

A fusion protein strategy for high-yield production of DOPA- modified recombinant mussel adhesive protein Pvfp-5P

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Abstract

Mussel adhesive proteins (MAPs), particularly Pvfp-5 from *Perna viridis*, are promising bio-adhesives for biomedical applications, but their natural extraction is inefficient and recombinant production often suffers from low yield and insufficient DOPA modification. Here, we developed a fusion protein strategy to overcome these limitations. A chimeric gene encoding Pvfp-5 β fused with a collagen-like preCol-P fragment (designated Pvfp-5P) was codon-optimized and expressed in *Pichia pastoris* GS115 together with a co-expressed tyrosinase from *Bacillus megaterium* for in situ conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA). After 48 h of methanol induction in shake flasks, the recombinant protein was secreted at approximately 300 mg/L and purified to > 90% purity by single-step Ni-NTA affinity chromatography. The DOPA content reached 2.5 wt% determined by colorimetric assay. Functional assays revealed that Pvfp-5P significantly promoted L929 fibroblast migration in a scratch wound healing assay, achieving 93.5% wound closure at 72 h (100 μ g/mL), outperforming the natural MAP (83.5%). It also exhibited strong coating ability on both hydrophilic (glass) and moderately hydrophobic (polystyrene) surfaces. Mechanical testing showed an extremely low elastic modulus of 2.34 kPa, indicating high flexibility and excellent interfacial conformability. This work provides a green, efficient, and scalable way for producing bioactive recombinant MAPs with promising applications in tissue repair, wound dressings, and surface engineering.

Introduction

Mussels are marine bivalves that attach firmly to various submerged surfaces, including rocks, ship hulls, and piers, withstanding constant wave action and tidal currents. This remarkable underwater adhesion is mediated by the byssus, a bundle of proteinaceous threads secreted by the mussel foot [1]. At the terminal end of each thread is a flat adhesive plaque that forms strong, durable bonds with the substrate. The proteins responsible for this adhesion are collectively known as mussel adhesive proteins (MAPs) or mussel foot proteins (MFPs). To date, at least eight distinct MAPs have been identified and characterized, including Mfp-1, Mfp-2, Mfp-3F, Mfp-3S, Mfp-4, Mfp-5, Mfp-6, and Mfp-7. In addition, five byssal thread matrix proteins (preCOL-D, preCOL-P, preCOL-NG, PTMP-1, and TMP-1) have been reported [2, 3].

The unique adhesive properties of MAPs are largely attributed to a post-translationally modified amino acid, 3,4-dihydroxyphenylalanine (DOPA), which is derived from tyrosine. DOPA contains a catechol side chain that can form strong bidentate hydrogen bonds, coordinate with metal ions, and undergo oxidation to reactive quinones that cross-link with surfaces or other proteins [4, 5]. Among all MAPs, Mfp-5 (known as Pvfp-5 in *Perna viridis*) is, together with Mfp-3, one of the smallest MAPs and possesses the highest DOPA content (up to 25.5 mol% in the *Mytilus* genus), making it a key component for interfacial adhesion [6]. Natural MAPs also exhibit excellent biocompatibility, low immunogenicity, and biodegradability, which have led to their application in wound dressings, surgical adhesives, cell culture coatings, and drug delivery systems [7–9].

Despite their enormous potential, the practical use of natural MAPs is severely limited by the inefficiency of extraction from natural sources. Consequently, recombinant DNA technology has become a major focus for MAPs production. Among various expression systems, the methylotrophic yeast *Pichia pastoris* (syn. *Komagataella phaffii*) is particularly attractive because it combines high-density fermentation, strong methanol-inducible AOX1 promoter, efficient secretion, and the ability to perform eukaryotic post-translational modifications such as glycosylation and disulfide bond formation [10–12].

However, several challenges remain in the recombinant production of functional MAPs. First, expression yields for many MAPs are relatively low, often below 100 mg/L, due to their repetitive sequences, high hydrophilicity, and susceptibility to proteolytic degradation [13]. Second, the critical DOPA modification is not efficiently performed by the yeast's endogenous enzymes; therefore, co-expression of a heterologous tyrosinase is required [14]. Third, the purification process often involves multiple chromatographic steps, increasing cost and time. To address these issues, various strategies have been explored, including fusing the target protein with a highly expressed carrier protein to improve stability and yield, performing codon optimization of the gene sequence to adapt to the host expression system, and optimizing fermentation conditions to enhance expression levels [15].

Recent studies have shown that *P. pastoris* exhibits a strong preference for proteins rich in Gly-X-Y repeat sequences, such as collagens and collagen-like proteins [16, 17]. For example, recombinant human collagen has been produced at yields exceeding 4 g/L in fed-batch fermentations [18]. Interestingly, the byssal thread matrix protein preCol-P contains a collagen-like domain with typical Gly-X-Y repeats [19]. This observation led us to hypothesize that fusing a target MAP (Pvfp-5) with a fragment of preCol-P might enhance its expression yield by exploiting the yeast's translational preference. Additionally, to achieve efficient in situ DOPA modification, we chose to co-express a tyrosinase from *Bacillus megaterium* in *P. pastoris*.

In this study, we constructed a fusion protein named Pvfp-5P, consisting of the full-length Pvfp-5 β from *P. viridis* and a partial peptide of preCol-P. The synthetic gene was codon-optimized for *P. pastoris* and placed under the control of the AOX1 promoter in the pPICZ α A vector. A second plasmid carrying the tyrosinase gene under the same promoter was introduced to generate a co-expression strain. The recombinant protein was produced in shake flasks, purified by one-step Ni-NTA affinity chromatography, and characterized for its DOPA content, cell migration-promoting activity, and surface coating ability. Our results demonstrate that this fusion strategy significantly improves production efficiency and yields a bioactive recombinant MAP with promising potential for biomedical applications.

Materials and methods

Strains, vectors, and chemicals

P. pastoris strain GS115 (his4) and expression vectors pPICZ α A and pPIC9K were purchased from Invitrogen. Restriction enzymes (*Xho*I, *Not*I, *Sac*I, *Sal*I), T4 DNA ligase, and PCR reagents were obtained

from Thermo Fisher Scientific. L-DOPA standard ($\geq 99\%$ purity), natural mussel adhesive protein (MAP, $> 90\%$ purity), nitroblue tetrazolium (NBT), bovine serum albumin (BSA), and Coomassie Brilliant Blue R-250 were purchased from MedChemExpress. Ni-NTA agarose (6FF) was purchased from GE Healthcare. All other reagents were of analytical grade and purchased from domestic suppliers.

