APPROACHES FOR THE PURIFICATION OF *E. COLI* B-CLAMP IN COMPLEX WITH THE C-TERMINAL DOMAIN OF THE A-SUBUNIT OF DNA POLYMERASE III.

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ABSTRACT: Understanding the structural organization of replisome components is essential to elucidate the coordinated processes of DNA replication and repair in bacteria. This study designed to purify complexes formed between the α subunit (DnaE) of replicative polymerase the and the β-clamp (DnaN). To this end, DnaN and a 255-amino-acid C-terminal fragment of DnaE tagged with 6xHis and containing the iCBM consensus sequence (referred to as DnaE905hM) were individually overexpressed in *E. coli* B834 cells. While DnaN was found in the soluble fraction, DnaE905hM was localized to the insoluble fraction. The insoluble DnaE905hM was solubilized under denaturing conditions, bound to an affinity column, and refolded on-column in the presence of the β-clamp. The resulting complex was further purified using size-exclusion chromatography. Based on molecular weight predictions, a complex comprising one dimeric β-clamp (81.2 kDa) and one DnaE905hM subunit (26 kDa) was expected to elute around 180 ml, while a complex containing two DnaE905hM molecules was predicted to elute at approximately 175 ml. A prominent elution peak was observed at 173 ml, along with broader secondary peaks at 155, 205, and 220 ml. SDS-PAGE and Western blot analyses, using mouse anti-6x-Histidine antibodies conjugated with alkaline phosphatase and rabbit polyclonal anti-β-clamp antibodies, showed that β-clamp was most abundant in fraction 21, while DnaE905hM was primarily detected in fraction 22 likely representing their monomeric forms. Fainter bands of both proteins across fractions 15 to 19 suggest a range of complex stoichiometries.

Key Words: Beta clamp, DnaE, DnaE-beta-clamp complex.

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INTRODUCTION

The faithful replication and inheritance of genetic material is essential for the survival of all living organisms. In all domains of life, DNA polymerases operate alongside a suite of accessory proteins together forming the replisome to ensure high-fidelity DNA replication (Kelman, 1995; Taft-Benz and Schaaper, 2004). Among these accessory proteins, circular sliding clamps also termed processivity factors play a pivotal role by anchoring DNA polymerases to the DNA template, thereby significantly boosting their processivity. In *Escherichia coli*, this function is carried out by the β-clamp, a homodimeric ring-like protein that encircles DNA and enhances the efficiency of polymerase action by several magnitudes (Kelman, 1995; Kuriyan and O'Donnell, 1993). *E. coli* possesses five distinct DNA polymerases (Heltzel et al., 2009), and substantial attention has been given to understanding how these enzymes interact with the β-clamp, particularly during polymerase switching events such as translesion synthesis (TLS). Genetic, biochemical, and structural analyses have consistently highlighted the central role of the β-clamp in coordinating these dynamic replication events (Heltzel et al., 2009; Sutton and Duzen, 2006). Yet, a comprehensive understanding demands detailed insight into the specific structural interactions between the β-clamp and its various partner proteins within the replisome.

Several co-crystal structures have been resolved to date, showing the β-clamp in complex with short synthetic peptides derived from various polymerases (Burnouf et al., 2004; Georgescu et al., 2008). While these studies have clarified the canonical clamp-binding motif (CBM), the limited size of the peptides used has hindered investigation into potential extended or secondary interaction regions (Bunting, Roe, and Pearl, 2003; Patoli, Winter, and Bunting, 2013). In *E. coli*, DNA polymerase III (Pol III) serves as the primary replicative enzyme, with its core composed of the ε, α, and θ subunits (Johnson and O'Donnell, 2005). The α subunit, encoded by *dnaE*, is responsible for the enzyme's polymerase activity and consists of 1160 amino acids, with an approximate molecular weight of 129.9 kDa (Welch and McHenry, 1982; EcoGene.org). On its own, the core Pol III exhibits low processivity, synthesizing only ~20 nucleotides per second and elongating DNA over short stretches of ~1–10 nucleotides per binding event (Johnson and O'Donnell, 2005). This processivity increases significantly up to ~750 nucleotides per second when the α sub-unit associates with β-clamp. Unlike most β-clamp-interacting proteins that possess a single CBM,

