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SHORT COMMUNICATIONS

Biosynthesis and Characterization of the Artificial Protein Consisting of Marine Mussel Adhesive Protein and Silk-Like Protein Sequences

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Mussel *Mytilus edulis* attach to solid surfaces in the turbulent environment by making a byssus, which consists of several redox-active proteins.[1] The first of these proteins to be characterized from the mussel *M. edulis* foot protein 1 (*mefp*-1) was found to consist of predominantly repeated decapeptide sequences.[2,3] The most frequently repeated sequence is AKPSYP*P**TY*K, where P*, P** and Y* denote *trans*-2,3-*cis*-3,4-dihydroxyproline (diHyp), 4-hydroxyproline (Hyp), and 3,4-dihydroxyphenylalanine (Dopa), respectively.[4,5] The Dopa residues are believed to result from the enzyme reaction of a catechol oxidase, which converts tyrosine residues to Dopa and subsequently to ortho-quinones.[6]

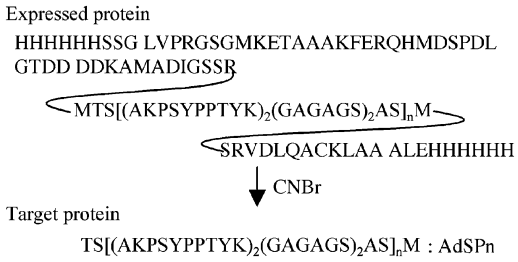
The Dopa residues are key components that are believed to be primarily responsible for chemisorption of the adhesion proteins to substrates underwater.[7] The ortho-quinone residues can react to lysine residues, which are active component of covalent cross-linking of the adhesive. The adhesive ability is influenced by secondary structure and increased with higher β-structure content.[8]

We attempted that the *Bombyx mori* silk fibroin GAGAGS repetitive sequence[9] was conjugated to the *mefp*-1 sequence in order to construct a novel adhesive protein with high β-structure content. Conjugated sequence GAGAGS is frequently found in the β-structure domain of silk fibroin. It was reported that a byssus contained fiber protein consisting of predominantly repeated sequence that combine collagen with flanking domains that resemble silk-fibroin or elastin.[10] But the fiber protein is one-component protein of byssus and is not conjugated to *mefp*-1 sequence. In this study, we attempted to construct the protein that is more adhesive materials than *mefp*-1 by introduction of higher β-structure, silk-sequence.

The novel adhesive protein was designed repetitive motif sequence, which is TS(AKPSYPPTYK)$_2$(GAGAGS)$_2$AS (AdSP1). The amino acid Pro and Tyr are the original amino acids of diHyp, Hyp and Dopa before post-translational modification, which are used instead of diHyp, Hyp and Dopa for AdSP.

Herein we describe the biosynthesis of 8 repeats AdSP polymer (AdSP8) by genetically engineered technique, and characterization of AdSP8.

AdSP8 was expressed as the fusion protein with terminal regions of histidine tagged sequence. The terminal regions can be removed by cyanogen bromide (CNBr) cleavage at flanking methionine residues. The target protein and the expressed proteins can be represented in Scheme 1. All recombinant techniques were used in general method and the polymerization of the DNA was accomplished by head-to-tail construction strategy method.[11] Protein expression was performed under control of T7 promoter. pET-30a and BL21(DE3)pLysS from Novagen were used as expression vector and host, respectively.



Scheme 1. The sequences of expressed protein and target protein.

The expressed protein was purified by nickel chelate affinity-chromatography by stepwise concentration of imidazole with 3 M Urea buffer.

Cleavage of the expressed protein was accomplished in peculiar condition of CNBr cleavage. Forty-five mg of expressed protein was dissolved in 5 mL of 20% formic acid with 50% acetic acid. The solution was deoxygenated with nitrogen, and 150 mg of CNBr crystals was added. The solution was stirred at 42 °C overnight, and then neutralized by ammonium aqueous solution. The neutralized solution was dialyzed against distilled water for 3 d and then lyophilized.

The obtained expressed protein and AdSP8 were analyzed by 12% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) as shown in Figure 1. Each purified protein was detected as a single band, the molecular weights being 38 kDa for expression protein (lane 2), and 30 kDa for AdSP8 (lane 3), respectively. The purified AdSP8 was identified by ^{1}H and ^{13}C NMRs.