Construction of expression vectors

The amino acid sequence of the fusion protein Pvfp-5P (Fig. S1a) was designed by linking the full-length Pvfp-5 β from the mussel *P. viridis* with a 171-amino-acid fragment from preCol-P. A (GGGGS)₃ flexible linker was inserted between the two domains. The nucleotide sequence encoding Pvfp-5P (Fig. S1b) was codon-optimized for *P. pastoris* using the GeneArt® GeneOptimizer software (Thermo Fisher) and synthesized by Sangon Biotech (Shanghai, China). The synthesized fragment was digested with XhoI and NotI and ligated into the pPICZαA vector (digested with the same enzymes) to generate pPICZαA-Pvfp5P. The construct was verified by DNA sequencing.

To convert tyrosine to DOPA in recombinant proteins, the gene encoding *B. megaterium* tyrosinase was codon-optimized and synthesized (Fig. S2a, b). The fragment was inserted into pPIC9K via XhoI and NotI to create pPIC9K-TYR, which was confirmed by sequencing.

Construction of Pvfp-5P single-expression strain

The pPICZαA-Pvfp5P plasmid (10 μ g) was linearized with SacI and purified by phenol-chloroform extraction. The linearized DNA (2 μ g) was electroporated into GS115 competent cells (1.5 kV, 25 μ F, 200 Ω). After recovery in 1 M sorbitol for 1 h, cells were plated on YPD agar containing 100 μ g/mL Zeocin and incubated at 30°C for 2–3 days. Colony PCR with primers 9654-F (5'-TATCATGGTGCTCCAGGTAGAC-3') and 3'AOX (5'-GCAAATGGCATTCTGACATCC-3') confirmed positive integrants by a ~ 500 bp amplicon.

Ten PCR-positive clones were cultured in BMGY at 28.5°C with shaking to OD₆₀₀ 2–6, then induced in BMMY with 1% methanol for 70 h. Culture supernatants were analyzed by SDS-PAGE and Western blotting using anti-His antibody. The clone showing the highest expression and fewest contaminant bands was designated GS115/Pvfp5P and selected for further co-expression.

Construction of co-expression strain

pPIC9K-TYR (10 μ g) was linearized with SalI, purified, and electroporated into GS115/Pvfp5P. Transformants were selected on MD plates (1.34% YNB, 4 $\times 10^{-5}\%$ biotin, 2% dextrose, 1.5% agar) containing 100 μ g/mL Zeocin. Colony PCR was performed using two primer pairs: (i) TYR-F (5'-GACGAGCAAGGTAACCCAT-3') / 3'AOX to amplify the tyrosinase gene, and (ii) Pvfp-5P-F (5'-TATCATGGTGCTCCAGGTAGAC-3') / 3'AOX to amplify the Pvfp-5P gene. Twenty clones were screened,

and those positive for both genes were subjected to small-scale co-expression in BMMY containing 100 μM CuSO_4 (as described above). The best co-expressing clone was named GS115/Pvfp5P/TYR.

Shake-flask production and Ni-NTA purification

A single colony of GS115/Pvfp5P/TYR was inoculated into 10 mL BMGY and grown overnight at 28.5°C with shaking. The overnight culture was diluted 1:1000 into 100 mL fresh BMGY and incubated under the same conditions until the OD_{600} reached approximately 5. Cells were harvested by centrifugation, resuspended in BMMY containing 100 μM CuSO_4 to an OD_{600} of 1.0, and induced for 48 h at 28.5°C with 1% methanol, which was replenished at 24 h together with CuSO_4 .

The culture supernatant was collected and loaded onto a Ni-NTA gravity column pre-equilibrated with binding buffer (20 mM sodium phosphate, 500 mM NaCl, 30 mM imidazole, pH 7.8). After washing with 10 column volumes of the same buffer, the bound protein was eluted with elution buffer containing 300 mM imidazole. Fractions containing the target protein were pooled, desalted using a 3 kDa ultrafiltration device, and lyophilized. Protein concentration was determined by the Bradford method using BSA as standard.

Mass spectrometry identification

The purified Pvfp-5P band was excised from SDS-PAGE and subjected to in-gel digestion with trypsin. The resulting peptides were analyzed by LC-MS/MS using an UltiMate 3000 RSLCnano system coupled to a Q Exactive HF mass spectrometer, with separation on a C18 analytical column at 300 nL/min. Data-dependent acquisition was employed: full MS1 scans were acquired at 60,000 resolution, and the top-20 precursors were fragmented by HCD with 28% collision energy. Raw data were searched with MaxQuant against a custom protein database. Variable modifications included carbamidomethylation of Cys, oxidation of Met, and acetylation of protein N-termini. Trypsin/P was used allowing up to two missed cleavages. Mass tolerances were 20 ppm for the first search and 4.5 ppm for the main search for MS1, and 20 ppm for MS2. Results were filtered at 1% FDR.

DOPA identification by NBT staining

The presence of DOPA was detected qualitatively by the nitroblue tetrazolium (NBT) method according to the Chinese pharmaceutical industry standard YY/T 1293.6–2020 [20]. Purified Pvfp-5P, natural MAP (positive control), and BSA (negative control) were dissolved in deionized water to 1 mg/mL. Two microliters of each solution were spotted onto a 0.2 μm nitrocellulose membrane (5 cm $\times$ 5 cm) and allowed to air-dry. The membrane was placed in a 500 mL beaker with 300 mL deionized water and sonicated for 10 min to remove unbound proteins. It was then transferred to a petri dish containing NBT staining solution (0.2 mg/mL NBT in 0.1 M glycine-NaOH, pH 10.0) and incubated in the dark for 45 min. After staining, the membrane was washed twice with boric acid solution (0.1 M, pH 8.5), soaked in

borate buffer overnight, and finally rinsed three times with deionized water. The appearance of a blue-purple spot indicated DOPA positivity.

DOPA quantification by colorimetric assay

DOPA content was determined by the nitrite-molybdate method as described in YY/T 1293.6–2020 [20]. L-DOPA standards (0–60 µg/mL) were prepared in 0.012 M HCl. Protein samples (natural MAP and Pvfp-5P) were dissolved in 0.012 M HCl at 0.5 mg/mL. One milliliter of each standard or sample was mixed with 0.5 mL of 0.516 M HCl and 1.5 mL of nitrite-molybdate reagent (1.45 M NaNO₂, 0.41 M Na₂MoO₄·2H₂O). After 5 min, 2 mL of 1 M NaOH was added, and the absorbance at 500 nm was measured immediately using a UV-Vis spectrophotometer (Ultrospec 2100, GE Healthcare). A standard curve was plotted (concentration vs. absorbance), and DOPA content in samples was calculated. Measurements were performed in triplicate.

