https://doi.org/10.31436/iiumecp.v1i1.4421

HYBRID MAGNETIC NANOPARTICLE-BASED AFFINITY PURIFICATION OF RECOMBINANT *Rhodopseudomonas palustris* COLLAGEN-LIKE PROTEIN

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ABSTRACT: Recombinant bacterial collagen-like proteins, particularly *Rhodopseudomonas palustris* collagen-like protein (RPCLP), represent a promising alternative to animal-derived collagens for pharmaceutical, cosmetic, and biomedical applications. This study investigates a novel purification strategy for histidine-tagged recombinant collagen-like protein (His-RPCLP) using functionalized magnetic nanoparticles in a hybrid affinity system, offering enhanced scalability and cost-effectiveness compared to conventional column-based affinity chromatography. The methodology encompassed three key stages: synthesis and functionalization of magnetic nanoparticles with metal-chelating ligands, development of a hybrid magnetic affinity purification protocol, and comprehensive characterization of the purified protein. Protein quantification was performed using Bradford assay, while purity assessment utilized sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) coupled with ImageJ®-based densitometric analysis. Process optimization was achieved through Face Centered Central Composite Design (FCCCD) under Response Surface Methodology (RSM) framework, targeting simultaneous maximization of protein yield and purity. The experimental design focused on two critical parameters: functionalized magnetic nanoparticle loading and imidazole concentration in the elution buffer. Thirteen experimental runs were systematically evaluated to establish optimal operating conditions. Initial screening experiments demonstrated successful nanoparticle functionalization with an average recovery of 0.0418 g, yielding 0.39 mg/mL of purified His-tagged protein at 26% purity. Statistical optimization revealed optimal conditions producing a maximum RPCLP yield of 0.314 mg/mL with significantly improved purity of 91.51%, representing a 3.5-fold enhancement in protein purity compared to non-optimized conditions. However, ANOVA analysis indicated statistical insignificance of the optimization models ($p > 0.05$), suggesting the need for expanded experimental design incorporating additional factors or extended parameter ranges in future investigations. Despite model limitations, this study successfully demonstrates the feasibility of magnetic nanoparticle-mediated affinity as a viable purification platform for non-enzymatic recombinant proteins.

KEYWORDS: *Magnetic Nanoparticles, Affinity Chromatography, Collagen-Like Protein, Rhodopseudomonas palustris, and Response Surface Methodology (RSM).*

1. INTRODUCTION

Collagen represents the most ubiquitous family of fibrous and structural proteins in animals. Apart from being a vital component for various tissues and processes, collagen is

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increasingly used in numerous industrial applications, such as food and beverages, healthcare, and cosmetics production. However, concerns over the halal status, sustainability, quality, and immunogenicity of animal-derived collagens have motivated the search for alternatives. Recombinant bacterial collagen-like proteins, particularly *Rhodopseudomonas palustris* collagen-like protein (RPCLP), represent a lucrative and halal alternative source to animal-derived collagens for pharmaceutical, cosmetic, and biomedical applications [1-3]. The use of recombinant *E. coli* BL21(DE3) strain for RPCLP production had been sought as it is a well-established organism for protein production with many advantages such as rapid growth and high expression levels. However, there is a need for affordable, biocompatible, rapid, reusable, and scalable protein purification techniques. Traditional affinity chromatography methods are costly and challenging to scale up due to the need for specialized columns, slow flow rates, and multiple centrifugation steps. The recent developments in the field of nanotechnology and magnetic nanoparticles in particular have been notable due to their various abilities including superparamagnetism, rapid and selective separation abilities, cost-effectiveness, environmental sustainability, and biocompatibility. These magnetic nanoparticles (MNPs) can be excellent alternatives to traditional protein separation as they have a large surface area to volume ratio. In addition, MNPs can be further surface modified and functionalized to produce functionalized MNPs (fMNPs) that precisely bind proteins and allow for their separation and purification [4]. However, few studies have applied fMNPs to protein purification [5-8]. This study investigates a novel purification strategy for histidine-tagged recombinant RPCLP (His-RPCLP) using fMNPs in a hybrid affinity system, offering enhanced scalability and cost-effectiveness compared to conventional column-based affinity chromatography. To our knowledge, this is the first study applying fMNPs to purify His-RPCLP from *E. coli*.

