iteration of the BST2.0 DNA polymerase, also required expression conditions of 18 °C for 16 hours. This is likely due to the large size of the enzyme (119 kDA). In addition, the cellulose immobilisation was found to be more selective than silica as shown in Figure 3.8. However, this iteration of the enzyme was found to have inconsistency activity and was not explored further.

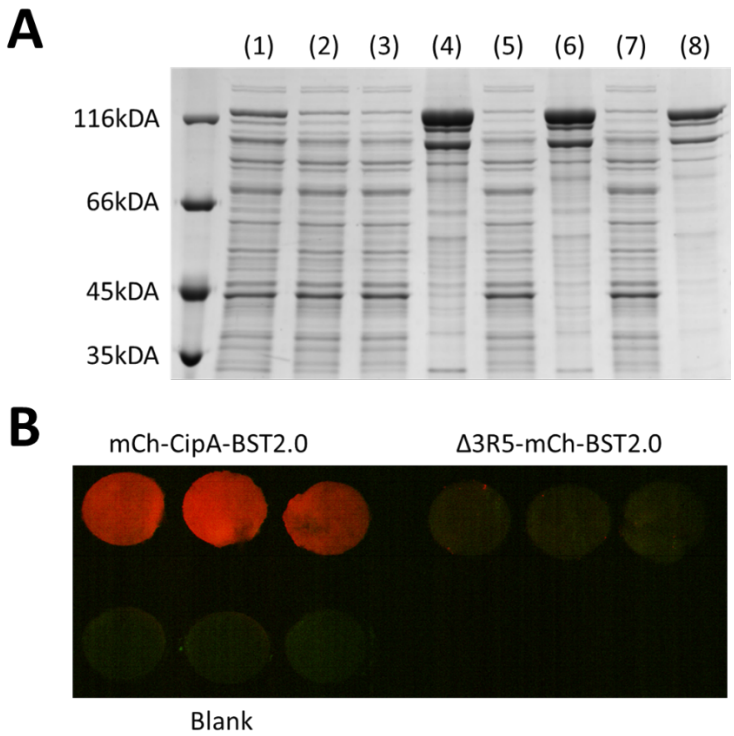

*Figure 3.8: **A** SDS-PAGE displaying the cell lysate from 18 °C expression (1) and 37 °C expression (2), 4.25mm diameter Whatman® filter paper flow through (3) and immobilisation (4), 3.25mm diameter Whatman® filter paper flow through (5) and immobilisation (6), and 2.25mm diameter Whatman® filter paper flow through (7) and immobilisation (8). **B** mCherry fluorescence image highlighting the selective absorption of only the CipA protein by cellulose.*

3.5 Expression and Purification SOP

Both the expression and purification SOP have been completed for the Boon2-BST protein. Both the Isopropyl ß-D-1-thiogalactopyranoside (IPTG) based expression and silica-based purification SOP have presented additionally in video format. Lastly, an SOP utilising auto-induction expression has also been delivered. Both the text and video protocols are available in the WP3 and WP8 dropbox.

The titles are the following:

- WP3.1 - Protein Expression Protocol of Engineered Boon2-BST.pdf
- WP3.1 – Protein Purification Protocol for Engineered Boon2-BST.pdf
- WP3.1 – Auto-induction Protein Expression Protocol for Engineered Boon2-BST.pdf
- WP3.1 – Boon2-BST Expression Training.mp4
- WP3.1 – Boon2.BST Purification Training Video.mp4