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Mussel adhesive proteins (MAPs) have been suggested as promising bioadhesives for diverse application fields, including medical uses. Previously, we successfully constructed and produced a new type of functional recombinant MAP, fp-151, in a prokaryotic Escherichia coli *expression system. Even though the* E. coli*-derived MAP showed several excellent features, such as high production yield and efficient purification,* in vitro *enzymatic modification is required to convert tyrosine residues to l-3,4-dihydroxyphenyl alanine (dopa) molecules for its adhesive ability, due to the intrinsic inability of* E. coli *to undergo post-translational modification. In this work, we produced a soluble recombinant MAP in insect* Sf9 *cells, which are widely used as an effective and convenient eukaryotic expression system for eukaryotic foreign proteins. Importantly, we found that insect-derived MAP contained converted dopa residues by* in vivo *post-translational modification. In addition, insect-derived MAP also had other post-translational modifications including phosphorylation of serine and hydroxylation of proline that originally occurred in some natural MAPs. To our knowledge, this is the first report on* in vivo *post-translational modifications of MAP containing dopa and other modified amino acid residues.* © 2011 American Institute of Chemical Engineers *Biotechnol. Prog.,* 27: 1390–1396, 2011
Keywords: mussel adhesive protein, post-translational modification, insect cells

Introduction

Mussel adhesive protein (MAP) is a distinguishable adhesive that has fascinating features such as strong and flexible adhesion on various materials, water resistance, biodegradability, biocompatibility, and even underwater adhesion, which is extremely difficult because water prevents direct contact between an adherent and adhesive.[1] For these reasons, MAP is regarded as the next generation of adhesive for aqueous environments including medical applications.[2,3] To date, six distinct types, from type 1 (fp-1) to type 6 (fp-6), of MAP families have been determined from mussel byssus and investigated for their roles in mussel adhesion. Notably, MAPs contain high portions of some sticky amino acids such as tyrosine, lysine, and histidine, which may contribute to adhesion by adsorption on hydrophilic surfaces with strong hydrogen bonding, electrostatic interaction, and metal ligand effects.[4,5] These MAPs also have strong amino acid components of aromatic and basic amino acids and contain high levels of l-3,4-dihydroxyphenyl alanine (dopa), which are produced by post-translational hydroxylation of tyrosine.[6–9] In addition, post-translational phosphorylation of serine, such as phosphoserine (pSer), and hydroxylation of proline, such as *trans*-4-hydroxyproline (HYP) and *trans*-2,3-*cis*-3,4-dihydroxyproline (diHYP), are found in some MAPs.[9–11] These composition biases and modifications of amino acids enhance strong hydrogen bonds with hydrophilic surfaces and form strong complexes with metal ions, metal oxides (Fe^{3+}, Mn^{3+}), and semimetals (silicon).[12–14] In particular, the dopa residues enable MAP molecules to cross-link each other by oxidative conversion to dopaquinone, and the reactive quinone has an important role in water-resistant mussel adhesion.[12–14]

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Recently, we developed a fusion MAP, fp-151, comprising six repeated fp-1 decapeptides on both termini of fp-5.[15] This new bioadhesive showed a significantly large production yield in *Escherichia coli* with strong adhesive ability (~1 MPa), simple purification, proper manipulation properties, and good biocompatibility.[3,15] Recombinant fp-151 overcame several limitations, such as high production cost and limited amounts of natural MAPs (mainly fp-1 and fp-2), from labor-intensive and inefficient extraction processes and unsuccessful functional productions of recombinant individual MAPs.[16–18] However, *E. coli*-derived MAP fp-151 still requires *in vitro* modification using tyrosinase for conversion of tyrosine residues to dopa molecules due to the intrinsic inability of *E. coli* to undergo *in vivo* post-translational modification. Even though some recombinant MAPs (mainly fp-1 or fp-1-derived polypeptides) have been expressed in yeast, they did not contain dopa and other modified residues such as hydroxylated proline.[19,20]

