

International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; 10(9): 505-509
www.biochemjournal.com
Received: 01-06-2026
Accepted: 06-07-2026

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Sub-cloning and expression of BuPAG-8: A potential antigen for buffalo pregnancy diagnosis

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i9g.10044>

Abstract

Background: Buffalo pregnancy-associated glycoprotein-8 (BuPAG-8) is a pregnancy-associated protein of potential utility in the development of pregnancy diagnostic assays in buffaloes. Recombinant production of BuPAG-8 is an important step toward generating specific antibodies and evaluating its diagnostic potential. The present study aimed to clone and express the BuPAG-8 open reading frame (ORF) in a prokaryotic expression system.

Methods: The 1105 bp BuPAG-8 ORF was amplified using primers containing *NdeI* and *NotI* restriction sites and cloned into the pET22b (+) expression vector. The recombinant pET22b(+)-BuPAG-8 construct was transformed into *Escherichia coli* BL21 (DE3) Lemo21 cells. Recombinant protein expression was induced using isopropyl β -D-1-thiogalactopyranoside (IPTG), and the expression profile was evaluated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Results: The recombinant pET22b (+)-BuPAG-8 construct was successfully generated and expressed in *E. coli* BL21 (DE3) Lemo21 cells following IPTG induction. SDS-PAGE analysis revealed a recombinant protein band of approximately 41 kDa. The expressed BuPAG-8 protein was predominantly detected in the inclusion-body fraction, with a smaller proportion detected in the soluble fraction.

Keywords: BuPAG-8, buffalo, cloning, recombinant protein, expression, pregnancy diagnosis

1. Introduction

Accurate and early pregnancy detection in buffaloes (*Bubalus bubalis*) is essential for efficient herd management, improved reproductive performance, and sustained milk production (Abdullah *et al.* 2017; Rajamanickam *et al.* 2022) ^[1, 14]. Delayed or inaccurate diagnosis leads to prolonged calving intervals, economic loss, and suboptimal reproductive efficiency (Batra *et al.* 2019; Van Hanh *et al.* 2020) ^[3, 20]. Conventional methods such as rectal palpation and ultrasonography, although widely used, have practical limitations in early gestation. Biochemical markers offer a more sensitive and non-invasive alternative, and pregnancy-associated glycoproteins (PAGs) have emerged as promising candidates for this role (Szenci 2021; Barbato *et al.* 2022; Ferraz *et al.* 2024) ^[18, 2, 4]. PAGs are secreted by binucleate trophoblast cells of the placenta and are released into maternal circulation shortly after implantation. Their presence in serum and milk reflects ongoing pregnancy, making them attractive biomarkers for diagnostic assays. In cattle, PAGs have been extensively studied and successfully applied in radioimmunoassay and ELISA-based tests (Holton *et al.* 2022; Kline *et al.* 2024) ^[5, 9]. However, in buffalo, despite their recognized importance, the diagnostic application of PAGs is complicated by the large number of isoforms and the limited understanding of their temporal expression patterns (Lotfan *et al.* 2018, 2019) ^[11, 12]. Recent studies have demonstrated that PAG concentrations in serum and milk can be used not only for early pregnancy detection but also for predicting embryonic mortality in buffaloes (Barbato *et al.* 2022) ^[2]. These findings highlight the potential of PAGs in improving reproductive efficiency. Still, a major limitation lies in the lack of purified isoform-specific antigens, which are essential for developing standardized immunoassays. Among the identified isoforms, BuPAG-8 has been recognized as a prominent candidate. The molecular identity of BuPAG-8 has been established through sequence characterization, but its recombinant expression has not yet been reported.

Recombinant protein expression in prokaryotic hosts such as *Escherichia coli* offers a cost-effective and scalable approach for generating antigenic proteins (Sandomenico *et al.* 2020; Huleani *et al.* 2022) [15, 6]. For PAGs, such an approach is particularly valuable as it would enable the production of large amounts of antigen for antibody generation and immunoassay development. In the present study, the BuPAG-8 open reading frame was subcloned from pJET1.2 into the pET2b + expression vector and expressed in *E. coli* BL21 (DE3) Lemo21 cells. The recombinant construct was verified by sequencing and colony PCR, while recombinant protein expression was confirmed by SDS-PAGE. This represents the first report of recombinant BuPAG-8 expression in *E. coli* and provides a foundation for its downstream use in developing immunodiagnostic assays for buffalo pregnancy detection.

