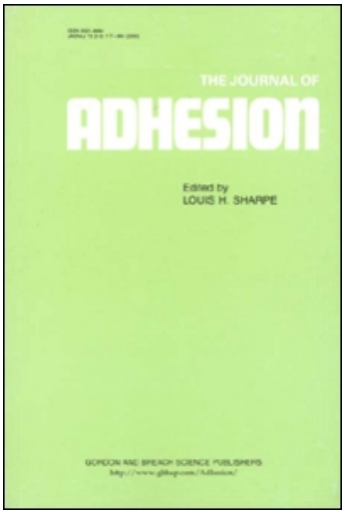

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Salt Effects on Aggregation and Adsorption Characteristics of Recombinant Mussel Adhesive Protein fp-151

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Mussel adhesive proteins (MAPs) are excellent bioadhesives. Although the role of L-3,4-dihydroxyphenyl alanine (DOPA) in water-resistant strong adhesion has been extensively studied, MAP aggregate formation and physical adsorption behavior have not been thoroughly explored. Here, we investigated the aggregation and attachment properties of a recombinant MAP with no DOPA residues. We found that chemical cross-linking of oxidized DOPA residues was not required for aggregate formation; increased anionic kosmotropy and cationic chaotropy allowed aggregation permitting strong adsorption onto hydrophilic surfaces. This study suggests that the initial step in MAP adhesion might thus be aggregation. Physical adsorption can be adjusted by varying the salt ions in water, to mediate initial efficient attachment followed by DOPA-mediated water-resistant strong adhesion.

Keywords: Adhesion; Adsorption; Aggregation; Mussel adhesive protein; Salt ions

INTRODUCTION

Mussel adhesive proteins (MAPs) are generally regarded as promising bioadhesives for many applications because of their unique properties, which include strong and flexible adhesion to any substrate in wet

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environments [1,2]. To date, six distinct types of MAPs have been isolated from mussel adhesive plaque, and a fundamental understanding of the adhesion mechanism has been sought. Notably, MAPs contain high proportions of aromatic and basic amino acids such as tyrosine, lysine, glycine, and histidine, which may contribute to adhesion by facilitating adsorption using strong hydrogen bonding, electrostatic interaction, and metal ligand effects [3–6]. Especially, high levels of L-3,4-dihydroxyphenyl alanine (DOPA), from modification of tyrosine, enable MAP molecules to cross-link by oxidative conversion of DOPA to DOPA-quinone; the reactive quinone is important in aggregate formation, water-resistant cross-linking, and development of strong adhesion [4,7,8].

In general, MAPs tend to easily aggregate in solution because of their high levels of aromatic and basic amino acids [9–12]; aggregate formation is increased with increasing pH, and discolored precipitates occur at pH 7.5–8.5 for MAP type 1 (fp-1) and type 3 (fp-3), where DOPA oxidation to quinone and eventual cross-linking have been mainly considered to lead to formation of initial aggregates. However, aggregate formation and multilayer adsorption of MAPs onto surfaces have not been fully investigated, whereas chemical cross-linking of oxidized DOPA residues has been extensively studied and is considered essential for strong adhesion. The presence of DOPA in naturally extracted MAPs, and the limited availability of MAPs, has hampered proper investigation of individual adhesion steps. Studies on model decapeptides from fp-1 have revealed that DOPA residues are essential for the attachment and film formation on surfaces, where decapeptides without DOPA did not show any adhesion properties [5,13,14]. Thus, it is now accepted that the unusual amino acid composition and oxidized DOPA residues are both involved in the remarkable adhesion properties of these proteins.