Pol III is unique in having two (Dohrmann and McHenry, 2005; López de Saro, Georgescu, and O'Donnell, 2003). A conserved CBM sequence, QL[SD]LF, was identified through bioinformatics, yeast two-hybrid assays, and peptide competition experiments (Dalrymple et al., 2001). Substitution of the internal CBM (iCBM) in DnaE with this consensus motif has been shown to improve β-clamp binding up to 120-fold, as demonstrated using Surface Plasmon Resonance and supported by *in vivo* data (Dohrmann and McHenry, 2005; Patoli, 2019). Since the C-terminal domain (CTD) of α sub-unit (DnaE) harbors both β-clamp binding sites (Dohrmann and McHenry, 2005; Lamers et al., 2006; López de Saro et al., 2003), structural studies of the DnaE-CTD in complex with the β-clamp are necessary to reveal the full scope of their interaction. In this study, we aimed to co-purify the DnaE-CTD with the β-clamp for further structural characterization. We also report on the purification strategies used and the different stoichiometric complexes formed during the process.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Primers.: The primers used in the current study were obtained from Eurofins MWG GmbH. The forward primers were designed to incorporate an N-terminal 6xHis tag specifically into the DnaE construct. The complete list of primer sequences is provided in Table 1. A plasmid (designated as pACYC11-dnaN) cloned with the gene encoding full-length β-clamp under the control of the pACYC184-11b promoter (Fribourg et al., 2001), generously provided by Dr. Karen A. Bunting (University of Nottingham, United Kingdom). Expression of the 6xHis-tagged DnaE905hM fragment was achieved using the pET11 vector (Novagen). *E. coli* DH5α cells were used for routine cloning, while *E. coli* B834 (DE3) cells, which carry an IPTG-inducible T7 RNA polymerase gene, were used for protein expression.

Table 1 Details of the primers used in this study.

Construct Name	*Primer Name*	Sequences of Primers
dnaEΔ905H	*E905h-f*	GGAATTCCATATGCATCATCATCATCATCATGCGTTAAAAGCGGCAG
905 – 1160 (768bp)	*E905h-r*	CCCGGATCCTTATTAGTCAAACTCCAGTTCC
dnaΔ905h-mut	*BC-f*	GCGGAAGCTATCGGTCAGCTGGATCTGTTCGGCGTGCTCGCCGAAG

Key: CATATG = *Nde*I, GGATCC = *Bam*HI

Amplification of DnaE905: A fragment encoding the C-terminal residues (a.a.=255) of DnaE were amplified from E. coli genomic DNA extending primers E905f and E905r (see Table 1). The forward primer included an N-terminal 6xHis tag. The amplified product was then cloned into the pET-11 vector at NdeI and BamHI restriction sites, producing the construct pAPdnaE905h. Cloning and plasmid propagation were performed in E. coli DH5α. Insert orientation and sequence fidelity were confirmed through DNA sequencing.

Site-Directed Mutagenesis of DnaE905h: Mutation-specific primer (i-CBMCf; Table 1) and the Quick-Change Multi-Site-Directed mutagenesis ready-kit (Stratagene), was used to mutate the internal clamp-binding motif (iCBM) of DnaE905h following the method of Atif A. Patoli (2019). The resulting construct containing the consensus motif was named pAP-dnaE905hM.

Over-Expression of protein

(a) **Over expression of the β-Clamp:** The plasmid **pACYC11-dnaN** was transformed into *E. coli* B834 (DE3) cells. Transformed cultures were grown in LB medium supplemented with 34 µg/ml chloramphenicol at 37 °C. When the optical density at 600 nm (OD_{600}) reached 0.6–0.8, protein expression was induced with 0.1 mM IPTG, followed by overnight incubation at 25 °C. Cells were harvested by centrifugation and lysed by sonication in Buffer A (200 mM NaCl, 20 mM imidazole, 50 mM HEPES, pH 7.0). The resulting lysate was clarified by centrifugation at 15,000 × g for 30minutesat 4 °C.

(b) Over expression and Solubilization of DnaE905hM.