2. Materials and Methods

2.1 Materials

E. coli TOP10 was used as the cloning host and *E. coli* BL21 (DE3) Lemo21 as the expression host. The BuPAG-8 gene was maintained in pJET1.2 and subcloned into the pET2b(+) expression vector. Restriction enzymes (*NdeI*, *NotI*), T₄ DNA ligase, and DreamTaq polymerase were obtained from Thermo Scientific (Germany). Plasmid isolation and gel extraction were carried out using the Wizard Plus SV Miniprep kit and Wizard SV Gel and PCR Clean-Up kit (Promega, USA). IPTG was procured from BR Biochem (India). All other standard chemicals and LB culture media components were of analytical grade.

2.2 Bacterial culture maintenance and plasmid isolation

E. coli TOP10 was used for cloning and plasmid propagation, while *E. coli* BL21 (DE3) Lemo21 served as the expression host. Both strains were routinely grown in LB broth at 37 °C, and glycerol stocks (25% final glycerol) were prepared from overnight cultures and stored at -80 °C for long-term preservation. Plasmid DNA was extracted from overnight-grown cultures using the Wizard Plus SV Miniprep DNA purification system (Promega, USA). Briefly, bacterial pellets were lysed, neutralized, and clarified, and the resulting supernatant was passed through silica spin columns. After washing, plasmid DNA was eluted in nuclease-free water and stored at -20 °C until further use.

2.3 PCR confirmation, restriction digestion, and ligation

The BuPAG-8 gene (1105 bp) was amplified using gene-specific primers carrying *NdeI* and *NotI* sites. Each 25 µl PCR reaction contained 1× DreamTaq buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 1 µl template DNA, and 1 U DreamTaq polymerase. Amplification conditions were: initial denaturation at 95 °C for 30 s; 30 cycles of denaturation (95 °C, 10 s), annealing (69 °C, 40 s), and extension (72 °C, 1 min); and a final extension at 72 °C for 5 min. The PCR-amplified BuPAG-8 fragment and pET2b(+) vector were digested with *NdeI* and *NotI* in Tango buffer at 37 °C for 4 h. Digested fragments were

purified from agarose gel and quantified. Ligation was carried out using T₄ DNA ligase (Thermo Scientific, USA) with a 3:1 insert-to-vector molar ratio in 1× ligation buffer. Reactions were incubated at room temperature for 6-8 h, and the ligation mix was used directly for transformation.

2.4 Bacterial transformation

Competent *E. coli* TOP10 cells were prepared using the CaCl₂ method. Briefly, exponentially growing cultures (OD₆₀₀ 0.25-0.3) were chilled on ice, harvested by centrifugation, and resuspended in ice-cold 0.1 M CaCl₂ followed by storage in 0.1 M CaCl₂ with 15% glycerol at -70 °C. For transformation, 5 µl of ligation mixture was added to 50 µl of competent cells, incubated on ice for 30 min, and subjected to heat shock at 42 °C for 90 s. Cells were immediately chilled, recovered in SOC medium for 45 min at 37 °C, and plated on LB agar containing ampicillin (100 µg/ml). Plates were incubated overnight at 37 °C, and transformants were further propagated in LB broth with ampicillin for plasmid isolation and verification.

2.5 Induction and verification of recombinant protein expression

Recombinant *E. coli* BL21 (DE3) Lemo21 harbouring pET2b(+)-BuPAG-8 was grown overnight in LB broth with ampicillin (100 µg/ml). A 1% inoculum was sub-cultured and grown at 37 °C until OD₆₀₀ reached 0.5-1.0. Expression was induced with 1.0 mM IPTG, while parallel uninduced cultures served as controls. Cultures were incubated for 4-5 h at 37 °C with shaking. Following induction, cells were harvested, resuspended in PBS, and lysed. Soluble and insoluble fractions were separated by centrifugation. Samples from uninduced and induced cultures, as well as soluble and pellet fractions, were mixed with SDS sample buffer, boiled for 3-5 min, and analysed by 12% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue to confirm BuPAG-8 expression and assess its solubility.

3. Results

3.1 Primer design and cloning of BuPAG-8

Specific primers containing *NdeI* and *NotI* restriction sites were designed to amplify the BuPAG-8 open reading frame (Table 1). PCR amplification yielded a single product of 1105 bp, which matched the expected size of the target gene (Fig. 1A). The fragment was ligated into the pET2b(+) vector under the control of the T₇ promoter and in-frame with the vector-encoded C-terminal His₆ tag, ensuring compatibility for downstream expression and purification (Fig. 1B). Recombinant plasmids were validated by restriction digestion, which released two distinct fragments corresponding to the 5375 bp pET2b(+) backbone and the 1105 bp BuPAG-8 insert (Fig. 1C). Colony PCR screening of randomly selected clones further confirmed the successful incorporation of the BuPAG-8 gene, with multiple colonies yielding the expected amplicon (Fig. 1D). These results confirmed the successful construction of the recombinant pET2b(+)-BuPAG-8 expression vector.