2. METHODS

The methodology encompassed three key stages; namely the synthesis and functionalization of MNPs with metal-chelating ligands, development of a hybrid magnetic affinity purification protocol, and characterization of the purified protein.All chemicals used were of analytical grade. SDS-PAGE reagents were purchased from Biorad Laboratories, USA.

2.1 Production and Functionalization of MNPs

Magnetic nanoparticles (MNPs) were synthesized via co-precipitation of 1.351 g ferric chloride and 0.6582 g ferrous sulfate in deionized water, using ammonium sulfate as the precipitating agent [5]. Ammonium hydroxide was added at room temperature until co-precipitation occurred. The resulting MNPs were washed repeatedly with deionized water to remove residual ions and maintain a neutral pH (pH 7). The nanoparticles were then filtered and dried at 37 °C for 3 hours. Dried MNPs were dispersed in Millipore-grade water using an ultrasonic probe (Misonix Sonicator 3000). Functionalization was carried out using nickel chloride ($NiCl_2$) with N-phosphomonoethyliminodiacetic acid (PMIDA) as the coupling agent, which served as a chelating ligand [5]. Dispersed MNPs were adjusted to pH 10, mixed with PMIDA for 12 hours, and then magnetically separated and washed. The particles were subsequently incubated with nickel chloride for 1 hour, magnetically separated again, and dispersed in 10 mL of water at 25 °C for storage.

2.2 Production and Purification of His-RPCLP

E. coli BL21(DE3) carrying pColdII (Takara Bio) with a gene coding for histidine-tagged *R. palustris* collagen-like protein (RPCLP) was cultivated in LB Vegitone (Sigma Aldrich). The culture and the induction conditions were as described by Gameil [3]. Following cell lysis, the cell lysate was collected and stored at –20 °C.

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For purification, 10 mg of Ni^{2+} functionalized MNPs (fMNPs) were sonicated in 50 mM sodium phosphate buffer containing 100 mM NaCl (pH 7.4) for 20 minutes. Then, the cell lysate was incubated with fMNPs for 1 hour at 37 °C. After binding, magnetic separation was used to isolate the fMNPs, which were washed with buffer containing 50 mM sodium phosphate, 100 mM NaCl, and 50 mM imidazole to remove unbound proteins. Next, elution was performed with a buffer containing 50 mM sodium phosphate, 100 mM NaCl, and 200 mM imidazole. The His-tagged RPCLP was recovered by magnetic separation again and stored for analysis. The supernatant containing the desired protein was then collected for analysis by Bradford assay and SDS-PAGE as described by Gameil [3]. Refer to Fig. 1 for a detailed schematic of the purification protocol.

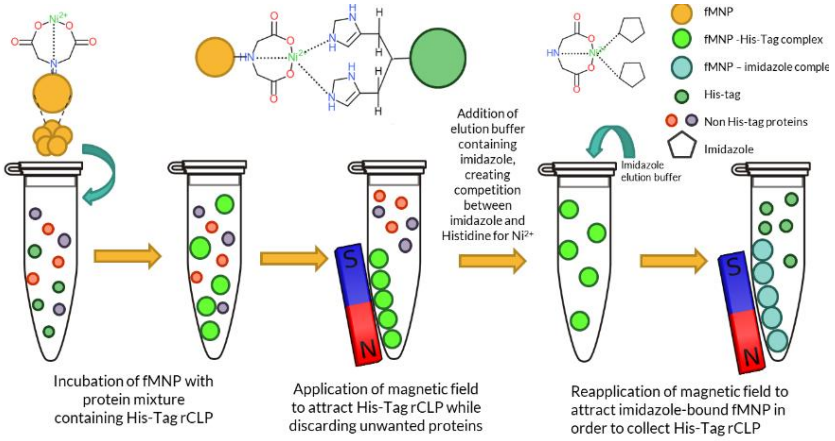

Fig. 1. Detailed illustration of purification of His-tagged CLP.

2.3 Protein Quantification and Purity Assessment

For quantitative protein detection, 5 µl of each sample (cell lysate, unbound protein, and purified His-tagged RPCLP) was mixed with 25 µl of Bradford dye reagent and absorbance was measured at 595 nm using a spectrophotometer (Multiscan Go™, Thermo Scientific, USA). A standard curve based on bovine serum albumin (BSA) was constructed and used to estimate the protein yield. By rearranging the linear equation of the standard curve, the protein concentration was determined from the absorbance at 595 nm.