In this work, we produced recombinant MAP fp-151 in an insect *Sf*9 cell system and investigated the potency of *in vivo* post-translational modifications including dopa conversion. The eukaryotic *Sf*9 cell system with recombinant baculovirus is effective and convenient for overproduction of foreign eukaryotic proteins due to its advantages such as correct functionality of foreign proteins,[21,22] high expression level,[21,23] and ability to undergo post-translational modifications.[24,25]

Materials and Methods

Construction of recombinant transfer vector and bacmid

To construct the transfer vector of recombinant baculovirus for MAP fp-151 expression in Sf9 cells, the fp-151 gene was amplified by polymerase chain reaction (PCR) from the pENG151 vector.[15] PCR primers (upstream 5'-ggagtcgacat gaggctcgccgtgggag-3' and downstream 5'-gcctctagaatgatgat gatgatga-3') were designed to allow cloning of the *Sal*I- and *Xba*I-digested amplified C-terminal hexahistidine tag-fused fp-151 fragment into the *Sal*I and *Xba*I sites of the pFastBac Dual vector (Invitrogen, San Diego, CA). This vector was denoted as pFBD/fp151-His. The nucleotide sequence of the inserted gene was verified by direct sequencing.

The purified recombinant transfer vector pFBD/fp151-His was transformed into DH10Bac™ *E. coli*-competent cells containing bacmid DNA using the manufacturer's standard protocols (Cat. No. 10359-016; Invitrogen). The recombinant transfer vector was transposed by the Tn gene (Tn*7L* and Tn*7R*) into the bacmid DNA during transformation. Transformed cells were incubated on Luria-Bertani agar plates containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100 μg/mL X-gal, and 40 μg/mL isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, MO) for 48 h. After selection of a single colony confirmed to have a white phenotype, we isolated recombinant bacmid DNA. Finally, we verified the presence of the target gene in the recombinant bacmid by PCR analysis using M13 sequences.

Generation of recombinant baculovirus

Insect *Sf*9 cells (#CRL-1711; American Type Culture Collection, Manassas, VA), which were originally established from ovarian tissue of the fall armyworm, *Spodoptera frugiperda*, were grown at 27°C in serum-free Sf-900 II SFM medium (Life Technologies, Carlsbad, CA) containing a 1% antibiotic–antimycotic mixture (Invitrogen). Cells were transfected with 1 μg purified bacmid DNA containing pFBD/fp151-His and Cellfectin reagent (Invtrogen). At 72 h after transfection, the supernatant containing recombinant baculovirus (denoted *v*PH-FP151) was harvested and this virus stock was then amplified three times to obtain a high titer: first, in a 6-well culture plate for 72 h; second, in 100-mm^2 culture dish for 48 h; and finally, in a 50-mL spinner flask for 48 h. The amplified recombinant baculovirus was titrated using end-point dilution and 50% tissue culture infectious dose ($TCID_{50}$) calculation. The titer of recombinant baculovirus *v*PH-FP151 was 1×10^9 pfu/mL. The recombinant baculovirus stocks were stored at 4°C and no further purification was performed.

Production and purification of recombinant MAP

Cell growth was monitored by measuring OD_{600} using a UV–visible spectrophotometer (Shimadzu). Dissolved oxygen during bioreactor culture was measured using an oxygen sensor (Mettler-Toledo Process Analytical). Concentration of fp-151 was quantified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the purified fp-151 protein as a standard. The cell pellets were mixed with 100 μL protein sample buffer (0.5 M Tris-HCl (pH 6.8), 10% glycerol, 5% SDS, 5% β-mercaptoethanol [Sigma], 0.25% bromophenol blue [Sigma]), incubated at 100°C for 5 min, centrifuged briefly, and loaded onto a 12% gel for electrophoresis. After electrophoresis, the gel was stained with Coomassie-blue (Bio-Rad). The stained gel was scanned and the image was analyzed using Gel-Pro Analyzer software (Media Cybernetics).