Recently, we constructed and produced a recombinant fusion protein MAP fp-151, with fp-1 decapeptide repeats at both the N- and C-termini of type 5 MAP (fp-5). The protein was synthesized by *Escherichia coli* in high yield ($\sim$1 g/L in a pilot-scale fed-batch bioreactor culture) and high purity ($\sim$90%), and demonstrated very good solubility ($\sim$300 g/L in aqueous solution) (Fig. 1) [15,16]. Interestingly, we have found that adhesion using unmodified recombinant MAP fp-151 (without DOPA residues) showed good shear strength, about 73% that of modified fp-151 (with DOPA residues, $\sim$30% modification yield) [17]. Therefore, we can surmise that other factors may also be important in MAP adhesion; for example, lysine may contribute to adhesion *via* ionic interaction to negatively charged surfaces, and glycine through the open, extended conformation on protein structures [14].

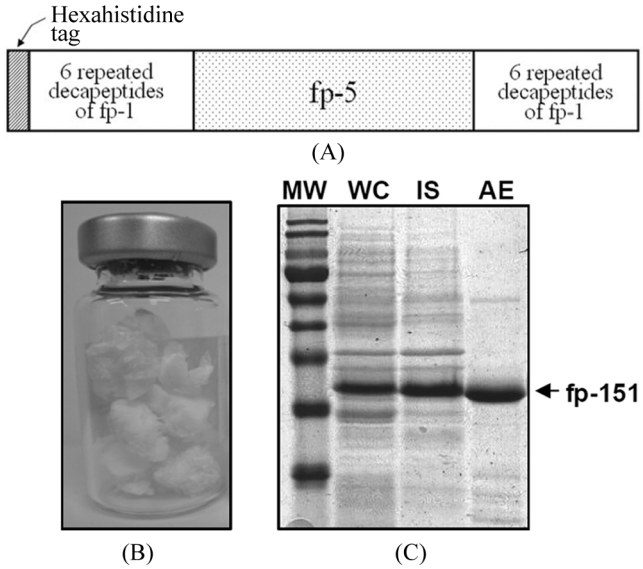

FIGURE 1 (A) Schematic structure and (B) powder sample of recombinant MAP fp-151. (C) Coomassie-Blue-stained SDS-PAGE analysis of purified fp-151. Lanes: MW, protein molecular weight marker; WC, whole-cell sample; IS, insoluble cell debris fraction; AE, fraction extracted with 25% (v/v) acetic acid. 12% SDS-PAGE gels were used for the analysis.

Thus, bulk production of MAP fp-151 without DOPA residues might allow investigation of MAP adhesion steps in more detail. The effects of aggregation and adsorption under various conditions, and the requirements for efficient adhesion, can be examined under conditions where chemical cross-linking does not occur. Moreover, because the *E. coli*-derived MAP fp-151 protein does not contain any DOPA residues, the importance of DOPA modification in the water-resistant strong adhesion of MAPs can be directly explored *in vitro*.

In the present work, we investigated the hydrodynamic radii and turbidities of fp-151 aggregates in solutions of different pH and in the presence of different salts of the Hofmeister series. This salt series is a qualitative list of anions and cations important in salt-induced protein precipitation, and considers the ability of any particular ion to alter the hydrogen bonding network of water [18–20]. Next, we investigated the adsorption of MAP aggregates onto hydrophilic glass and hydrophobic polystyrene surfaces. Relative water tolerances of adsorption were determined and compared, based on the effectiveness of salt ions as protein precipitants.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant fp-151

Expression and purification of recombinant fp-151 have been previously described; the procedure involves extraction with 25% (v/v) acetic acid [16]. *E. coli* BL21 (DE3) cells containing plasmid pENG151 encoding recombinant fp-151 were grown in 3 L of Luria-Bertani (LB) medium with 50 μg/mL ampicillin (Sigma, St. Louis, MO, USA) at 37°C and 250 rpm. The inducer isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM final concentration) was added at an OD_{600} value of 0.2–0.5 and incubation continued at 37°C for 5 h. Cells were harvested by centrifugation at 18,000 g for 10 min at 4°C, and the pellet was resuspended in 5 mL lysis buffer (10 mM Tris-Cl, 100 mM sodium phosphate; pH 8.0) per gram wet weight. Cells were lysed by a cell disruption system (Constant Systems Ltd., Daventry, UK) at 20 kpsi. The lysate was centrifuged (18,000 g, 20 min, 4°C) and cell debris was collected, washed in lysis buffer, and resuspended in 25% (v/v) acetic acid for fp-151 extraction. Protein purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) as standard.