The **pAP-dnaE905hM** construct was transformed into *E. coli* B834 (DE3) cells. Transformed colonies were grown in LB medium containing 100 µg/ml ampicillin at 37 °C. When the culture reached an OD_{600} of 0.6–0.8, protein expression was induced with 0.1 mM IPTG, followed by overnight incubation at 25 °C. Cells were harvested and lysed using the same procedure as described above in Buffer A. The insoluble fraction containing DnaE905hM was isolated by centrifugation and subsequently solubilized in a denaturing buffer composed of 1 M NaCl, 40 mM imidazole, 50 mM HEPES (pH 7.0), and 6 M urea.

Affinity Chromatography for Refolding and Complex Formation: Purification using nickel-based affinity was performed on a HisTrap (5 ml.) column (GE Healthcare). The binding buffer (1 M NaCl, 50 mM HEPES pH 7.0, 40 mM imidazole, 6 M urea) was used to equilibrate column. Denatured DnaE905hM was loaded, and on-

column refolding was conducted by gradually reducing the urea concentration using a gradient of refolding buffer (same composition, without urea) managed by an ÄKTA Prime Plus system. Following refolding, 10 ml of clarified β-clamp lysate was manually injected at 0.5 ml/min to allow complex formation. The refolding buffer was used to wash column for the removal of unbound proteins, and the complex was eluted with 5 column volumes of elution buffer (1 M NaCl, 300 mM imidazole, 50 mM HEPES pH 7.0) using the same system.

Size Exclusion Chromatography: Preparative gel filtration was performed using a Superdex 200 (26/60) column (GE Healthcare) pre-equilibrated with size exclusion buffer (250 mM NaCl, 50 mM HEPES). The chromatography run was executed on the ÄKTA Prime Plus using a predefined program.

SDS-PAGE and Western Blotting: Samples were resolved on 10% SDS-PAGE gels with 1X SDS buffer. For this each sample (15 µl) was mixed with 5 µl protein loading dye. This was then boiled, loaded and electrophoresed at 125V for 1 hour 20 minutes. Protein transfer (western blotting) was carried out using the Xcell II Blot Module (Invitrogen) onto a nitrocellulose membrane in 1X transfer buffer at 25 V for 1.5 hours. Membranes were blocked in 5% milk in PBS-T for 15 minutes. For detecting His-tagged DnaE905hM, membranes were incubated with mouse anti-His alkaline phosphatase-conjugated antibody (Sigma) at 1:1000 for 1 hour, followed by three PBS washes and one PBS rinse. Detection was carried out using BCIP/NBT substrate (Sigma). For β-clamp detection, the membrane was incubated with rabbit polyclonal anti-β-clamp IgG (from Dr. Jody Winter) for 1 hour, washed, and then treated with a donkey anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody. BCIP/NBT was used for detection as earlier.

RESULTS

Overexpression of Proteins. Initial efforts focused on independently overexpressing and purifying DnaE905 for subsequent in vitro complex formation with the β-clamp. *E. coli* B834 (DE3) cells were transformed with the plasmid pAPdnaEΔ905h and grown in 50 ml LB medium added with suitable antibiotics. Cultures were grown at 37ºC to an OD_{600} of ~0.6, then induced using 0.1 mM IPTG at 25ºC for 4 hours. A modest reduction in growth rate was noted after induction, consistent with the expected cellular burden from overexpressed protein. This could also indicate potential disruption of replication processes, possibly due to sequestration of native β-clamp by the overproduced DnaE905h, which contains two clamp-binding motifs. Cell lysis was performed via sonication in 50 mM HEPES and 200 mM NaCl a buffer, followed by collection of soluble fraction. SDS-PAGE analysis of samples taken before and after induction revealed that DnaE905h was predominantly localized in the insoluble fraction (Figure 1), likely due to rapid accumulation or its intrinsically disordered character (Sutton and Duzen, 2006). Various induction parameters and temperatures were tested, but none yielded soluble DnaE905h. Consequently, a co-expression approach was adopted, wherein β-clamp (DnaN) was co-expressed with DnaE905h, following protocols established by Patoli (2019) and Patoli & Patoli (2019). This co-expression strategy resulted in both proteins being present in the soluble fraction (Figure 1). Soluble expression was maximal after 4 hours and/or overnight induction with 0.1 mM IPTG at 25ºC, suggesting that interaction with β-clamp would have stabilize DnaE-CTD and enhanced its solubility.