SDS-PAGE, Western blot, and AU-PAGE analyses

For SDS-PAGE analysis, samples were mixed with sample buffer (0.5 M Tris-HCl [pH 6.8], 10% glycerol, 5% SDS, 5% β-mercaptoethanol, 0.25% bromophenol blue), boiled for 5 min at 95°C, resolved by 12% SDS-polyacrylamide gel and detected using Coomassie blue staining (Bio-Rad).

For Western blot analysis, 12% SDS-polyacrylamide gel was electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell BioScience, Keene, NH). After blocking for 1 h in TBS buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) containing 5% non-fat dry milk, the membrane was then incubated for 1 h at room temperature in antibody solution (1% non-fat dry milk in TTBS [TBS with 0.05% Tween-20]) containing an anti-hexahistidine antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and probed with anti-mouse immunoglobulin G conjugated with alkaline phosphatase (Sigma). After successive washing with TTBS and TBS, 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP; Roche, Welwyn Garden City, UK) and nitroblue tetrazolium chloride (NBT; Roche) were added for colorimetric detection. The detected membrane was scanned, and the digitized images were stored and analyzed by Gel-Pro Analyzer software (Media Cybernetics, Bethesda, MD). The quantity of fp-151 was determined using purified *E. coli*-derived fp-151[15] as a calibration standard for Western blot.

Recombinant proteins (*Sf*9-derived, *E. coli*-derived, and *in vitro* dopa-modified *E. coli*-derived fp-151 MAPs) were analyzed by acid-urea PAGE (AU-PAGE) with redox cycling staining involving NBT and glycinate to determine the presence of dopa or dopaquinone residues.[26,27] *In vitro* dopa modification of *E. coli*-derived fp-151 was processed overnight at

37°C with shaking in 1× phosphate-buffered saline (2.68-mM KCl, 13.7-mM NaCl, 1.47-mM KH_2PO_4, and 0.875-mM Na_2HPO_4) with 25-mM ascorbic acid and 50 μg/mL mushroom tyrosinase (Sigma). Modified *E. coli*-derived fp-151 was dialyzed twice against distilled water. The final modified products were freeze-dried and stored at -80°C.

Liquid chromatography-mass spectrometry analysis for post-translational modifications

In-solution digestion was performed as in a previous study.[28] The purified fp-151 protein (20 μg) was resolved in a digestion solution (6 M urea and 40 mM ammonium bicarbonate dissolved in high-performance liquid chromatography-grade water). Protein reduction was performed with 5 mM tris(2-carboxyethyl)phosphine hydrochloride for 1 h, followed by an alkylation step with 25 mM iodoacetamide in the dark for 30 min at room temperature. The sample was digested in-solution with 5 ng/mL sequencing grade modified trypsin (Promega, Madison, WI) for 16 h at 37°C. All digested proteins were collected and desalted with the C-18 spin column (Thermo, Rockford, IL). Tryptic peptides were repeatedly analyzed (five times) by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) on an LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA). MS/MS data were analyzed by a computational proteomics analysis system using X!! Tandem search engine[29] with the database from Uniprot.[30]

Quantitative phosphorylation analysis

For removing false-positive signals, recombinant MAP fp-151 was delipidated and desalted by the chloroform-methanol precipitation method.[31] Shrimp alkaline phosphatase (Fermentas, Vilnius, Lithuania) was added to *Sf*9-derived fp-151 protein pellets at 37°C for 30 min to obtain phosphates. *E. coli*-derived fp-151 was treated by an equal procedure to set the zero point. Phosphates from each substrate were measured in triplicate by P_i ColorLock™ Gold (Innova Biosciences, Cambridge, UK).