Preparation of Various Salt Solutions

Anionic salts (all 0.1 M) of NaSCN, $NaClO_4$, NaI, $NaNO_3$, NaCl, NaHCOO, NaH_2PO_4, and Na_2SO_4, and cationic salts (also all 0.1 M) of NH_4Cl, KCl, NaCl, $CaCl_2$, and $MgCl_2$ were dissolved in 5% (v/v; 0.84 M) acetic acid (final concentration), and pH was adjusted using sodium hydroxide. Protein fp-151 solutions of 0.5 and 100 mg/mL, in the presence of various salts and at different pH values, were prepared in experiments exploring aggregation and adsorption behaviors, respectively.

Determination of Aggregate Size and Measurement of Turbidity

Each fp-151 solution (0.5 mL) was transferred into a spectroscopic plastic cell. Aggregate size distribution was determined at room temperature using an electrophoretic light scattering spectrophotometer (Photal ELS-8000; Otsuka Electronics, Osaka, Japan). For turbidity determination, 180 μL amounts of fp-151 solutions were loaded into a 96-well plate. Absorbance at 570 nm was determined using a

multi-well plate reader (Victor 3 multilabel reader; PerkinElmer Inc., Waltham, MA, USA). Each measurement was repeated three times independently and the data averaged.

Adsorption of fp-151 onto Surfaces and Measurement of Water Tolerance

Five microliter drops of fp-151 solutions at a concentration of 100 mg/mL were spotted on hydrophilic glass (Paul Marienfeld GmbH, Lauda-Königshofen, Germany) and hydrophobic polystyrene surfaces (100π non-treated polystyrene petri dish; Doowon Meditech Corp., Yongin, Korea). The two surface materials were directly used without any modification. Lysozyme (Bio Basic Inc., Ontario, Canada) was used as a negative control. All samples were dried at room temperature for 2 h and immersed in at least 10 volumes of distilled water on a slowly rotating platform for 10 min at room temperature. After removal of washing solution, residual surface material was stained using Coomassie Blue (Bio-Rad) and quantitated using a Gel-Pro Analyzer™ (Media Cybermetics Inc., Bethesda, MD, USA). Each measurement was repeated at least eight times independently and mean values are shown.

RESULTS AND DISCUSSION

Size Distribution of fp-151 Aggregates at Different pH Values

The size distribution of fp-151 aggregates at various pH values was first determined (Table 1). Natural MAP fp-1 can be easily extracted using 5% (v/v) acetic acid and 4 M urea [21]. Recombinant fp-151 was also solubilized, by acetic acid extraction, from inclusion bodies. Thus, MAP samples at different pH values were prepared, beginning

TABLE 1 Hydrodynamic Radius of Recombinant MAP fp-151 Aggregates in Different pH Solutions. The Radius was Determined by Using an Electrophoretic Light Scattering Spectrophotometer

Dissolving solution for fp-151 power	Hydrodynamic radius (nm)	Polydispersity
0.1 M HCl, pH 1.1	229.9	0.251
0.84 M acetic acid, pH 2.5	250.2	0.170
0.84 M acetate buffer, pH 3.9	252.3	0.232
0.84 M acetate buffer, pH 4.8	Precipitation	–
0.84 M acetate buffer, pH 5.8	Precipitation	–

with a 5% (v/v; 0.84 M) acetic acid solution. We noticed that unmodified fp-151 formed a precipitate at physiological pH values and aggregated at low pH, as did fp-1 and fp-3 [9]. Aggregation increased with rising pH and was reversible. Previously, it was suggested that aggregation of fp-1 was driven mainly by cross-linking of DOPA residues and decreased repulsion of fp-1 molecules after dissociation of protons from lysine and phenolic side chains [10,11]. However, aggregate formation of unmodified fp-151 was achieved using non-covalent interactions of protein, ions, and water. As fp-151 showed good adhesive ability and aggregated effectively, the first step in MAP adhesion may, thus, be aggregation involving physical interactions such as hydrogen bonding, electrostatic forces, and metal ligand effects, without the need for oxidation of DOPA.