Purification of β-Clamp with DnaE905h. Initial attempts to purify the DnaE905h–β-clamp complex at small scale used batch Talon affinity chromatography. Elution fractions, wash steps, and flow-through were analyzed by SDS-PAGE. However, DnaE905h failed to co-elute with β-clamp, which was predominantly found in the wash fractions, while DnaE905h remained on the resin. These results implied that, the interaction between DnaE-CTD and β-clamp was insufficiently stable under the tested conditions (Figure 1).

Purification of β-clamp in complex with DnaE905hM (Mutant): To strengthen the interaction, the internal clamp-binding motif (CBM) of DnaE905h was substituted with a consensus sequence (QL[S/D]LF), which has been previously believed to enhance clamp binding (Dohrmann and McHenry, 2005; Patoli and Patoli, 2019). The resulting mutant, DnaEΔ905hM, was generated via site-directed mutagenesis as described earlier. Successful co-purification of β-clamp with DnaE905hM was achieved using Talon affinity resin (Figure 1). The method was progressed to scale up, and the complex was subjected to size exclusion chromatography on a Superdex (26/60) preparative column. The chromatogram showed a sharp void peak, that was then analyzed through SDS-PAGE and Western blotting using anti-6xHis and anti-β-clamp antibodies. Both β-clamp and DnaE905hM were detected in this early eluting peak, rather than in the expected region around 180 ml, which corresponds to a complex of one β-clamp in a dimeric form (~81.2 kDa) and one DnaE905hM (~26 kDa) (Figure 2).

Given that the OB-fold domain of DnaE905hM contributes to its interaction with β-clamp (Georgescu et al., 2009), it was hypothesized that single-stranded or primed DNA might further stabilize the complex. To test this, agarose gel electrophoresis was used to examine whether DNA co-eluted with the protein complex, and DNA was indeed present in void peak fractions (Figure 3). To eliminate this variable, the eluate was treated with

Benzonase (Novagen) to degrade contaminating DNA. A marked decrease in absorbance at 280 nm was observed after treatment (Figure 2), consistent with DNA removal, and coinciding with increased absorbance in later-eluting fractions. Nonetheless, even after optimization, no distinct peak consistent to the β-clamp / DnaE905hM complex was observed.