Direct surface coating analysis

Amine glass (Superamine Substrates; Arrayit Corp., Sunnyvale, CA) was used to investigate surface coating ability of *E. coli*- and *Sf*9-derived fp-151 proteins. Each lyophilized protein powder was dissolved in 100 mM acetate buffer (pH 5.0) to make 1 g/L sample solution. One microliter drop of each sample solution was spotted 11 times on amine surface and cured at 30°C and 90% of humidity for 4 h. Adhered samples were washed in at least 10 volumes of 1× phosphate buffered saline (PBS; 2.68 mM KCl, 13.7 mM NaCl, 1.47 mM KH_2PO_4, and 0.875 mM Na_2HPO_4) for 10 min at room temperature. Residual surface proteins were stained by Coomassie blue and quantified by a Gel-Pro Analyzer. Numerated intensity values of each dot were averaged and normalized.

Results and Discussion

Expression and purification of recombinant MAP in Sf9 cells

The system for production of fusion MAP fp-151 fused with a C-terminal hexahistidine affinity ligand was successfully constructed in recombinant baculovirus under the control of a strong polyhedrin (Polh) promoter. The expression of recombinant fp-151 in suspended *Sf*9 cells was investigated in spinner flask cultures (Figure 1). Before infecting the recombinant baculovirus *v*PH-FP151, *Sf*9 cells were healthy and showed over 95% viability (Figure 1A). Cell viability decreased rapidly after baculoviral infection, whereas non-infected cells maintained their good viability during the culture period. According to cell viability, viable cell numbers also decreased largely after infection (Figure 1B). Figure 1C shows the recombinant fp-151 expression profile, as determined by Western blot. The fp-151 expression level was minimal until 48 h post-infection time (hpi). After 48 hpi, fp-151 was highly expressed and infected *Sf*9 cells showed the highest expression at 72 hpi. This expression profile occurred because of the intrinsic property of baculoviral Polh promoter having very late expression; this profile was identical with profiles reported in the previous study using other recombinant protein.[32] In particular, a strong Polh promoter can accumulate heterologous protein over 50% of total cellular proteins.[33,34] The maximum concentration of hybrid fp-151 expressed was 23 μg/mL in the 150-mL spinner flask culture. Note that the production yield of fp-151 in *E. coli* was ~100 μg/mL when cultured in a 5-L batch bioreactor.[15]

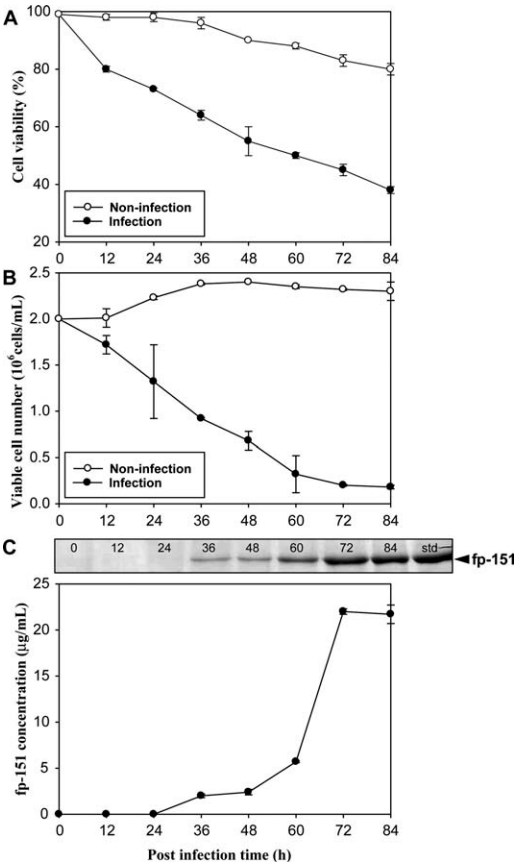

Figure 1. Time course of (A) cell viability, (B) viable cell number, and (C) MAP fp-151 concentration in *Sf*9 cells infected by recombinant baculovirus *v*PH-FP151 and uninfected *Sf*9 cells.