The hydrodynamic radius of fp-151 aggregates was about 200–300 nm at pH values below 4, but rose sharply above this pH, resulting in relatively rapid precipitation (Table 1). Protein solubility depends on several factors such as pH, surface hydrophobicity, surface-charge distribution, size, and type and concentration of salts [22]. The relatively high hydrophobicity of fp-151 seems to allow facile aggregate formation, even in HCl solutions. As the protein has a high basic amino acid content (the protein pI value is 10.3), the protonation states of most charged residues do not change below pH 6. The carboxyl groups of the only two glutamates of fp-151 can be neutralized below pH 4 (the carboxyl group of glutamate has an intrinsic pKa of 4.25). This increases the net charge of fp-151 and may facilitate protein solubility below pH 4. In addition, NaOH was used to adjust pH values of the acetate buffer. Because the pKa of acetic acid is 4.76, approximately 99% of acetate ions are neutralized at pH 2.5, ~90% at pH 3.9, ~50% at pH 4.8, and only ~10% at pH 5.8. Acetate buffers at higher pH, thus, contain more Na^+ ions, and such increased salt concentrations can also effectively precipitate fp-151.

Degree of fp-151 Aggregation in Different Salt Solutions

To explore ion-specific salt-induced aggregation of recombinant MAP fp-151, the Hofmeister salt ion series was selected for investigation; this series ranks the relative influences of ions on a wide variety of aqueous processes including protein aggregation [18–20]. The typical orders of the anionic and cationic series are $SO_4^{2-} > H_2PO_4^- > HCOO^- > Cl^- > NO_3^- > I^- > ClO_4^- > SCN^-$, and $Mg^{2+} > Ca^{2+} > Na^+ > K^+ > NH_4^+$, respectively. In general, species nearer the left of these two subseries are termed kosmotropes, showing high protein salting-out effects, and species nearer the right are chaotropes, demonstrating

salting-in behavior. As the Hofmeister effects become important at moderate-to-high salt concentrations (0.01 to 1 M) [23], 0.1 M salt and 0.5 mg/mL fp-151 were dissolved in 0.84 M (final concentration) acetate buffer, pH 3.9. Table 2 shows the hydrodynamic radii of fp-151 in different salt solutions. Aggregation size fell with increasing anionic kosmotropy, except in the case of Na_2SO_4, and rapid precipitation by chaotropic anions was observed. However, the degree of aggregation in the presence of cations rose with increasing kosmotropy. Turbidity was also measured, to explore the effects of salt ions on precipitation (Fig. 2). The turbidity of fp-151 in different anionic salt solutions clearly decreased with increasing kosmotropy, in a manner similar to what was seen when aggregation size distribution was measured. However, such correlation between aggregation size and turbidity was not observed when cationic salt solutions were tested. Within monovalent or divalent ion classes, low correlations were noted.

The effects of salt ions on fp-151 solution behavior were more pronounced when the anionic salts were explored (compared with the cationic group), and were in the reverse order of the Hofmeister series. It has been reported that the solubility of lysozyme, a basic protein (with a pI value of 11.1) similar to fp-151 [24], also showed reverse Hofmeister series behavior [25–27]. Salt ion effects on biopolymer processes result from ion-specific interactions with both amide groups and a nonpolar surface [28]. Different ions have distinct charge densities and show very specific dispersion potentials near an interface

TABLE 2 Hydrodynamic Radius of Recombinant MAP fp-151 Aggregates in Different Salt Solutions Based on the Hofmeister Series. The Radius was Determined by Using an Electrophoretic Light Scattering Spectrophotometer