For qualitative assessment and protein purity analysis, sample was assessed via SDS-PAGE at constant voltage (240 V) for one hour. The gel was analyzed densitometrically by using ImageJ® software to quantify protein bands around 32 kDa. The areas under the highest peaks of each plot were recorded for calculating the yield (refer to Eq. (1)).

$$Yield = \left(\frac{Area\ of\ Purified\ Protein}{Area\ of\ Total\ Protein}\right) \times 100\% \quad (1)$$

2.4 Optimization

The optimization process targeted the maximization of both RPCLP yield and purity. Design Expert® version 13 was used to implement a Face Centered Central Composite Design experimental design. Two factors, namely fMNP loading and imidazole concentration in the elution buffer, were varied at three levels (7.5, 10, 12.5 mg and 150, 200, 250 mM respectively). The response variables were RPCLP yield (mg/mL) and purity (%). Thirteen experimental runs with five (5) center points were systematically evaluated to establish optimal operating conditions.

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3. RESULTS

3.1 Synthesis of Magnetic Nanoparticle and Functionalization

Magnetic nanoparticles (MNPs) were synthesized following a modified version of Sahu's [5] protocol. While the original method yielded 0.3 g of solid MNPs, the modified procedure produced 22.5 mL of MNPs in liquid suspension. This adaptation facilitated functionalization, as sonication is most effective in the liquid phase. In total, 67.5 mL of magnetic nanoparticles were successfully synthesized and functionalized.

3.2 Preliminary Analysis via Bradford Assay and Densitometric Quantification

An average of 0.0418 grams of produced magnetic nanoparticles were recovered and were successfully functionalized. Preliminary purification experiments resulted in 0.39 mg/ml purified His-tagged protein with 26% purity.

3.3 Optimization and ANOVA Analysis by Design Expert® Software version 13

Fig. 2 and 3 show the SDS-PAGE results of all 13 experimental runs and the densitometric analysis in ImageJ® software, respectively, to determine purity (refer to Table 1 for the purity values). The yield values were calculated from the Bradford assay and the results are given in Table 1. The experiments aimed to maximize both the yield and purity of His-tagged RPCLP. Optimization results indicated that higher imidazole concentrations and greater fMNP amounts led to improved purity and yield. The 3D surface plot in Fig. 4 shows that purity was highest (91.51%) at 250 mM imidazole concentration and 12.5 mg fMNPs (Run 3), while Fig. 5 indicates the highest yield (0.315 mg/mL) was at 200 mM imidazole concentration and 12.5 mg fMNPs (Run 9).

ANOVA results indicated that both the models were not statistically significant ($F = 1.99$ and 3.23 for yield and purity respectively, p-values > 0.05, and R^2 values < 0.7). This is possibly due to a high variability in RPCLP concentration. Similarly, the parameters and their interactions were also not significant ($p > 0.05$). This suggests the need for further refinement of the design space and consideration of additional variables to improve model robustness. Despite model statistical insignificance, the data obtained is relevant and informative. Design Expert® software identified eight optimization solutions, with the most desirable condition identified as 12.5 mg fMNPs and 244.02 mM imidazole concentration. This supports the hypotheses that increased fMNP loading enhances nickel binding, and higher imidazole concentrations promote competitive elution of His-tagged proteins.

SDS-PAGE and densitometric analysis using ImageJ® software were successfully used to assess the purity of RPCLP during the purification (See Fig. 2 and 3). Fig. 3 shows the densitometric line profile analysis of gel band, taken from the SDS-PAGE intensity analysis. Representative line-scan plots demonstrating the quantification of a single band from an electrophoretic gel. The peak corresponds to the signal intensity of the band, while the surrounding fluctuations represent background noise. In the top and middle panels, baseline points are defined on either side of the peak to establish background levels for area integration. The bottom panel shows the final measured peak area highlighted in yellow, representing the integrated density used for quantitative comparison. Consistent baseline selection was applied to ensure accurate and reproducible densitometric measurements.This establishes the usefulness of this method for non-enzymatic protein purity assessment such as for other collagen-like proteins. Although the purity achieved exceeded 90%, the yield remains suboptimal. The imidazole concentration may still be insufficient to fully disrupt nickel-histidine interactions. Future work should explore higher imidazole concentrations and

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additional purification runs to enhance yield. Further optimization will be necessary to fully validate the purification platform for industrial-scale applications.