*Sf*9 cells were infected with the recombinant baculovirus of MOI 100 and grown at 27°C. Quantification of *Sf*9-derived fp-151 was performed by Western blot analysis using *E. coli*-derived fp-151 as a standard protein (std; 25 μg/mL). Each measured value is shown as an average of six independent culture experiments.

Because fp-151 protein was fused with hexahistidine affinity ligand, we performed purification using affinity

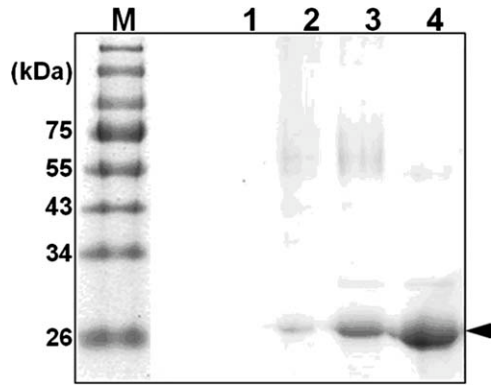

Figure 2. SDS-PAGE analysis for purification of recombinant MAP fp-151 in *Sf*9 cells.

Lane M: protein molecular weight marker; (1) *Sf*9 cell debris; (2): supernatant cell culture medium; (3) 10× concentrated cell culture medium by ultrafiltration; and (4) purified sample with IMAC.

chromatography. IMAC purification gave a yield of 32.4% and purity of ~96% for hybrid fp-151 (Figure 2, lane 4). The matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrum of fp-151 after purification showed the molecular weight to be almost identical to the predicted size of 23.6 kDa (data not shown). Interestingly, the apparent molecular weight of recombinant fp-151 from *Sf*9 cells on an SDS-PAGE gel was smaller (~25.5 kDa) than the *E. coli*-derived one (~30 kDa),[15] and this is much closer to the predicted molecular mass (23.6 kDa). Note that the apparent molecular weight of *E. coli*-derived fp-151 on an SDS-PAGE gel was greater than the predicted size, which is consistent with results for other MAPs.[6,15,35–37] We, therefore, surmised that the difference in the apparent and actual masses of MAPs might result from the protein being basic (proteins with higher pI values tend to bind more SDS molecules). We suspect that this lower apparent molecular weight of *Sf*9-derived fp-151 compared with the *E. coli*-derived protein might result from a decrease of basicity and/or a conformational change from complex with SDS due to post-translational modification of its amino acids.

Dopa modification of Sf9-derived recombinant MAP

Dopa, which is a hydroxylated form of tyrosine, is a key material for adhesion in underwater conditions because of its reversible adhesive properties and strong non-covalent bond with half-strong covalent bonding with a wet metal oxide surface.[38] Dopa is able to make many kinds of cross-linking for hardening of MAPs by making a covalent bond with dopa itself, dopaquinone (an oxidized form of dopa), amine, and thiol groups.[39,40] These chemical properties give high durability to the mussel foot through strangle with MAPs by cross-linking.[12,41] Among several MAPs, fp-1 and fp-5 proteins have high portions of dopa, about 13% and 30%, respectively.[1]

Because dopa and dopaquinone can be detected by redox-cycling staining using glycine and NBT,[26] the NBT staining method is widely used for detecting dopa in MAPs.[27] Thus, we investigated the existence of dopa and/or dopaquinone in Sf9-derived MAP fp-151 using NBT staining (Figure 3). Unmodified *E. coli*-derived fp-151 and *in vitro* tyrosinase-modified *E. coli*-derived fp-151 were used as negative and positive controls, respectively. Although all protein bands were clearly detected by Coomassie staining (Figure 3A), the bands of *Sf*9-derived fp-151 (Figure 3B, lane SF-151) and