Salt in 0.84 M acetate buffer, pH 3.9	Hydrodynamic radius (nm)	Polydispersity	Remarks
0.1 M Na_2SO_4	883.1	0.403	Kosmotrope
0.1 M NaH_2PO_4	269.8	0.073	
0.1 M HCOONa	214.2	0.221	
0.1 M NaCl	796.5	0.154	For anionic effect
0.1 M NaI	Precipitation	–	
0.1 M $NaNO_3$	Precipitation	–	
0.1 M $NaClO_4$	Precipitation	–	
0.1 M NaSCN	Precipitation	–	Chaotrope
0.1 M $MgCl_2$	2047.4	0.326	Kosmotrope
0.1 M $CaCl_2$	1311.6	0.366	For cationic effect
0.1 M NaCl	796.5	0.154	
0.1 M KCl	625.3	0.322	
0.1 M NH_4Cl	512.3	0.252	Chaotrope

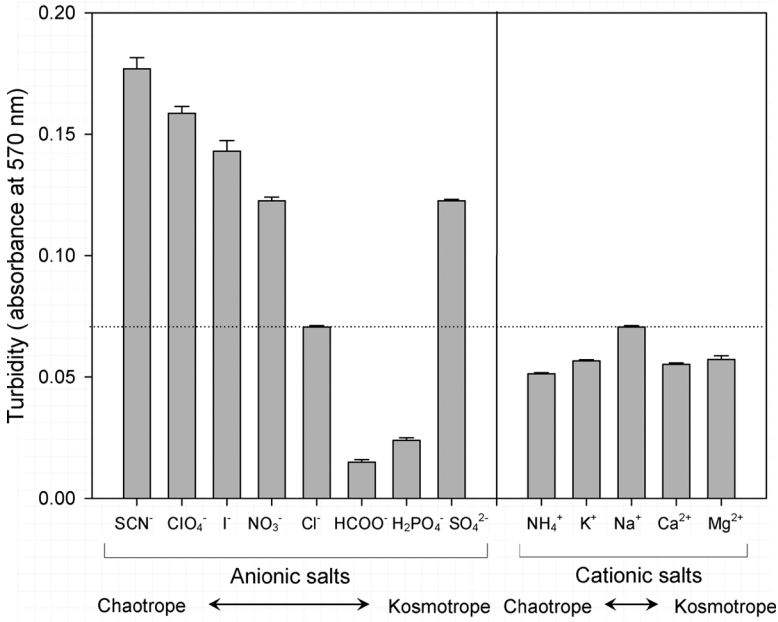

FIGURE 2 Turbidity of recombinant MAP fp-151 solutions in various anionic and cationic salts of the Hofmeister series. Protein (0.5 mg/mL) and various salts (all 0.1 M) were dissolved in 0.84 M acetate buffer (pH 3.9). Absorbance at 570 nm was determined, to measure turbidity. Each measurement was repeated three times independently and averages are shown.

[19,29,30]. Thus, varying ionic interaction strengths can explain why aggregation of fp-151 follows the Hofmeister series generally. The positively charged residues of Lys, His, and Arg in fp-151 at pH 3.9 interact mainly with surrounding anions and water molecules by electrostatic interaction. However, the ionic dispersion force is more attractive for chaotropes (weakly hydrated ions of low charge density) than for kosmotropes (strongly hydrated ions of high charge density) [19,31]. Thus, chaotropic anions such as SCN^- and NO_3^- bind readily to the positively charged residues of fp-151, whereas kosmotropic anions such as $HCOO^-$ and $H_2PO_4^-$ do not associate so easily with the protein. Aggregation of fp-151 can, thus, be more readily achieved by chaotropic anions rather than kosmotropes. Unexpectedly, particle size and turbidity in Na_2SO_4 were very high, compared with parameter values seen using kosmotropic anions. However, in this case, the amount of Na^+ added to the solution was twice that of the (equimolar) SO_4^{2-} concentration, which possibly increased aggregation and

turbidity. In fact, the turbidity of fp-151 increased with additional NaCl addition (Fig. 3).