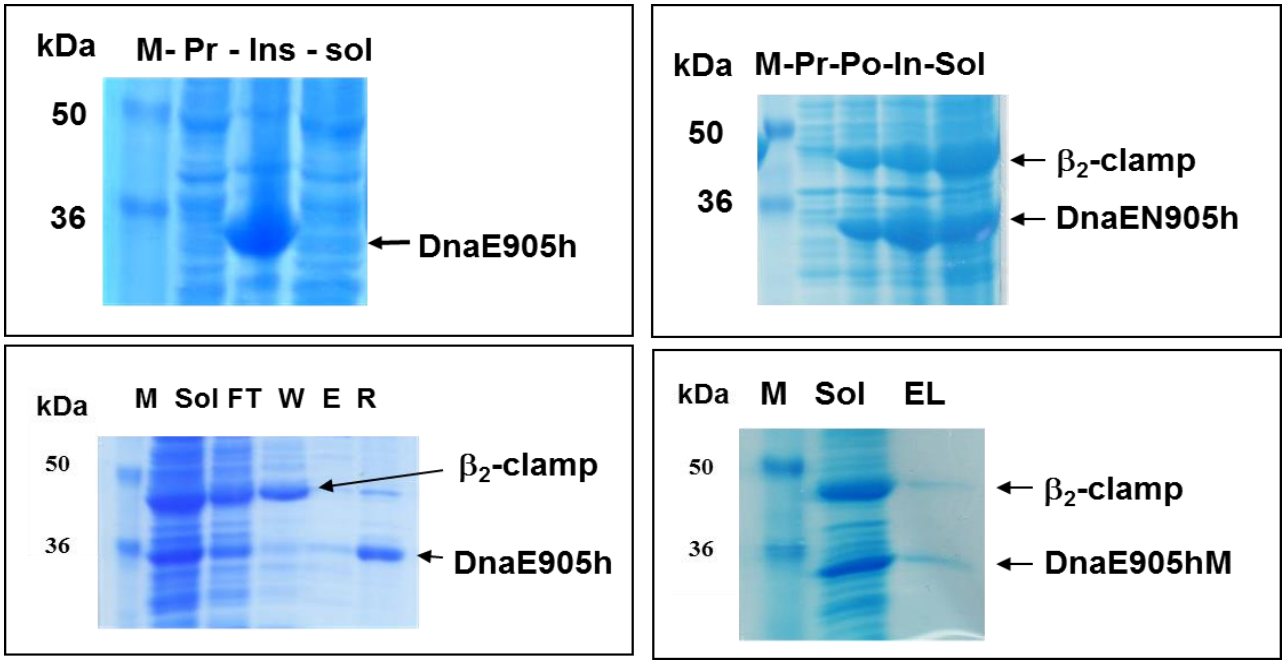

Figure 1. SDS-PAGE analysis displaying the insoluble and soluble fractions of DnaE905h (*Top Left*), co-expression of DnaE905h with β-clamp (*Top Right*), retention of DnaE905h on Talon resin and dissociation of β-clamp from the complex during wash steps (*Bottom Left)* and the presence of both DnaE905hM and β-clamp following affinity chromatography (*Bottom Right*). M = Molecular weight marker, Pr = Pre-induction, Po = Post-induction, Ins = Insoluble fraction, Sol = Soluble fraction, FT = Flow-through, S = Soluble fraction, W = Wash, E = Eluent, R = Resin after elution.

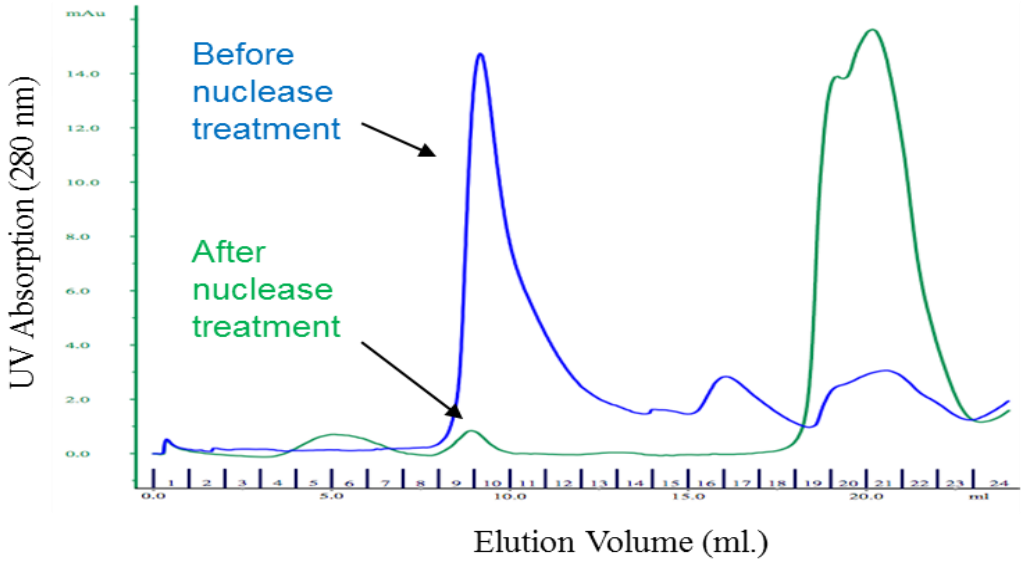

Figure 2. Size-exclusion chromatography (SEC) trace of the DnaE905hM–β-clamp complex (*Blue*), showing a prominent void peak. *(Green)* SEC trace following nuclease (Benzonase) treatment, demonstrating DNA degradation. The reduced absorbance at the void peak indicates the removal of co-eluting DNA by the nuclease.