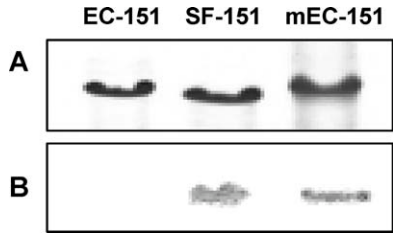

Figure 3. AU-PAGE analysis for dopa modification with (A) NBT and (B) Coomassie blue staining of *E. coli*-derived fp-151 (EC-151), *Sf*9-derived fp-151 (SF-151), and *in vitro* tyrosinase-modified *E. coli*-derived fp-151 (mEC-151).

modified *E. coli*-derived fp-151 (Figure 3B, lane mEC-151) were observed on NBT-stained AU-PAGE except for unmodified *E. coli*-derived fp-151 (Figure 3B, lane EC-151). Thus, we were convinced that recombinant MAP from insect *Sf*9 cells has *in vivo*-modified dopa and/or dopaquinone residues.

For more specific analysis of dopa and dopaquinone in *Sf*9-derived recombinant MAP, LC-MS/MS analysis was performed. We found that KPSYPPTYK and AKPSYPPTYK, which are peptide fragments from *Sf*9-derived fp-151 by trypsin digestion, have dopa and dopaquinone (dq) at the first tyrosine position, respectively (Figures 4A,B). Because MAP fp-1 has a repeated decapeptide (AKPSYPPTYK), two fragments, AKPSYPPTYK and KPSYPPTYK, can be regarded as the same fragment in the fp-1 region of recombinant fusion MAP fp-151. Interestingly, dopa and dopaquinone were detected at the same fifth position of the decapeptide. However, original dopa conversion in mussel is more frequently seen at position 9 than position 5.[10] A similar phenomenon was observed on synthetic decapeptide conversion using mushroom tyrosinase.[42] Thus, we suspect that the different preferences of hydroxylation activity may be caused by differences in hydroxylase. However, comparison of hydroxylases is not currently possible because genes for hydroxylases in mussel and insect *S. frugiperda* have not been reported yet.

Other post-translational modifications of Sf9-derived recombinant MAP

Interestingly, fp-1 and fp-5 have other post-translational modifications, namely phosphorylation on serine (pSer) and hydroxylation on proline (diHyp and Hyp) as well as dopa. Roles of pSer and hydroxylated prolines are not clearly known yet, but it was assumed that pSer supports adhesion on the mussel shell made of calcium carbonate, which is easily attached by pSer residues[9] and hydroxylated forms of proline give proper confirmation for efficient interaction of hydroxylated and phenolic side chains in dopa residues with the surfaces.[42]

We also found pSer, another post-translational modification product, on KPSYPPTYK, part of the fp-1 decapeptide, by LC-MS/MS (Figure 4B). Previously, pSer was revealed on natural fp-5 protein.[9] Quantitative analysis of pSer was performed for pSer in the fp-5 region of recombinant fp-151. Phosphates were collected from *Sf*9-derived fp-151 by shrimp alkaline phosphatase and their amount was measured by the commercially available phosphate measurement kit. To eliminate false-positive signals, the *E. coli*-derived fp-151 sample was also identically processed and set to zero; 18.0 ± 2 of phosphates were detected on *Sf*9-derived fp-151 protein. Phosphorylation in eukaryotic cells can occur on