Cell migration (scratch wound healing) assay

L929 mouse fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5% CO₂ humidified incubator. For the scratch assay, cells were seeded into 6-well plates at 2×10⁶ cells per well and cultured to a confluent monolayer. A straight scratch was created using a sterile 200 µL pipette tip. Detached cells were removed by washing three times with PBS. Then, serum-free DMEM containing 100 µg/mL of either Pvfp-5P or natural MAP was added. Wells with serum-free DMEM alone served as the blank control. The plates were incubated under standard conditions. Phase-contrast images of the scratch were taken at 0, 24, 48, and 72 h using an inverted microscope (Nikon Eclipse Ti-U, Japan). Wound areas were measured using ImageJ software (NIH, Bethesda, MD, USA). The wound healing rate was calculated as [(initial area – area at time t) / initial area] × 100%. Each condition was tested in triplicate.

Surface coating ability assay

Glass (hydrophilic) and polystyrene (moderately hydrophobic) were used as substrates. Glass slides were cleaned by sequential sonication in acetone, ethanol, and water for 15 min each; Polystyrene dishes were rinsed with water. Protein solutions (1.0 mg/mL) of Pvfp-5P, natural MAP (positive control), and BSA (negative control) were prepared. Ten microliters of each protein were spread over a 1 cm × 1 cm area on each substrate (triplicate), incubated for 30 min, then washed three times with PBS (pH 7.4) and once with water. Coomassie Brilliant Blue R-250 staining solution (0.25% R-250 in 45% ethanol and 10% acetic acid) was applied to cover the coated areas and incubated for 30 min. Destaining solution (acetic acid:ethanol:water = 1:2:7, v/v) was then added and changed every 30 min until the background became colorless. Digital images were captured under constant lighting conditions. The mean gray value (MGV) of each stained spot was measured using ImageJ after converting images to 8-bit grayscale. The net gray value (NGV) was calculated by subtracting the MGV of an uncoated background area. In this study, the relative coating activity (RCA) was defined as NGV of the test protein divided by NGV of BSA. Based

on our preliminary experimental results, an RCA ≥ 2.0 was empirically set as the threshold for significant coating ability.. Three independent experiments were performed.

Mechanical characterization of adhesion

To further evaluate the adhesive performance of Pvfp-5P, mechanical testing was performed using a Mach-1 V500c multi-axial mechanical testing system (Biomomentum, Canada) equipped with a 1.5 N single-axis force sensor (SN: 149610). A flat-ended cylindrical indenter with an equivalent spherical contact model was used (indenter radius: 1.000 mm). The Poisson's ratio of the sample was set to 0.5. Lyophilized Pvfp-5P was reconstituted in deionized water to a concentration of 10 mg/mL, and a uniform layer (thickness approximately 0.4 mm) was applied onto a glass substrate. Compression mode was employed with the following parameters: contact trigger threshold, 0.5025 gf; compression loading rate, 0.1000 mm/s; maximum compression limit, 1.000 mm; upper load limit for curve fitting, 32.9500 gf. A third-order low-pass filter with a cutoff frequency of 20 Hz was applied. The elastic modulus was derived from the force-displacement curve using the Hertzian contact model. The mean squared error (MSE) between the experimental data and the theoretical fit was calculated to assess data reliability.

Statistical analysis

All quantitative data are presented as mean $\pm$ standard deviation (SD) from at least three independent replicates. Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test in GraphPad Prism 8.0. A p-value < 0.05 was considered statistically significant.

Results

Construction and screening of Pvfp-5P single-expression strain

We first expressed the non-hydroxylated form of Pvfp-5P (Tyr-form) in *P. pastoris*. After electroporation of linearized pPICZαA-Pvfp5P into GS115, approximately 80 Zeocin-resistant colonies appeared on YPD plates. Twenty randomly selected clones were screened by colony PCR using primers specific for the Pvfp-5P gene. All 20 clones yielded an amplified product of the expected size (~ 500 bp), indicating a 100% positive integration rate (Fig. 1a). Ten positive clones were cultured in BMGY, induced with methanol for 70 h, and the supernatants were analyzed by SDS-PAGE. As shown in Fig. 1b, all clones exhibited a major protein band with an apparent molecular mass of 34–43 kDa, which is slightly higher than the theoretical molecular mass of 27.76 kDa, likely due to N-glycosylation (the sequence contains three predicted N-glycosylation sites). Clone #4 showed the highest expression level and few contaminant bands and was therefore selected for further experiments. Western blotting with anti-His antibody confirmed that the 34–43 kDa band indeed represented the His-tagged Pvfp-5P (Fig. 1c). This strain was named GS115/Pvfp5P.

Construction and screening of co-expression strain

To confer physiological activity, the unmodified adhesive protein was co-expressed in *P. pastoris* with tyrosinase from *B. megaterium*, a well-characterized enzyme with high catalytic efficiency[21]. To enable DOPA modification, pPIC9K-TYR was electroporated into GS115/Pvfp5P. Transformants were selected on MD plates containing Zeocin. Twenty colonies were picked, and PCR was performed with two primer pairs. All 20 clones were positive for both genes (Fig. 2a), indicating successful co-integration. Ten of these double-positive clones were induced in BMMY containing 100 μ M CuSO₄. SDS-PAGE analysis showed that, in addition to the Pvfp-5P band at 34–43 kDa, an extra band (corresponding to the tyrosinase-Flag fusion protein, theoretical 35.35 kDa) appeared upon induction (Fig. 2b). Western blotting using anti-His antibody (for Pvfp-5P) and anti-Flag antibody (for tyrosinase) confirmed the presence of both proteins (Fig. 2c, d). Clone #1 exhibited the highest expression of both proteins and the lowest amount of contaminating bands; it was designated GS115/Pvfp5P/TYR and used for all subsequent experiments.

Production and purification of recombinant Pvfp-5P

To produce sufficient quantities of Pvfp-5P for functional assays, we performed 48 h inductions in shake flasks (100 mL scale). The culture supernatant was collected and subjected to one-step Ni-NTA affinity chromatography under native conditions. SDS-PAGE analysis of the purification fractions is shown in Fig. 3. The culture supernatant (lane 1) contained a major band at 34–43 kDa. Most of this protein bound to the Ni-NTA resin, as evidenced by its near-complete absence in the flow-through (lane 2). After washing with 30 mM imidazole (lane 3), most contaminating proteins were removed. Elution with 300 mM imidazole (lanes 4–14) yielded a single major band at the expected size, with an estimated purity > 90% by densitometry. The yield of purified Pvfp-5P was approximately 300 mg per liter of culture (determined by Bradford assay). This high yield from a simple shake-flask cultivation demonstrates the effectiveness of the preCol-P fusion strategy.