Salt-induced interactions mediated by cations differed from those caused by anions. Cations do not directly interact with MAP fp-151 because the protein lacks negatively charged residues and can only change the hydrogen bonding network between fp-151 and water. Positively charged amino acid residues are weakly hydrated [32]. Thus, strongly hydrated kosmotropic cations can more easily influence water molecule orientation at the protein surface, inhibiting the formation of hydrogen bonds between fp-151 and water. As a result, the solubility of fp-151 in the presence of kosmotropic cations was lower than when chaotropic cations were available, and particle size increased with rising kosmotropy of different cationic salt solutions (Table 2). In addition, differences in aggregate sizes in various cationic Hofmeister series solutions weakly influenced turbidity, but anionic salts did not show this property. This can be explained by indirect interaction of cations with the water layer at the protein surface; such water molecules are not directly replaced by cations in fp-151 solutions and the water shell can, thus, be conserved in the presence of various cations.

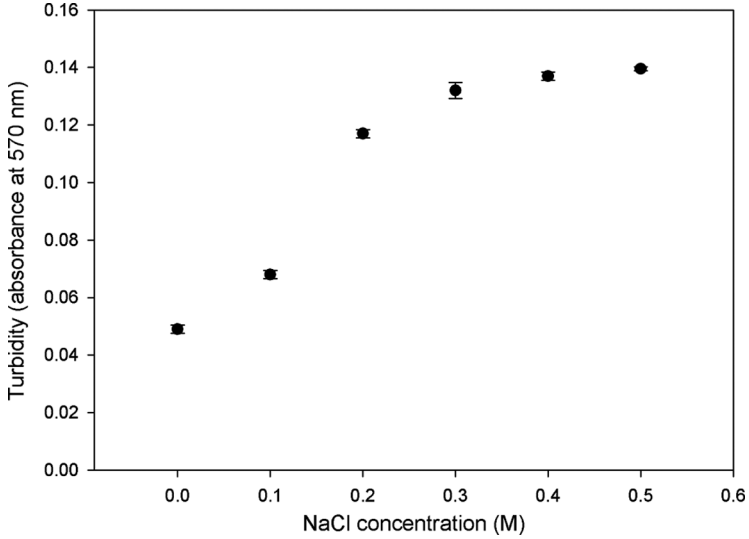

FIGURE 3 Turbidity of recombinant MAP fp-151 in 0.84 M acetate buffer (pH 3.9) with different NaCl concentrations. Protein (0.5 mg/mL) and various concentrations of NaCl were dissolved in 0.84 M acetate buffer (pH 3.9). Absorbance at 570 nm was determined, to measure turbidity. Each measurement was repeated three times independently and averages are shown.

Surface Adsorption and Water Tolerance of fp-151

We found that recombinant fp-151, like other MAPs, existed as aggregates in aqueous solutions. If rapid aggregate formation is the first step in strong MAP adhesion, protein aggregates will be well adsorbed to various substrates. The adsorption behavior of unmodified fp-151 was investigated using hydrophilic glass and hydrophobic polystyrene surfaces, and lysozyme was used as a negative control because it is very basic, as is fp-151. First, fp-151 and lysozyme (both at 100 mg/mL) were dissolved in 0.84 M acetate buffer (pH 3.9), spotted onto the surfaces, and dried. All samples were washed in distilled water and residual protein amounts determined after staining. On the