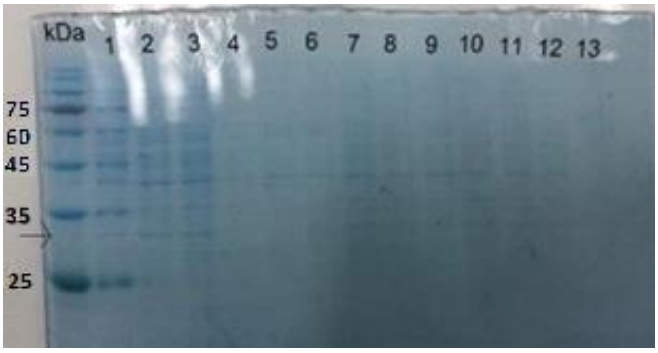

Fig. 2. SDS-PAGE gel showing the protein ladder and the bands for the thirteen runs (Lanes 1 to 13). The arrow indicates the protein band of interest (RPCLP).

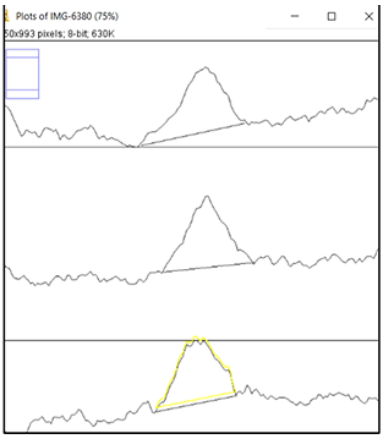

Fig. 3. An example of how curves are constructed from SDS-PAGE gel highlights using ImageJ® software. The band intensity was measured by using ImageJ® software, by selecting the area under the curve of the band. Area for total protein: 9317.03 unit2. The protein purity was then be calculated by dividing area under the graph of highest peak with the area of total protein. For e xample, $Purity_{Run\ 1}$ =(2154.39/9317.03) ×100% =23.12%

Table 1: FCCCD Design showing the experimental design and the results for each run

Run	Std	Mass of fMNP [mg]	Concentration in elution buffer [mM]	RPCLP yield [mg/ml]	RPCLP Purity [%]
1	11	10	200	0.12	23.12
2	12	10	200	0.02	9.38
3	4	12.5	250	0.16	91.51
4	10	10	200	0.04	37.38
5	13	10	200	0.16	43.47
6	9	10	200	0.13	18.14
7	3	7.5	250	0.10	10.78
8	5	7.5	200	0.20	76.61
9	6	12.5	200	0.32	75.85
10	1	7.5	150	0.07	17.76
11	7	10	150	0.04	7.13
12	2	12.5	150	0.06	10.06
13	8	10	250	0.11	58.29

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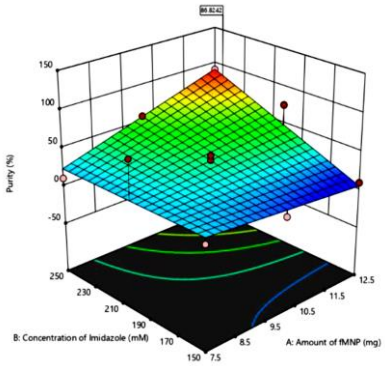

Fig. 4(a). 3D Surface graph of optimization of purity.

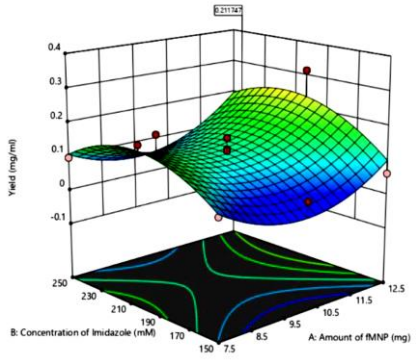

Fig. 4(b). 3D Surface of optimization of yield.

4. CONCLUSION

This study demonstrated the successful purification of His-RPCLP using fMNP-based affinity with 91.5% purity and 0.314 mg/mL yield, using SDS-PAGE and densitometric analysis for purity assessment. The method offers key advantages over traditional systems, including faster processing, minimal equipment, and automation potential. While the statistical models were not significant, the findings demonstrate that the approach is feasible for non-enzymatic recombinant proteins and facilitates CLP production for industrial applications. Future work should enhance model robustness, explore alternative functionalization chemistries, and evaluate the method for other proteins to fully realize its commercial potential.

ACKNOWLEDGEMENT

This study was funded by the Transdisciplinary Research Grant Scheme (TRGS/1/2018/UIAM/01/1/3) by the Ministry of Higher Education, Malaysia.

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