Mass spectrometry confirmation of Pvfp-5P identity

The Ni-NTA-purified Pvfp-5P band was excised and analyzed by LC-MS/MS as described. The MaxQuant search results (Table 1) identified a total of 16 proteins with 90 unique peptides. Among these, 27 unique peptides matched Pvfp-5P, covering 72.6% of its full-length amino acid sequence. Representative peptides included GVYYPNPCSPYPCR from the Pvfp-5 core region, GDEGPMGMIGMSGLK from the preCol-P fusion region, and KYGGGGSGGGGSGGGGSGEPGYHGAPGRP GQK from the linker region (Supplementary Table S1). The protein score for Pvfp-5P was 323.31, confirming the reliable sequence match of the recombinantly expressed Pvfp-5P.

Table 1

Summary of mass spectrometry identification results for the recombinantly expressed proteins.

Accession	Score	Sequence coverage (%)	Unique sequence coverage (%)	Peptides	Unique peptides
Pvfp-3P	323.31	72.6	60.4	28	27
TYR	149.48	38.7	36.7	20	13
A0A1B2JHD1	81.735	22.7	22.7	11	10
A0A1B2JII2	32.522	13.3	5.6	5	5
A0A1B2JHJ8	12.809	15	15	5	5
A0A1B2JGG7	49.739	17.8	17.8	4	4
A0A1B2JAL9	25.383	30.3	30.3	4	4
A0A1B2JFK5	29.35	11.5	11.5	4	4
A0A1B2J755	18.156	13.8	13.8	4	4
A0A1B2JBG9	28.465	12.1	12.1	3	3
A0A1B2J758	15.815	4.7	4.7	2	2
A0A1B2JCC4	19.952	8.2	8.2	2	2
A0A1B2J841	20.075	5	5	2	2
A0A1B2JC66	1.1577	2.7	2.7	2	2
A0A1B2JBI0	7.9902	16.9	16.9	2	2
A0A1B2J5A4	3.8621	2.6	2.6	1	1

Identification and content determination of DOPA in Pvfp-5P

As the physiological activity of the adhesive protein is dictated by its DOPA content [4], we quantified the DOPA level in the purified recombinant protein. The presence of DOPA in Pvfp-5P was initially assessed by NBT staining. As shown in Fig. 4a, both natural MAP (positive control) and purified Pvfp-5P produced intense purple spots on the nitrocellulose membrane, whereas BSA (negative control) showed no color development. This qualitative result confirmed that the co-expressed tyrosinase successfully hydroxylated tyrosine residues to DOPA during fermentation.

Quantitative analysis using the nitrite-molybdate colorimetric method (Fig. 4b, c) revealed that natural MAP contained 3.3 wt% DOPA, while the recombinant Pvfp-5P contained 2.5 wt%. Both values meet the ≥ 1.5 wt% requirement specified in the T/CASME 530–2023 standard for recombinant mussel adhesive protein used in dressings [22]. Although the DOPA content of our recombinant protein is slightly lower

than that of the commercial natural product, it is well within the range considered functionally effective for many biomedical applications.

Promotion of L929 cell migration by Pvfp-5P

To evaluate the effect of Pvfp-5P on cell migration, we performed a scratch wound healing assay on L929 fibroblasts. Representative images at 0, 24, 48, and 72 h are shown in Fig. 5a. At 0 h, the scratch widths were similar across all groups. After 24 h, the blank control (serum-free DMEM) showed only a few cells migrating into the scratch, whereas both Pvfp-5P- and natural MAP-treated groups exhibited significant cell migration. Notably, the Pvfp-5P group displayed more extensive migration than the natural MAP group. At 48 h, the scratch in the Pvfp-5P group was almost closed, while the blank control still had a wide gap. By 72 h, the wound healing rate in the Pvfp-5P group reached $93.5 \pm 2.1\%$, significantly higher than that of the natural MAP group ($83.5 \pm 3.2\%$) and the blank control ($32.3 \pm 4.5\%$) ($p < 0.05$ for both comparisons) (Fig. 5b). These results demonstrate that recombinant Pvfp-5P possesses excellent cell migration-promoting activity, even outperforming the natural MAP at the same concentration.

Surface coating ability of Pvfp-5P

To evaluate the adhesive property of Pvfp-5P on different materials, we performed a simple coating assay on glass (hydrophilic) and polystyrene (moderately hydrophobic) surfaces. After washing and Coomassie Blue staining, both Pvfp-5P and natural MAP produced clearly visible blue spots on both substrates, while BSA (negative control) yielded only very faint or no staining (Fig. 6a). Quantitative analysis of net gray values (NGV) from ImageJ measurements (Fig. 6b) showed that Pvfp-5P achieved NGV of 3.45 ± 0.21 on glass and 2.68 ± 0.18 on polystyrene, both significantly higher than those of BSA ($p < 0.01$). The relative coating activities (RCA = NGV of test protein / NGV of BSA) were 3.5 and 2.8, respectively, both exceeding the threshold of 2.0. These data indicate that Pvfp-5P possesses strong coating ability on both hydrophilic and moderately hydrophobic surfaces, comparable to that of natural MAP.

Mechanical characterization of adhesion

To assess the mechanical properties and interfacial behavior of the recombinant adhesive protein, we performed compression testing using a Mach-1 mechanical testing system. The elastic modulus values of the three protein samples are summarized in Table 2. The BSA exhibited a high elastic modulus of 12.436 MPa, classifying it as a rigid protein material. In contrast, natural MAP showed an elastic modulus as low as 0.001228 MPa (1.23 kPa), indicating high flexibility. The inventive Pvfp-5P also exhibited a very low elastic modulus of 0.002335 MPa (2.34 kPa), demonstrating that this protein is a highly compliant soft material with mechanical properties similar to those of natural soft biological tissues.