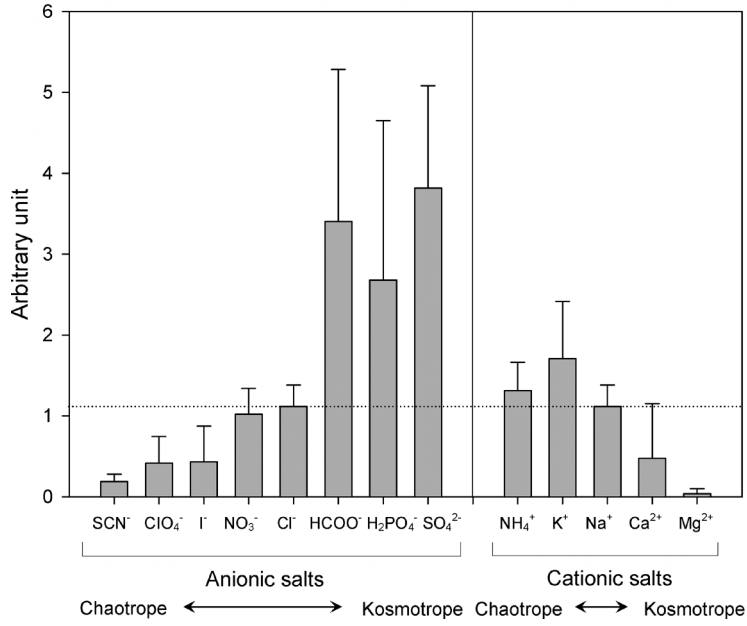

FIGURE 4 Adsorption of recombinant MAP fp-151 onto a hydrophilic glass surface in the presence of various anionic and cationic salts of the Hofmeister series. Protein (0.5 mg/mL) and various salts (all 0.1 M) were dissolved in 0.84 M acetate buffer (pH 3.9). Five microliter drops of each solution were spotted onto the hydrophilic glass surface and dried for 2 h at room temperature. After washing with distilled water, residual fp-151 was assayed by Coomassie-Blue staining and the relative intensity was analyzed. Arbitrary unit on y-axis means relative amount of the residual protein on the glass surface. Each measurement was repeated eight times independently and averages are shown.

hydrophobic polystyrene surface, most fp-151 protein was washed away, as was lysozyme (data not shown). Therefore, we surmise that DOPA residues may be critical for adhesion to hydrophobic surfaces. It has been reported that DOPA facilitated a high-strength yet fully reversible, non-covalent, interaction of MAP with a surface [33]. On the other hand, the adsorption of unmodified fp-151 to a hydrophilic glass surface was remarkable (data not shown). We assumed that the degree of aggregation and presence of salt ions influenced the water tolerance of adsorption. Therefore, fp-151 samples were prepared in the different salt solutions of the Hofmeister series, and adsorption tests on a hydrophilic glass surface were performed. Figure 4 shows the degree of attachment from different anionic and cationic salt solutions. Attachment of fp-151 was well conserved as anionic kosmotropy and cationic chaotropy varied. Importantly, the correlation was considerably similar to the case of particle size distribution in different salt solutions. Protein fp-151 with smaller particle size distribution showed better adsorption behavior. Larger aggregates of fp-151 might reduce their polar nature and decrease attachment strength by non-covalent interaction between ionic surfaces and aggregates. These results suggest that the attachment of fp-151 without DOPA only depends on non-covalent interaction, and the effectiveness of an ion in altering non-covalent interactions influences the attachment strength of fp-151, as well as the degree of aggregation.

CONCLUSIONS

Unmodified recombinant MAP fp-151 rapidly formed aggregates, without the need for DOPA-mediated chemical cross-linking. The degree of aggregation varied with pH and salt type. Attachment strength correlated with the degree of aggregation, and aggregates showing efficient adsorption were easily prepared in kosmotropic anionic and chaotropic cationic solutions. The results suggest that intrinsic physical properties of MAPs, such as aggregation ability and adsorption by non-covalent interaction, may contribute to initial strong attachment, where DOPA residues for the formation of initial aggregates may not be crucial. Chemical cross-linking of DOPA residues may be involved mainly in water-resistant adhesion and optimized curing. The *E. coli*-derived recombinant MAP fp-151 will be most helpful in the further elucidation of the adhesion mechanism, and will receive *in vitro* modification to permit chemical cross-linking of oxidized DOPA residues, yielding water-resistant strong adhesion.

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