

SspB Peptide Assay Reveals Saliva-Mediated *Porphyromonas gingivalis* Attachment

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Received 17 October 2015; accepted 10 November 2015; published 13 November 2015

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Abstract

Background: *Porphyromonas gingivalis* is a major periodontal pathogen that binds efficiently to *Streptococcus gordonii*, which in turn binds to salivary agglutinin (gp340). The SspB of *S. gordonii* appears to mediate this association. We previously reported that the streptococcal SspB peptide analog, designated SspB (390-T400K-402), showed high binding activity with saliva. To understand the three-way interaction among *S. gordonii*, *P. gingivalis* and salivary gp340 as a unit, we established a peptide binding assay using SspB (390-T400K-402). **Methods:** The binding activity of the SspB (390-T400K-402) to *P. gingivalis* was detected by ELISA. Ninety-six well plates were coated with whole bacterial cell (*P. gingivalis* strains ATCC 33277, and W83; *S. gordonii* DL1) in Na₂CO₃ coating buffer. After blocking, bacterial cells were incubated with saliva or salivary agglutinin peptide (SRCRP2). Biotinylated SspB (390-T400K-402) was applied and incubated with 1:1000 streptoavidin-conjugated alkaline phosphatase. After development, A₄₀₅ was recorded. **Results:** *P. gingivalis* 33277 showed the highest binding activity of the tested bacteria, whereas *P. gingivalis* W83, which was deficient in Mfa1 fimbriae, exhibited poor binding activity, as did *S. gordonii*. The binding of SspB (390-T400K-402) peptide in saliva- or SRCRP2-treated *P. gingivalis* was significantly higher than that in non-treated cells. **Conclusion:** The SspB (390-T400K-402) peptide binding assay revealed that initial attachment of *P. gingivalis* to the substrata of *S. gordonii* may require gp340-mediated SspB-Mfa1 interactions. The assay is available to assess the relationships among SspB, Mfa1 and salivary gp340 as a unit.

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Keywords

SspB, Biofilm, *Porphyromonas gingivalis*, Saliva, gp340

1. Introduction

Saliva-coated teeth and epithelium (cheeks, gums, and tongue) provide a variety of surfaces for bacterial attachment and colonization, followed by dental plaque maturation [1]. Dental plaque is a complex biofilm composed of early and late colonizing bacteria [2] [3]. Attachment of early colonizing bacteria as typified by *Streptococcus gordonii* to salivary components, specifically targeting innate immunity scavenger receptor glycoprotein-340 (gp340) [4], is a key event in oral biofilm formation [5] [6]. The periodontitis-associated pathogen *Porphyromonas gingivalis*, one of the later biofilm inhabitants, binds and forms biofilms on the antecedent organisms, such as *S. gordonii* [7].

SspB protein, a streptococcal adhesin [8], mediates attachment of *S. gordonii* to saliva-coated enamel surfaces and has been shown to bind to Mfa1 fimbriae of *P. gingivalis* [9]. The binding domain of SspB to *P. gingivalis* has been mapped [10]. In addition, a discrete structural region in SspB that confers the adherence phenotype has been identified [11] [12]. However, few assays are available to assess the relationships among SspB, Mfa1 and salivary gp340 as a unit.

We previously demonstrated that analogous SspB (390-T400K-402) peptide [a substitution of threonine for lysine at 400, SspB (390-402) peptide] had the highest binding activity to the salivary components among several analogous SspB peptides [13]. The positively charged amino acid residue, *i.e.* lysine, is essential for binding to the negatively charged salivary components [14] and gp340 peptide (designated SRCRP2) [15].

Salivary gp340 has a high bacteria-binding capacity, and recognizes different bacterial receptors based on whether gp340 is in the fluidphase or is bound to the hydroxyapatite surface [16]. Furthermore, the fimbriae of *P. gingivalis* also bind to salivary proteins, such as proline-rich salivary protein 1 and statherine [17]–[19]. Therefore, we hypothesized that *P. gingivalis* was able to bind to the salivary gp340 and to the SRCRP2 peptide simultaneously with Mfa1-SspB interaction.

In the present study, to establish an assay that enables us to understand a three-way interaction among *S. gordonii*, *P. gingivalis* and salivary gp340 as a unit, and to examine whether *P. gingivalis* is able to bind to salivary gp340 and to SRCRP2, we evaluated the binding activity of the streptococcal peptide analog SspB (390-T400K-402) to *P. gingivalis* in the presence of saliva or gp340 peptide SRCRP2. An understanding the association between these molecules and periodontal bacteria is essential for elucidating the mechanisms of supra- and sub-gingival oral biofilm formation.

2. Materials and Methods

2.1. Bacterial Culture

Porphyromonas gingivalis strains 33277 and W83 (afimbriated) were grown in brain heart infusion (BHI) broth supplemented with 1 µg/ml hemin (final concentration), and 5 µg/ml menadione (final concentration) (BHI-HM) under anaerobic conditions (10% CO₂, 10% H₂, and 80% N₂) at 37°C for 48 h. *Streptococcus gordonii* DL1, *Streptococcus mutans* MT8148, and *Actinomyces naeslundii* X600 were cultured in BHI broth at 37°C for 24 h.