Table 2
Elastic modulus test results of different proteins

Sample	Sample thickness (mm)	Elastic modulus (MPa)	Mean squared error	Contact trigger force (gf)
BSA	0.418	12.436	0.1852	0.508
MAP	0.412	0.001228	0.0182	0.503
Pvfp-5P	0.415	0.002335	0.0203	0.503

For protein gels or coating materials, a low elastic modulus corresponds to excellent deformability and interfacial conformability, which is crucial for establishing robust contact with various substrates [23, 24]. Both Pvfp-5P and natural MAP were detected by the system at a minimal contact trigger force of only 0.503 gf. This indicates that the recombinant protein surface exhibits sensitive interfacial response, good wetting and adhesion, and possesses significant adhesion tendency.

The MSE values for Pvfp-5P and natural MAP were 0.0203 and 0.0182, respectively, both much lower than that of BSA (0.1852). This demonstrates that the experimental force-displacement curves fit the theoretical Hertzian contact model well, confirming the high reproducibility and reliability of the measurements.

Discussion

Mussel adhesive proteins have attracted considerable interest for biomedical applications, but their industrial production has been hindered by low natural abundance and inefficient recombinant expression. In this study, we addressed these challenges by developing a fusion protein strategy combined with tyrosinase co-expression in *P. pastoris*. The key findings are: (i) fusion of Pvfp-5 β with a preCol-P fragment dramatically increased secretion yield to ~ 300 mg/L in shake flasks; (ii) co-expression of *B. megaterium* tyrosinase enabled in situ DOPA modification, achieving 2.5 wt% DOPA; (iii) the purified Pvfp-5P significantly accelerated L929 fibroblast migration and exhibited strong surface coating ability comparable to natural MAP.

The yield improvement achieved by fusing with preCol-P is consistent with the reported preference of *P. pastoris* for Gly-X-Y repeat-rich sequences [11]. The collagen-like domain of preCol-P likely enhances translation efficiency or protein stability. Codon optimization further contributed to high expression. Compared to previous reports on recombinant MAPs, which often yield less than 100 mg/L in shake flasks [25, 26], our 300 mg/L represents a substantial advancement. Moreover, our one-step Ni-NTA purification yielded $> 90\%$ pure protein, simplifying downstream processing.

The in situ DOPA modification by co-expressed tyrosinase is a notable advantage over post-purification chemical modification, which often requires harsh conditions and leads to low yields [14, 27]. The DOPA content of 2.5 wt% is slightly lower than that of commercial MAP (3.3 wt%), possibly due to suboptimal

copper concentration, inadequate oxygen supply in shake flasks, or restricted accessibility of tyrosine residues in the folded protein. We anticipate that DOPA content could be further increased by optimizing fermentation parameters (e.g., fed-batch with controlled dissolved oxygen, higher Cu²⁺ concentration, or extended induction time) and engineering the tyrosinase for higher activity.

The scratch wound healing assay revealed that Pvfp-5P promotes fibroblast migration even more effectively than commercial MAP. This surprising result might be explained by several factors: (i) the fusion protein may have a more favorable three-dimensional structure for cell interaction; (ii) the preCol-P domain might possess intrinsic cell adhesion motifs; (iii) the higher purity of the recombinant protein (no contaminating proteases or other biogenic factors) could enhance its bioavailability. The exact mechanism warrants further investigation.

The surface coating experiments demonstrated that Pvfp-5P adheres strongly to both hydrophilic glass and moderately hydrophobic polystyrene. This broad-spectrum adhesion is characteristic of DOPA-containing MAPs, which can form hydrogen bonds, metal-ion coordination, and hydrophobic interactions [28, 29]. The RCA values of 3.5 (glass) and 2.8 (polystyrene) indicate a robust coating ability, making Pvfp-5P suitable for functionalization of various medical device surfaces.

Several limitations of this study should be noted. First, expression and functional assays were performed at shake-flask scale. Fed-batch fermentation in bioreactors could potentially increase yield by several-fold. Second, the molecular mechanism by which preCol-P fusion enhances expression remains speculative; experiments with truncated variants or site-directed mutagenesis would help delineate the critical regions. Third, while we demonstrated cell migration promotion, *in vivo* wound healing studies are needed to validate therapeutic efficacy. Fourth, the long-term stability and shelf life of the lyophilized protein were not evaluated.

Nevertheless, the present work provides a practical, scalable, and cost-effective platform for producing bioactive recombinant mussel adhesive protein. The expression strategy (collagen-like fusion + tyrosinase co-expression) can likely be extended to other MAPs (e.g., Mfp-3, Mfp-6) that are also difficult to produce in high yields. Moreover, the DOPA-modified Pvfp-5P has immediate potential applications as a wound dressing component, tissue adhesive, or cell culture coating matrix.

Conclusion

We have successfully constructed a *P. pastoris* co-expression system for the production of a DOPA-modified fusion mussel adhesive protein, Pvfp-5P, by fusing Pvfp-5 β with a preCol-P peptide and co-expressing *B. megaterium* tyrosinase. The recombinant protein was secreted at approximately 300 mg/L, purified to > 90% purity by one-step Ni-NTA chromatography, and contained 2.5 wt% DOPA. Functional assays revealed that Pvfp-5P significantly accelerates L929 fibroblast migration and exhibits strong coating ability on both hydrophilic and hydrophobic surfaces. This work provides a green, efficient, and scalable way to produce bioactive recombinant MAPs with promising applications in tissue repair, wound healing, and surface engineering.

Declarations

Findings

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Competing interests

The authors declare no competing interests.

Ethical approval and consent to participate

This article does not contain studies with human participants or animals performed by any of the authors.

Author Contribution

Z.W. and L.Z. conceived and designed the study; J.H., Y.C., and Z.W. performed the experiments; T.L. analyzed the data; J.H. and T.L. wrote the manuscript.

Data availability

The data that support the findings of this study are available within the article and its supplementary information. Raw data pertaining to this work are available from the corresponding authors upon reasonable request.