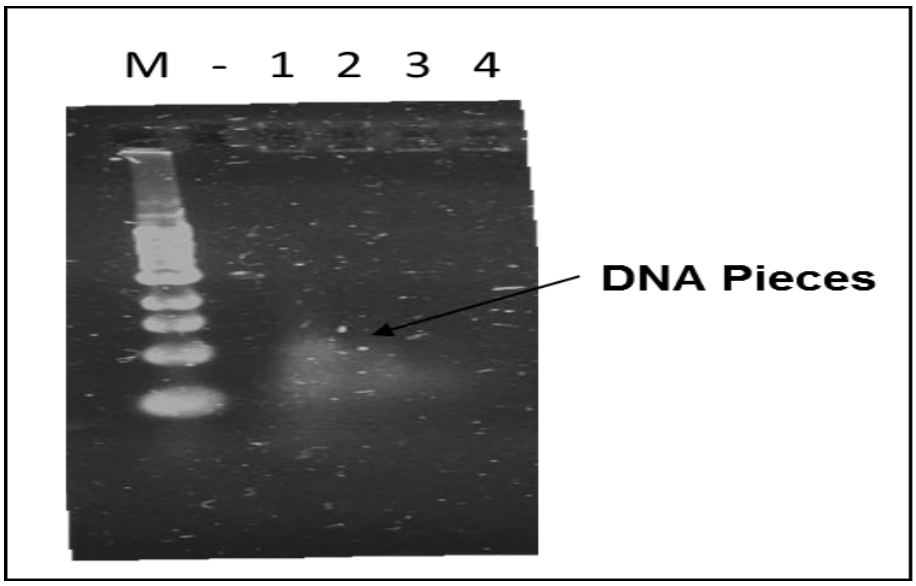

Figure 3 Agarose gel confirming the presence of DNA fragments in the void volume. M=Marker, E=Eluent, Fractions 1-4.

On-Column Refolding of the DnaE905hM and its Binding to β-Clamp. To circumvent the issue of aggregation linked to DNA binding, an on-column refolding strategy was implemented. Insoluble DnaEΔ905hM was solubilized in 6 M urea and applied to a nickel based affinity column at denaturing conditions to strip away bound DNA. Refolding was then achieved by gradual reduction of urea concentration on the column. Clarified lysate containing overexpressed, untagged β-clamp was then applied to the same column to allow binding. The complex was eluted using a buffer containing 300 mM imidazole, 50 mM HEPES, and 200 mM NaCl. SDS-PAGE confirmed the presence of both proteins in the eluate (Figure 4).