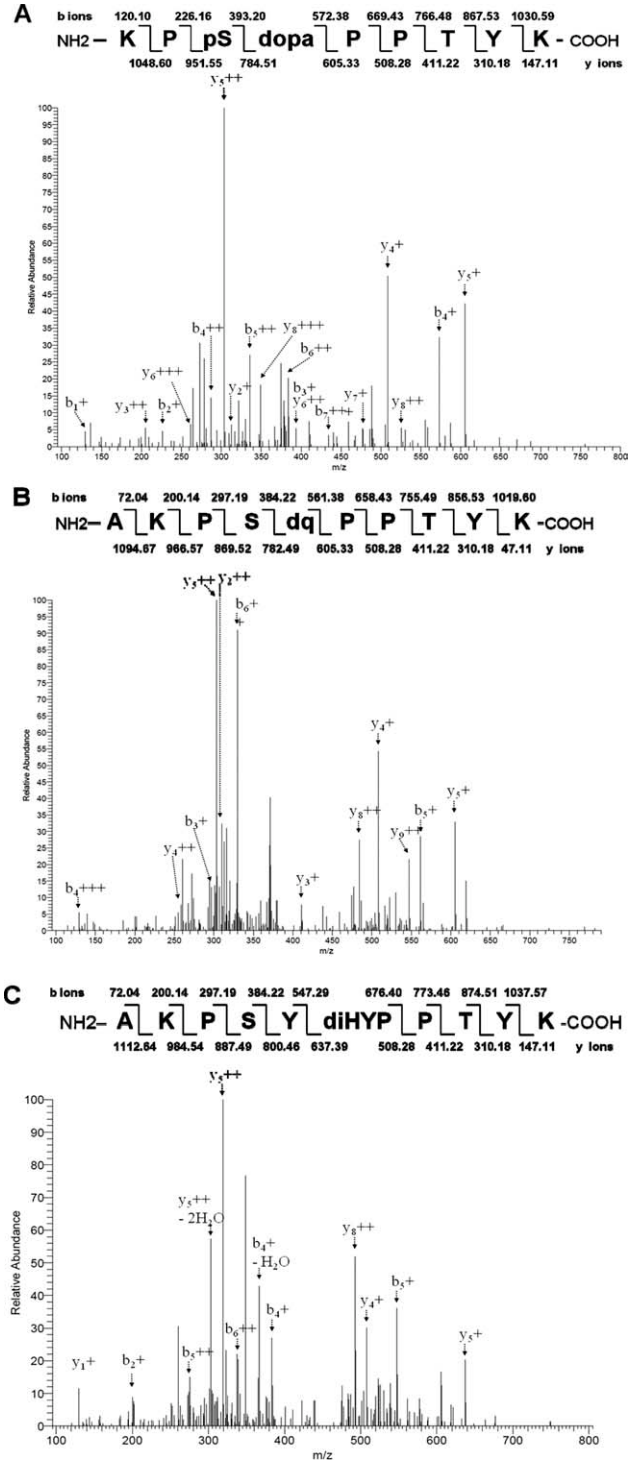

Figure 4. LC/MS-MS analyses for post-translational modifications of *Sf*9-derived MAP fp-151. (A) dopa, (B) dopaquine (dq) and phosphoserine (pS), and (C) *trans*-2,3-*cis*-3,4-dihydroxyproline (diHYP).

Trypsin-digested partial fp-151 peptides, KPSYPPTYK and AKPSYPPTYK, were detected and analyzed for A, B, and C.

tyrosine and threonine as well as serine. Thus, LC-MS/MS analysis was performed to confirm that phosphate molecules in *Sf*9-derived fp-151 were only from serine residues. Phosphothreonine and phosphotyrosine were not detected by LC-MS/MS. Thus, we can assume that almost every serine included in the fp-5 region in fp-151 was converted to pSer because *Sf*9-derived fp-151 has 20 serine residues, which is nearly equal to 18.0 ± 2 phosphate molecules.

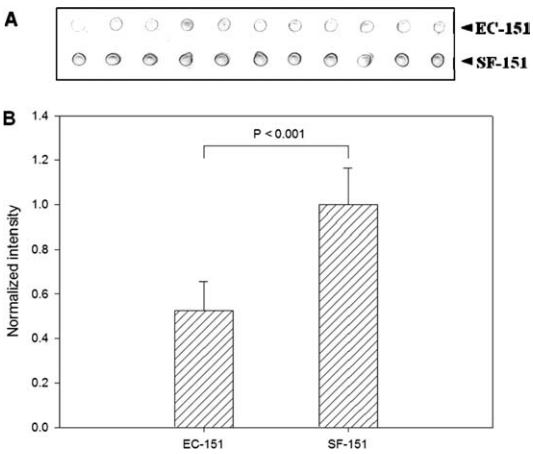

Figure 5. Direct coating analysis of *E. coli*-derived fp-151 (EC-151) and *Sf*9-derived fp-151 (SF-151) on amine glass surface.