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Figures

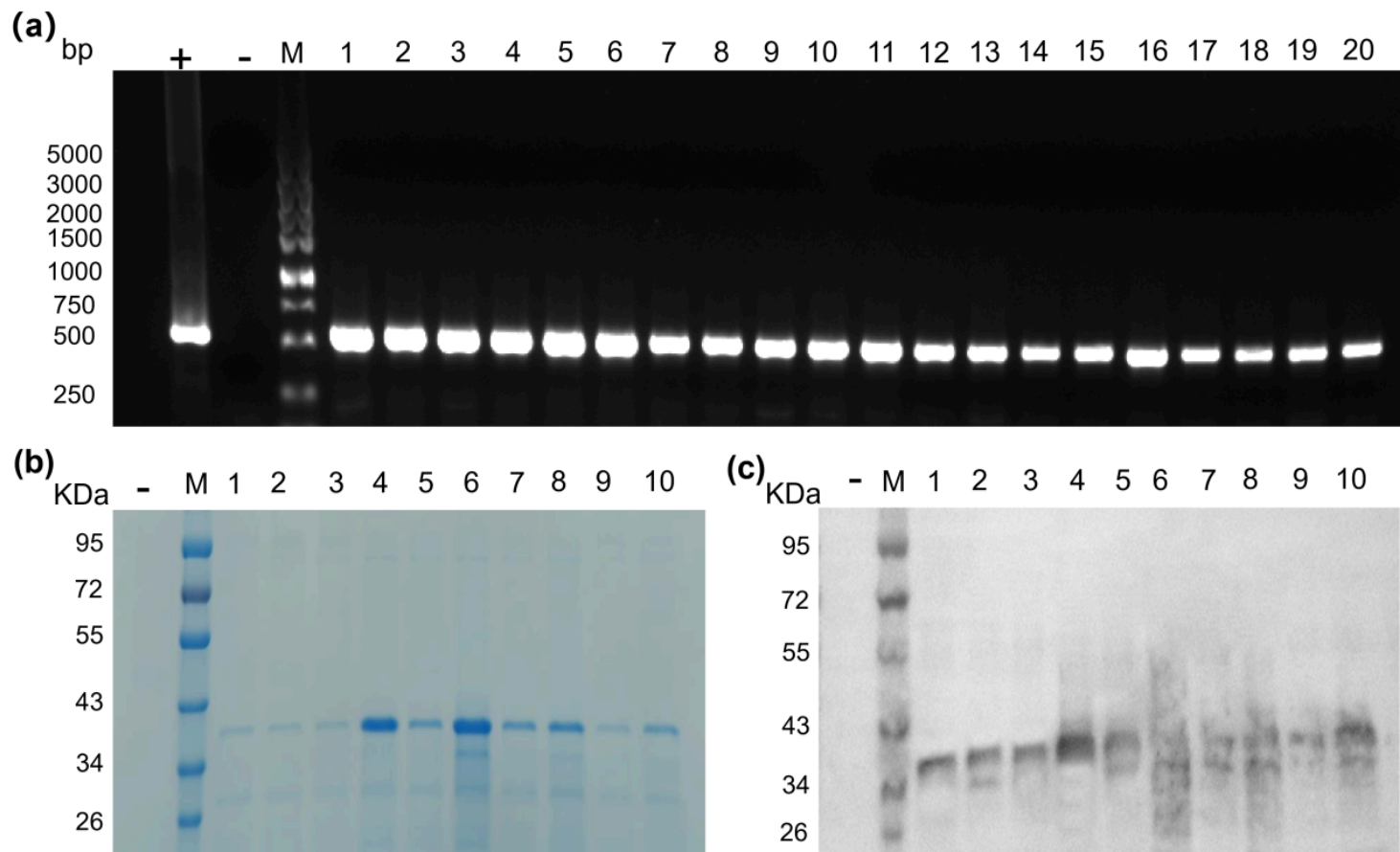


Figure 1

Screening of Pvfp-5P single-expression strain. (a) PCR identification of Pvfp-5P integrants. Lane M: DL5000 DNA marker; lanes 1-20: individual clones; -: blank control; +: positive control, the recombinant plasmid pPICZα-Pvfp5P. (b) SDS-PAGE of culture supernatants after 70 h induction. Lane M: protein marker; lanes 1-10: positive clones; -: uninduced control. (c) Western blot using anti-His antibody confirming expression.