Table 1: Oligonucleotide primer sequences used for amplification of the BuPAG-8 gene.

Sr. No.	Restriction Enzyme	Primer name	Sequence (5'-3')	Tm
1	<i>NdeI</i>	BuPAG-8 Forward	ACGCCATATGATAGTCAAAATACCTCTAAGGC	60 °C
2	<i>NotI</i>	BuPAG-8 Reverse	TAATGCGGCCGCTGCTCGTGCCAGGCCAAT	58 °C

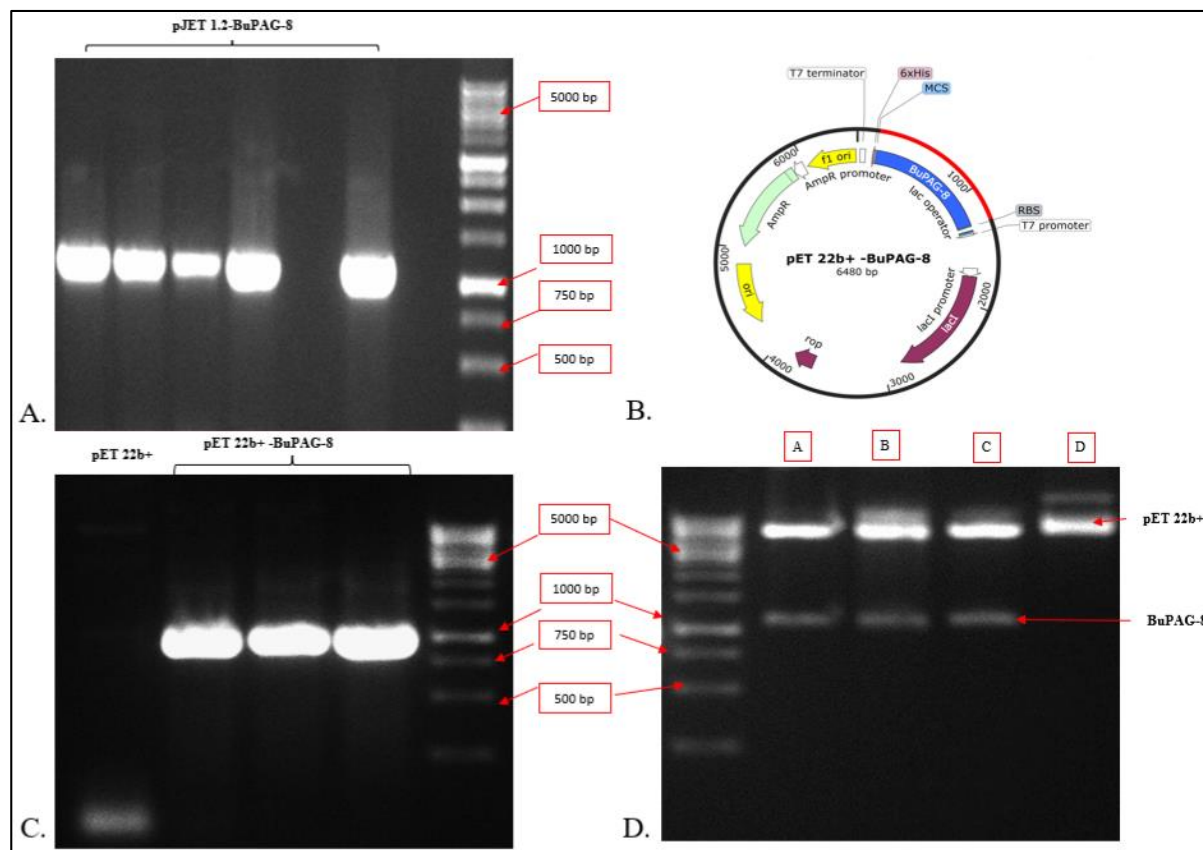


Fig 1: Construct verification of BuPAG-8 cloning.

(A) PCR confirmation of BuPAG-8 (1105 bp) amplified from the recombinant pJET_{1.2}-BuPAG-8 plasmid.