(A) Dot-blotted feature and (B) normalized quatification of residual proteins after washing. Values and error bars represent the means of samples and standard deviations with statistical significance (*$P < 0.001$).

Both hydroxylated prolines, diHyp and Hyp, were detected on natural fp-1 protein.[10,11] In *Sf*9-derived fp-151, only diHyp was detected on AKPSYPPTYK by LC-MS/MS analysis (Figure 4C). This result suggests the possibility that *S. frugiperda* does not have 4-prolylhydroxylase or 4-prolylhydroxylase is not actively expressed. Interestingly, *Sf*9-derived fp-151 has diHyp on only position 6 of the fp-1 decapeptide, which is the identical position to the original fp-1 decapeptide.[11] Therefore, we might assume that the proline dihydroxylase of *S. frugiperda* has similar preference with the enzyme in the mussel foot.

Direct surface coating analysis of Sf9-derived recombinant MAP

To investigate the effect of post-translational modification on activity of MAP, we used direct surface coating analysis. Both *E. coli*- and *Sf*9-derived fp-151 proteins dissolved in acetate buffer were coated on amine-modified glass surface, and each drop was cured on the surface in a 90% humidity condition. Because dopa, one of the modified amino acid products found in *Sf*9-derived fp-151, is known as the essential component in underwater adhesion,[1] curing was performed in a wet environment. After curing and washing, residual surface proteins were stained (Figure 5A) and quantified (Figure 5B). The *Sf*9-derived MAP fp-151 showed about 2-fold higher coating ability compared with the *E. coli*-derived protein (Figure 5B). It is known that dopa facilitates adhesion on wet surfaces,[1] and dopaquinone has the ability of cross-linking with amine groups.[12] We confirmed the existence of both dopa and dopaquinone in *Sf*9-derived fp-151 using LC-MS/MS analysis (Figure 4). Thus, we surmise that significantly enhanced surface coating ability of *Sf*9-derived recombinant fp-151 was a result of *in vivo* modification of tyrosine residues in dopa and dopaquinone.

Conclusions

To achieve successful *in vivo* modification, recombinant fusion MAP fp-151 was expressed in insect *Sf*9 cells using a recombinant baculovirus system. Optimal expression of *Sf*9-

derived fp-151 proteins was at 72 h after baculoviral infection and protein was efficiently purified using affinity chromatography. Occurrence of post-translational modifications of recombinant fp-151 in *Sf*9 cells was identified mainly by LC-MS/MS analyses. Fragments of fp-1 decapeptide, which were obtained by trypsin digestion of whole fp-151 protein, were detected, and we found that dopa and dopaquinone existed on position 5 of the fp-1 decapeptide instead of position 9 unlike the original fp-1. pSer, which was originally identified on natural fp-5, was verified on the same fp-1 decapeptide fragment by LC-MS/MS, and serine phosphorylation in the fp-5 region was checked through quantitative analysis of phosphate. Among the hydroxylated prolines observed in natural fp-1, recombinant Sf9-derived fp-151 had only diHyp in the fp-1 region, but its conversion position was identical with the original fp-1. *E. coli*- and *Sf*9-derived fp-151 proteins were compared by direct surface coating analysis on amine-modified glass surface under humidity controlled conditions, and we found highly enhanced (~2-fold) coating ability of *Sf*9-derived fp-151. These findings suggest the possibility of *in vivo* modifications of recombinant MAPs in eukaryotic expression systems.

Acknowledgments

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