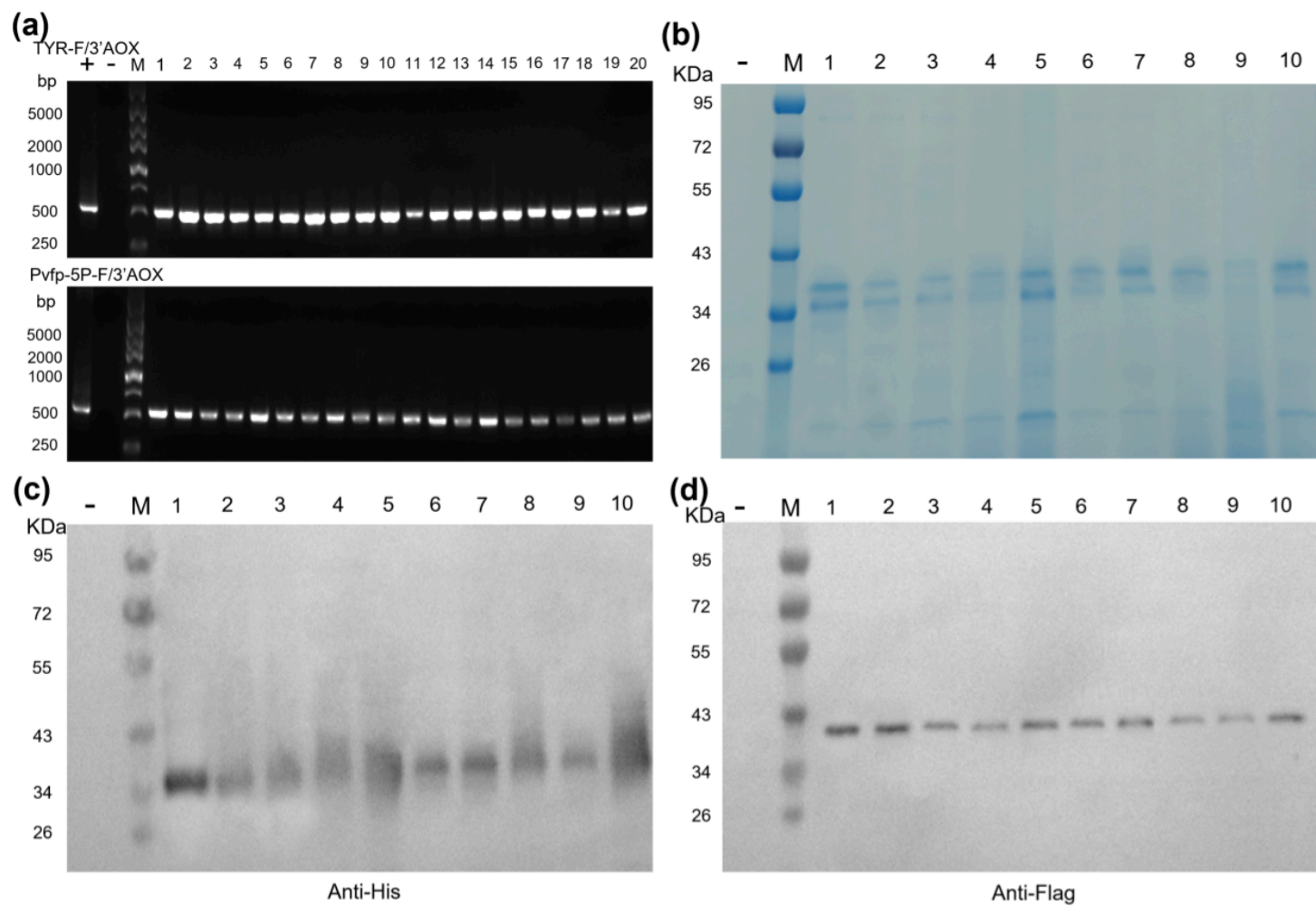


Figure 2

Screening of co-expression strain. (a) PCR verification of co-integration. Lane M: marker; lanes 1-20: clones; -: blank control; +: positive control, the recombinant plasmid. Upper panel: tyrosinase gene; lower panel: Pvfp-5P gene. (b) SDS-PAGE of culture supernatants from co-expression clones after 70 h induction. Lane M: marker; lanes 1-10: clones; -: uninduced control. (c) Western blot (anti-His) for Pvfp-5P. (d) Western blot (anti-Flag) for tyrosinase.

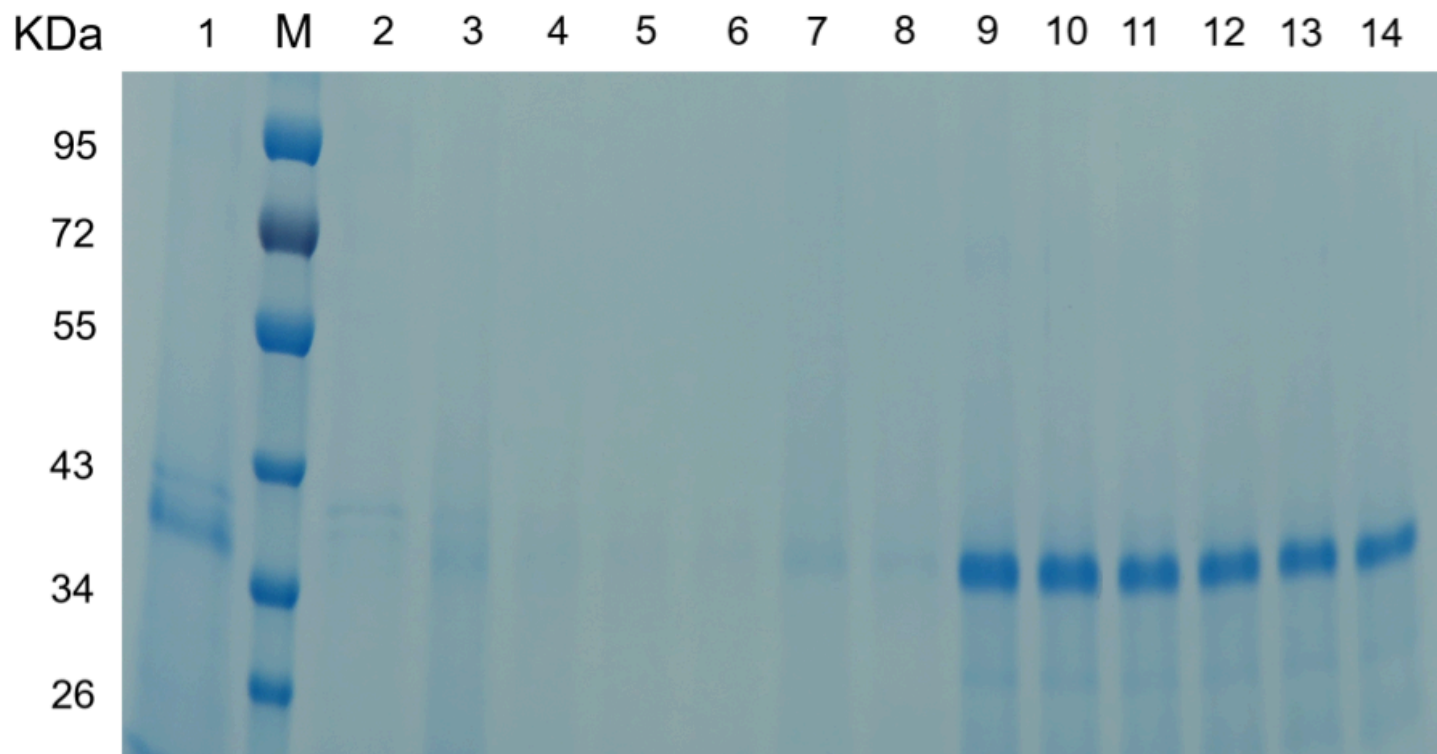


Figure 3

SDS-PAGE analysis of the Pvfp-5p by Ni-NTA affinity chromatography. Lane M: protein marker; lane 1: culture supernatant; lane 2: flow-through fraction; lane 3: wash fraction; lanes 4-14: elution fractions.