(B) Schematic representation of the recombinant pET_{22b}(+)-BuPAG-8 construct showing the BuPAG-8 insert downstream of the T₇ promoter and in-frame with the vector-encoded His₆ tag.

(C) Restriction digestion of the recombinant pET_{22b}(+)-BuPAG-8 construct with *NdeI* and *NotI*, releasing the expected 5375 bp pET_{22b}(+) backbone and 1105 bp BuPAG-8 insert.

(D) Colony PCR screening of recombinant pET_{22b}(+)-BuPAG-8 clones, showing amplification of the BuPAG-8 insert (1105 bp).

3.2 Expression of recombinant BuPAG-8

The verified construct was transformed into *E. coli* BL21 (DE3) Lemo21 for protein expression. Following induction with IPTG, SDS-PAGE analysis revealed a distinct band at ~41 kDa, corresponding to the theoretical molecular weight of BuPAG-8 (Fig. 2). The intensity of this band was markedly higher in induced cultures compared with

uninduced controls. However, a faint band of similar size was also detected in uninduced lanes, indicating basal or leaky expression from the T₇ promoter, which is commonly observed in strong expression systems. This confirmed that BuPAG-8 was successfully expressed in *E. coli* in a controlled, IPTG-inducible manner.

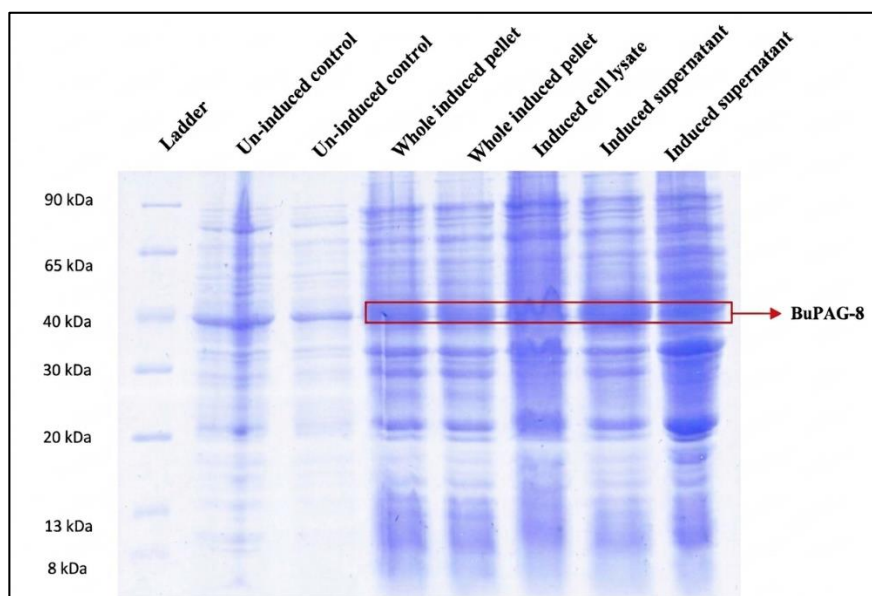


Fig 2: Expression and solubility analysis of recombinant BuPAG-8 in *E. coli* BL21 (DE3) Lemo21.

SDS-PAGE showing induction and solubility of recombinant BuPAG-8 (41 kDa). Lane M: protein molecular weight marker Lane 1-2: uninduced control. Lanes 3-4: induced whole-cell pellet. Lanes 5: induced cell lysate. Lanes 6-7: induced soluble supernatant. A prominent band of 41 kDa, corresponding to BuPAG-8, is observed in induced fractions compared with the uninduced control.

3.3 Solubility analysis of recombinant protein

Fractionation of cell lysates revealed that BuPAG-8 was present in both soluble and insoluble fractions. The recombinant protein was detected predominantly in the whole-cell pellet fraction, suggesting aggregation into inclusion bodies, a common outcome for eukaryotic proteins expressed in bacterial systems. Nevertheless, a weaker band of ~41 kDa was also visible in the soluble supernatant (Fig. 2), indicating that a fraction of the recombinant protein was expressed in a properly folded, soluble form. The recovery of soluble BuPAG-8 is an encouraging result, as it may retain structural features closer to the native protein, providing a suitable basis for antibody generation and future immunodiagnostic applications.