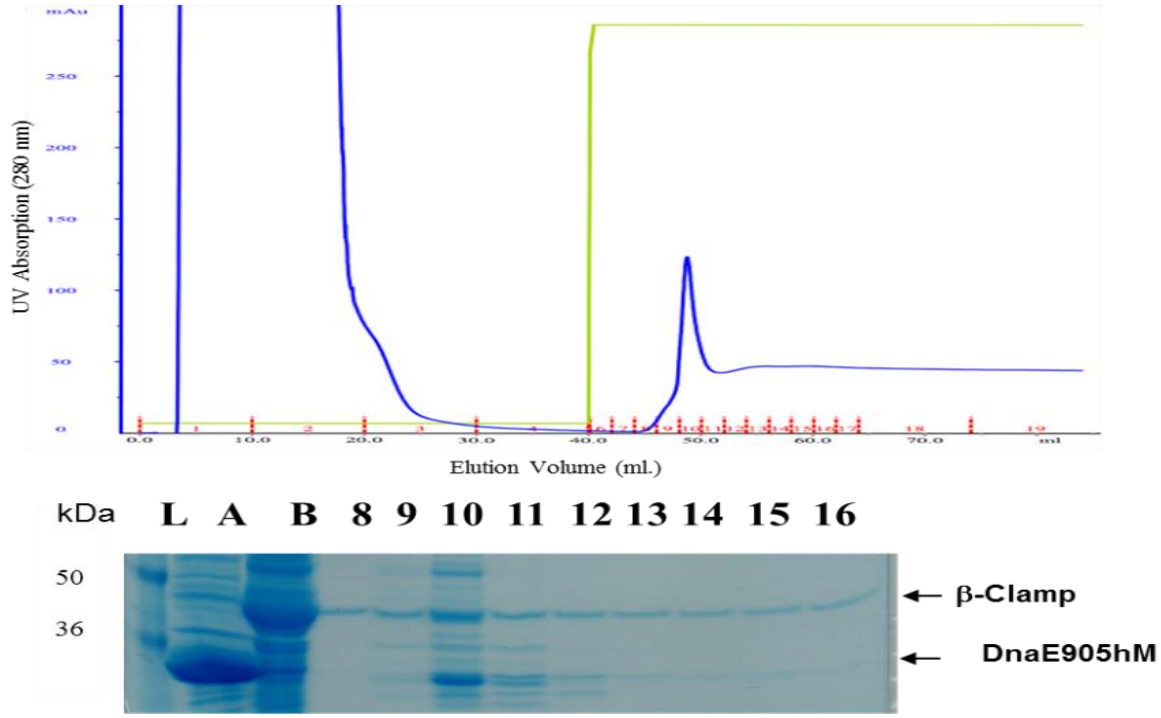

Figure 4. Chromatography trace from nickel affinity purification showing an absorbance peak at fraction 10 *(Top)*. SDS-PAGE analysis confirming the presence of DnaE905hM and β-clamp in the elution fractions following affinity chromatography *(Bottom)*.

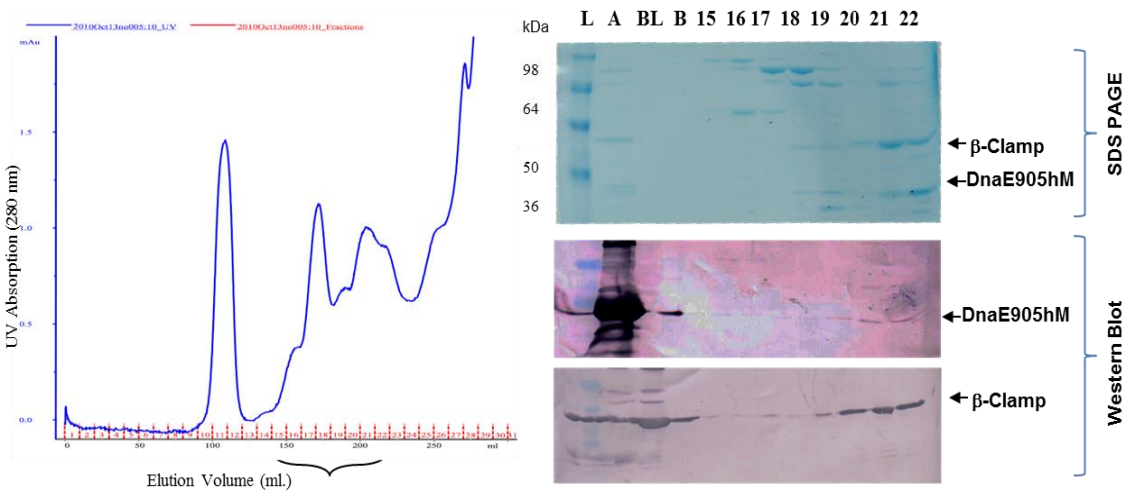

Figure 5. ***(Left)*** **Size-exclusion chromatography trace showing purification of the DnaE905hM/β-clamp complex, with increased UV absorbance observed across a broad elution volume range. SDS-PAGE analysis displaying the occurrence of DnaE905hM and β-clamp across multiple fractions. Western blotting confirms the identity of DnaE905hM and β-clamp using anti-His and anti-β-clamp antibodies, respectively** ***(Right)*****.**

Fractions 10 to 16 were mixed and used for size exclusion chromatography applying on a Superdex 200 10/300 column. The resulting chromatogram displayed a broad UV absorbance profile (150–220 ml), with a distinct peak at 173 ml. Notably, the void peak was reduced, indicating decreased aggregation (Figure 5). Elution volumes for various complex forms are summarized in Table 2. Predicted complex configurations include a β-clamp dimer with one DnaE905hM (~180 ml elution) and a dimer with two DnaE905hM molecules (~175 ml). A sharp peak was observed at 173 ml, with additional peaks at 155, 205 and 220 ml. Interpretation was complicated by the presence of co-purifying contaminants from the β-clamp lysate. SDS-PAGE showed intense β-clamp bands in fraction 21 and DnaE905hM in fraction 22, likely corresponding to their monomeric forms (Figure 5). Western blotting detected weaker signals for both proteins in fractions 15–19, suggesting the existence of multiple stoichiometric forms of the complex (Figure 5).