The secondary structure was identified by FT-IR and protein secondary structure analysis program SSE-4000 from JASCO. Figure 2 shows the IR spectra of the AdSP8 and peak fit to yield of α-helix, β-sheet, β-turn and other structures. The structure content of AdSP8 was 21% α-helix, 35% β-sheet, 21% β-turn and 23% other structures from result. The β-structure content of AdSP8 was higher than that of *mefp*-1 and *mefp*-1 model peptide, which contained about 20% and 16% of β-structure content.[12,13]

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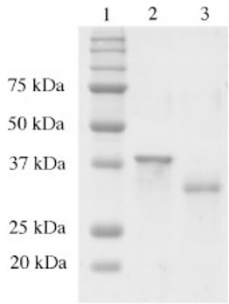

Figure 1. SDS-PAGE analysis of expressed protein after purification (lane 2) and AdSP8 (lane 3), with protein markers (lane 1).

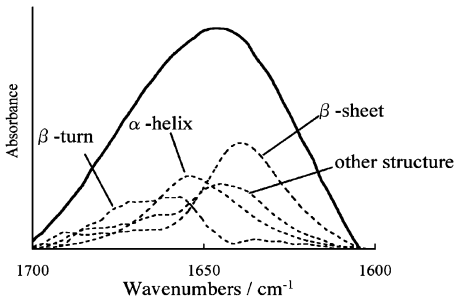

Figure 2. The amid I band of FT-IR spectrum and curve fitting spectra assigned to α-helix, β-turn, β-sheet, and other structure for AdSP8.

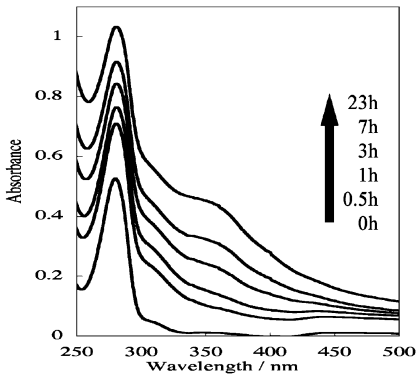

Figure 3. UV/VIS spectra of AdSP8 hydroxylation of the tyrosine residue to Dopa and *ortho*-quinone.

The hydroxylation of AdSP8 was accomplished by tyrosine oxidase. Approximately 5 mg of AdSP8 was dissolved in 8 mL and H_2O of Millipore quality. The solution was bubbled with air, and 200U of enzyme was added. The hydroxylation of AdSP8 can be monitored by spectroscopic analysis.[14,15] The absorption spectra of AdSP8 hydroxylation were shown in Figure 3, where the absorbances at 285, 350 and 395 nm increased with reaction time because absorption at 280 nm of tyrosine residues changed to 285 nm of Dopa, subsequently to 395 nm of ortho-quinone[14] and to 350 nm of cross-linking. For evaluating adhesive properties of hydroxylated AdSP8, the hydroxylation was reacted in 1% AdSP8 solution of H_2O at 25 °C with 400U of enzyme for 24 h. On this reaction, the precipitates were observed in the solution after 23 h. But precipitates weight was lower than 1% of solved AdSP8 weight.

Adhesive properties were evaluated by thermo chemical estimation method.[16] To calculate the work of adhesion (W_A), we measured surface tension and contact angle for 0.4%, 1% of AdSP8 and 1% of hydroxylated AdSP8 in aqueous solution on various substrates (glass, Nylon, PET, Teflon). The Young-Dupre equation was used to calculate W_A that is $W_A = \gamma L (1 + \cos\theta)$, wherein γL and θ are surface tension and contact angle, respectively. The results are summarized in Table I. The contact angle on organic substrates improved with AdSP8 concentration increasing, but the surface tension lowered. At influence of hydroxylation, the surface tension and contact angle on glass of hydroxylated AdSP8 were better than those of AdSP8. The work of adhesion increased with hydroxylation. From results, AdSP8 was hydrophobic protein that had good adhesive property on organic substrates from concentration comparison results. The polarity and adhesive properties increased by hydroxylation process for AdS8. Compared with report,[13] the work of adhesion of hydroxylated AdSP8 were better than those of modified *mefp*-1 model peptide that are 92.5 mN/m on glass, 67.4 mN/m on PET, and 47.4 mN/m on Teflon.

Table I. Results of surface tension, contact angles, and work of adhesion (W_A) on various substrates

	γL	contact angle (degree)				W_A (mN/m)			
	(mN/m)	glass	Nylon	PET	Teflon	glass	Nylon	PET	Teflon
0.4% AdSP8	49.9	26.0	54.0	79.0	97.0	95.0	79.0	60.0	44.0
1.0% AdSP8	48.5	34.0	48.0	71.0	95.0	90.0	84.0	70.0	52.0
1.0% modified AdSP8	52.7	28.0	49.0	73.0	98.0	100.0	91.0	74.0	54.0

In summary, novel adhesive protein with high with β-structure, AdSP8 was synthesized by genetically engineered technique. After hydroxylation, AdSP8 has better adhesive properties than *mefp*-1 model peptide. Our results could advocate new adhesive material and new attempt of material developments.

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