4. Discussion

Pregnancy-associated glycoproteins (PAGs) are important placental proteins secreted by mononucleate and binucleate trophoblast cells in ruminants (Mohanty *et al.* 2022; Wiltbank *et al.* 2023) [13, 22]. Their presence in maternal circulation has prompted their use as biomarkers for pregnancy diagnosis in cattle and buffalo. However, the multiplicity of PAG isoforms and variation in their temporal expression patterns complicate the identification of a single reliable diagnostic candidate. Among these, BuPAG-8 has been reported to be highly expressed during early gestation (Lotfan *et al.* 2018; Wiedemann *et al.* 2018) [11, 21], making it a promising biomarker for early pregnancy detection. In this study, the BuPAG-8 open reading frame was successfully amplified, cloned into the pET₂b(+) vector, and expressed in *E. coli* BL21 (DE3) Lemo21 cells. Construct verification through PCR and restriction digestion confirmed the integrity of the recombinant plasmid. Following IPTG induction, SDS-PAGE analysis revealed a band at 41 kDa, corresponding to the expected molecular weight of BuPAG-8. Interestingly, a faint band was also visible in uninduced cultures, indicating leaky expression, a well-documented feature of T₇ promoter-driven systems (Studier and Moffatt 1986) [17]. While leaky expression may pose problems for toxic proteins, BuPAG-8 did not appear to hinder bacterial growth, suggesting that it is well tolerated in *E. coli*. The T₇ promoter-based expression system is one of the most powerful tools for recombinant protein expression in *E. coli*. For this study, the pET₂b + vector was selected which provides a favourable environment for proper folding and disulfide bond formation due to the presence of oxidizing conditions and folding enzymes such as disulfide oxidoreductases and isomerases (Sokolosky and Szoka 2013; Taherian *et al.* 2019) [16, 19]. The vector also carries a C-terminal His-tag that greatly facilitates detection and purification of the recombinant protein through Ni-IMAC, while minimizing the recovery of truncated products that may arise from premature termination of translation (Kashani and Moniri 2015) [8]. The use of a C-terminal tag also allows the native N-terminus to remain unaltered, reducing interference with normal folding pathways. The construct was maintained in *E. coli* Top10 cells, which offer

high transformation efficiency and support a high plasmid copy number. This ensured that sufficient quantities of recombinant plasmid DNA were available for sub-cloning and further manipulations (Liu *et al.* 2018; Ibrahim *et al.* 2022) [10, 7]. Care was taken to avoid nucleotide insertions or deletions during cloning, as such changes could alter the open reading frame and compromise protein structure. For expression, the construct was introduced into *E. coli* Lemo21 (DE3), a strain derived from BL21 (DE3) and engineered to lack Lon and Omp proteases. The absence of these proteases prevents degradation of recombinant proteins and enhances overall yield. Optimal expression was achieved with induction using 1 mM IPTG for 4-5 hours at 37 °C in small-scale culture tubes. This condition allowed efficient accumulation of BuPAG-8 protein, consistent with expression strategies commonly employed for mammalian proteins in bacterial systems. The recombinant BuPAG-8 protein was successfully expressed in good quantity and was detected in both soluble and insoluble fractions, as verified using the Qproteome cell lysis system. This confirmed the effectiveness of the chosen vector-host combination and the optimized culture conditions for bacterial expression of BuPAG-8.

5. Conclusion

The present study successfully demonstrated the subcloning and overexpression of buffalo pregnancy-associated glycoprotein-8 (BuPAG-8) in *E. coli* using the pET₂b + vector system. The recombinant protein was efficiently expressed in both soluble and insoluble fractions, providing the first step toward its purification and further characterization. This work establishes BuPAG-8 as a promising candidate for developing species-specific immunoassays aimed at early pregnancy detection in buffalo, a field where reliable biomarkers are still limited. The production of recombinant BuPAG-8 thus opens opportunities for antibody generation and downstream applications that may enhance reproductive management in buffalo.

6. Credit authorship contribution statement

Neeraj K. Pawaskar: Conceptualization, Methodology, Investigation, Formal analysis, Writing-original draft, Writing-review & editing.

Ashok K. Mohanty: Supervision, Conceptualization, Resources, Investigation, Validation.

Sudarshan Kumar: Supervision, Conceptualization, Methodology, Validation.

Jai K. Kaushik: Supervision, Conceptualization, Methodology.

Munna Lal Yadav: Supervision, Conceptualization, Methodology.

Rashi Rastogi: Methodology, Formal analysis, Investigation.

7. Conflict of interest

The authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

8. Acknowledgment

The authors thank the Director, ICAR-NDRI, Karnal, India, for providing the necessary infrastructure and research facilities.

Funding

This research was funded by Indian Council of Agricultural Research; National Agricultural Science Fund (ICAR-NASF), Government of India.

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