Table 2. Predicted elution volumes and fractions for various components and expected DnaE-Beta-clamp complexes on a size exclusion Superdex (26/60) column.

Expected complexes and individual components	Description	MW (kDa)	Superdex column (26/60)		Expected complexes and individual components	Description	MW (kDa)	Superdex column (26/60)	
			A	B				A	B
	β-clamp monomer	40.6	~205	21		β-clamp dimer and DnaE905hM	107.2	~180	19
	β-clamp dimer	81.2	~190	20		β-clamp dimer and 2 x DnaE905hM	133.2	~175	18
	DnaE905hM	26	~217	22		2 x β-clamp dimers and DnaE905hM	188.4	~170	18
	β-clamp monomer and DnaE905hM	66.6	~200	21		3 x β-clamp dimer and 2 x DnaE905hM	295.6	~160	17
	β-clamp dimer and DnaE905hM	107.2	~180	19		4 x β-clamp dimer and 3 x DnaE905hM	402.8	~150	16

DISCUSSION

DnaE905hM and β-clamp in the absence of DNA form complexes of varying stoichiometry: In the absence of DNA, DnaE905hM forms complexes with β-clamp that display variable stoichiometries. Given the highly dynamic and intricate nature of DNA replication, it is essential to gain structural insights into the interaction interfaces between replisome components to fully understand the mechanisms underlying coordinated polymerase activity and switching. Since β-clamp has been previously purified (Patoli, Winter, and Bunting, 2013), initial efforts were directed toward the purification of the DnaE905 domain of DNA polymerase III. For easier detection and purification, a 6xHis tag was introduced at the N-terminus of DnaE905. However, repeated attempts revealed that His-tagged DnaE905 remained predominantly insoluble. This led to a shift in strategy—co-expressing DnaE905h with β-clamp which successfully yielded soluble DnaE905h, most likely due to stabilizing interactions between the two proteins.

Despite achieving solubility, downstream purification proved difficult due to complex instability. Stability was improved by introducing a mutation in the internal clamp-binding motif (iCBM) of DnaE905h, resulting in the mutant DnaE905hM. However, purification remained complicated by the presence of contaminating DNA, which promoted aggregation and hindered recovery of the desired complex. To address this, a refolding protocol for DnaE905hM was implemented, followed by on-column complex formation with β-clamp. This approach successfully eliminated DNA contamination. Nevertheless, size exclusion chromatography revealed increased absorbance over a broad elution range, indicating the formation of complexes with heterogeneous stoichiometries in the DNA-free condition.

Although the OB-fold mutant and wild-type DnaE905 exhibit comparable binding affinities to β-clamp in the absence of DNA (Georgescu et al., 2009), data from Dohrmann and McHenry (2005) suggest that, without DNA, the external clamp-binding motif (eCBM) interacts with β-clamp weaker than the internal motif (iCBM). Thus, in DNA-free conditions, the eCBM may contribute little to complex stability. This raises the possibility that only one of the two clamp-binding sites in DnaE905hM engages with β-clamp, forming a minimal complex (MW ~107.2 kDa) as outlined in Table 2. Due to the similar molecular weights, complexes engaging either one or both binding motifs would likely elute in the same fraction (fraction 19, Table 2). However, if only one site is occupied, the remaining β-clamp interface could potentially recruit a second DnaE905hM molecule, giving rise to a higher-order complex predicted to elute in fraction 18.

Indeed, SDS-PAGE and Western blot analysis confirmed the presence of both DnaE905hM and β-clamp in fraction 18, consistent with this model. However, SDS-PAGE also revealed significant contamination by unrelated proteins in the same fraction, complicating precise interpretation of the complex's composition.

Authors' contributions: Project was designed by A.A. Patoli. Experimental work was performed by A.A. Patoli and B. Patoli. Manuscript was written with the contribution of both the authors.

Ethics approval: We declare that none of the human participants or animal models is used in this research work.

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