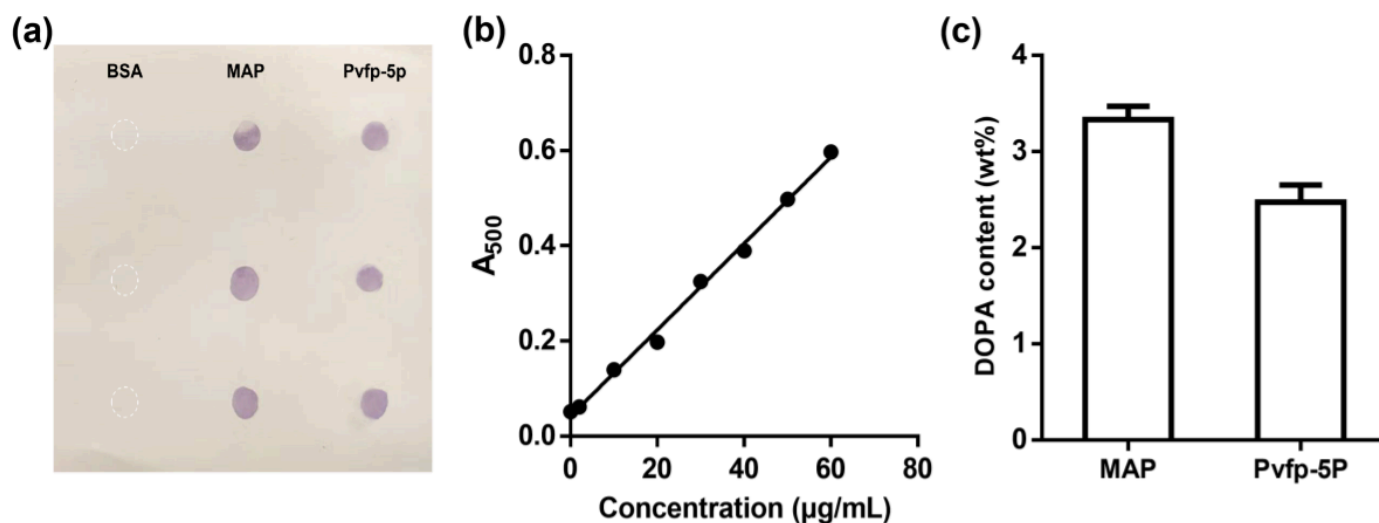


Figure 4

Identification and quantification of DOPA in the recombinant Pvfp-5P. (a) NBT staining of proteins spotted on nitrocellulose membrane. (b) Standard curve of L-DOPA. A_{500} : the absorbance at 500 nm. The standard curve equation: $y = 0.009073x + 0.04201$, $R^2 = 0.9955$. (c) DOPA contents calculated in MAP and Pvfp-5P (mean $\pm$ SD, $n=3$).

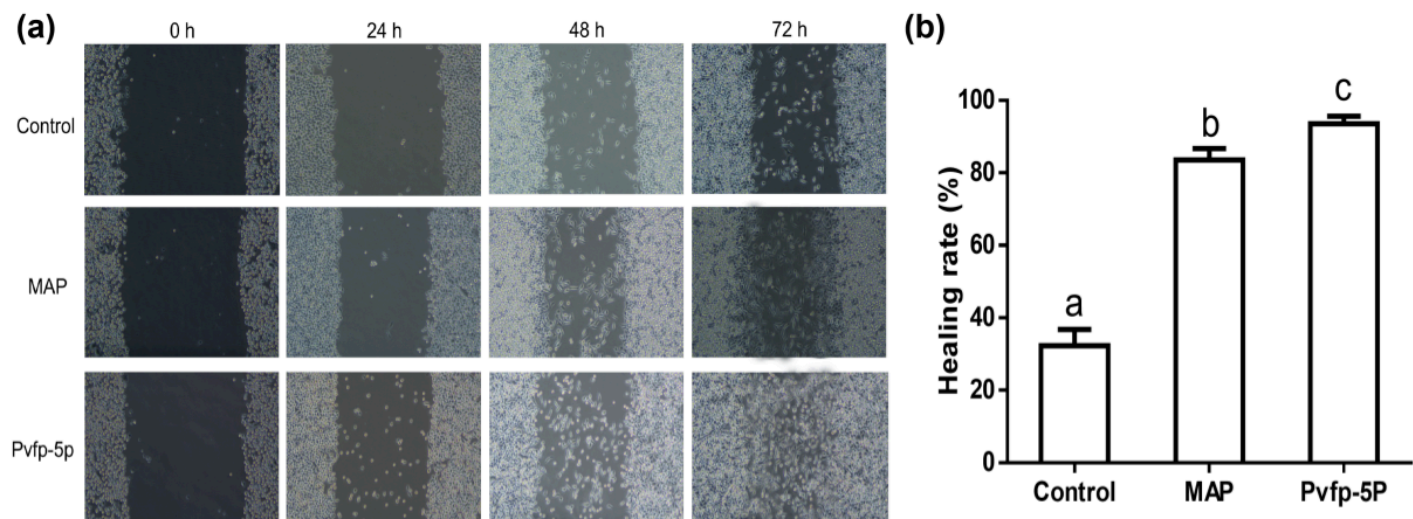


Figure 5

Promotion of L929 cell migration by Pvfp-5P. (A) Representative micrographs of scratch wounds at 0, 24, 48, and 72 h for blank control, natural MAP (100 µg/mL), and Pvfp-5P (100 µg/mL). (B) Quantitative wound healing rates at 72 h (mean ± SD, n=3). Different letters above the bars denote significant differences among treatments ($p < 0.05$, Tukey's test).

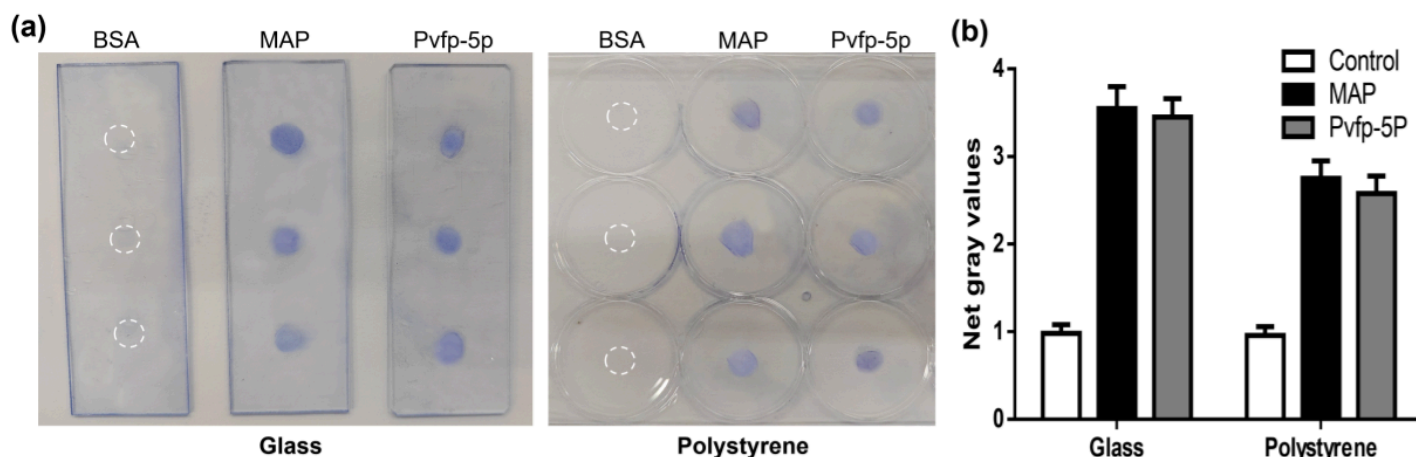


Figure 6

Surface coating ability assay. (a) Coomassie Blue-stained protein spots on glass and polystyrene surfaces. (b) Net gray values (NGV) for each protein on glass and polystyrene (mean ± SD, n=3).

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