2.2. Peptide Synthesis

The SspB peptide analog, in which the T (threonine) at position 400 has been replaced with K (lysine) in SspB (390-402), resulting in the SspB (390-T400K-402) peptide, DYQAKLAAYQKEL, and scavenger receptor cysteine-rich domain peptide 2, designated SRCRP2, QGRVEVLYRGSWGTVVC, on salivary gp340 [20] were synthesized at 95% purity by Scrum, Inc. (Tokyo, Japan). Peptides were suspended in sterile distilled water (DW) at the desired concentration immediately before use.

2.3. Human Saliva Collection

As described previously [21], saliva samples were collected from volunteers in good oral health, after stimula-

tion with chewing paraffin gum. Volunteers had refrained from eating, drinking and brushing for at least 2 h prior to collection. Saliva was placed into ice-chilled sterile bottles for 5 min, followed by centrifugation at $10,000 \times g$ for 10 min at 4°C in order to remove cellular debris. Supernatant was filter sterilized through a 0.22- μ m Acrodisc filter (Pall Corporation, Ann Arbor, MI) for peptide binding assay. After filtration, samples were pooled and stored at -20°C until use.

2.4. ELISA

2.4.1. Peptide Binding Assay

Binding activity of SspB (390-T400K-402) peptide to saliva and to *P. gingivalis* were detected by enzyme-linked immunosorbent assay (ELISA). A previously reported method [21] was used, with some modifications. Ninety-six well H-plates (Sumitomo Bakelite, Tokyo, Japan) were coated for 1 h at 37°C with 100 μ l of whole bacterial cells (*P. gingivalis* strains 33277 and W83, *S. gordonii* DL1, *S. mutans* MT8148, and *A. naeslundii* X600) in Na₂CO₃ coating buffer at an optical density of 0.40 at 600 nm. After washing three times with PBS containing 0.1% Tween 20 (PBST), wells were blocked with 200 μ l of 3% bovine serum albumin (BSA) in PBST at 4°C overnight. Biotinylated SspB (390-T400K-402) peptide (12.5, 25, 50 and 100 μ g/ml) in 100 μ l of sterile DW was applied to the wells, followed by incubation at 37°C for 1 h. Wells were then washed three times with PBST, and were further incubated for 1 h at 37°C with 1:1000 streptoavidin-conjugated alkaline phosphatase (Invitrogen Corp., Carlsbad, CA). After development, absorbance at 405 nm was measured. For sandwich ELISA, we sandwiched the bacteria between biotinylated and non-biotinylated SspB (390-T400K-402) peptides. Briefly, the non-biotinylated SspB (390-T400K-402) peptide (12.5, 25, 50 and 100 μ g/ml) was coated to the ELISA plate. After blocking, 100 μ l of *P. gingivalis* 33277 cell suspension was applied to each well, followed by incubation at 37°C for 1 h. After washing three times with PBST, 100 μ l of biotinylated SspB (390-T400K-402) peptide (25 μ g/ml) was applied, followed by further incubation at 37°C for 1 h. Subsequent steps were as described above.

2.4.2. Responses of Salivary Immunoglobulins with *P. gingivalis*

In order to determine whether human saliva contains *P. gingivalis*-specific secretory IgA (sIgA) and/or IgG, we compared the ability of anti-IgA and IgG labeled antibodies to bind to *P. gingivalis* coated with saliva. Bacterial cells were deposited into the wells of ELISA plates. After blocking with 1% skim-milk and washing, 1:10 saliva diluted with sterile DW was added to the wells. Alkaline phosphatase-conjugated goat anti-human immunoglobulins (SIGMA) (1:1000 IgA or 1:4000 IgG) were reacted after the wells had been incubated with 1:10 saliva. Reactions were detected as mentioned above. All experiments were performed independently at least in triplicate.

2.4.3. Statistical Analyses

Data are expressed as means standard deviation. GraphPad Prism version 5.0 d for Mac OS X (GraphPad Software, San Diego, CA) was used to assess significance. The statistical significance of differences between two groups was determined by unpaired *t*-test. For comparisons between multiple groups, one-way analysis of variance (ANOVA) and Tukey's test were used. *P*-values less than 0.01 or 0.05 were considered to be statistically significant using two-tailed comparisons. All experiments were repeated and analyzed independently.

3. Results

3.1. Binding Properties of SspB (390-T400K-402) Peptide

In order to establish a peptide binding assay using ELISA, we examined the binding properties of SspB (390-T400K-402) peptide at various concentrations (12.5, 25, 50 and 100 μ g/ml) to *P. gingivalis* (Figure 1(a)) or to saliva (Figure 1(b)). The binding of SspB (390-T400K-402) peptide to *P. gingivalis* cells at 25 μ g/ml was significantly higher than that at 12.5 μ g/ml. At peptide concentrations of 50 or 100 μ g/ml, the peptide binding properties to *P. gingivalis* were comparable to those at 25 μ g/ml (Figure 1(a)). Thus, SspB at 25 μ g/ml was used for further studies. A binding reaction to saliva was observed at 25 μ g/ml, whereas no reaction was detected when the peptide was not applied (no peptide), thus suggesting that SspB (390-T400K-402) peptide has a binding capacity for saliva (Figure 1(b)).

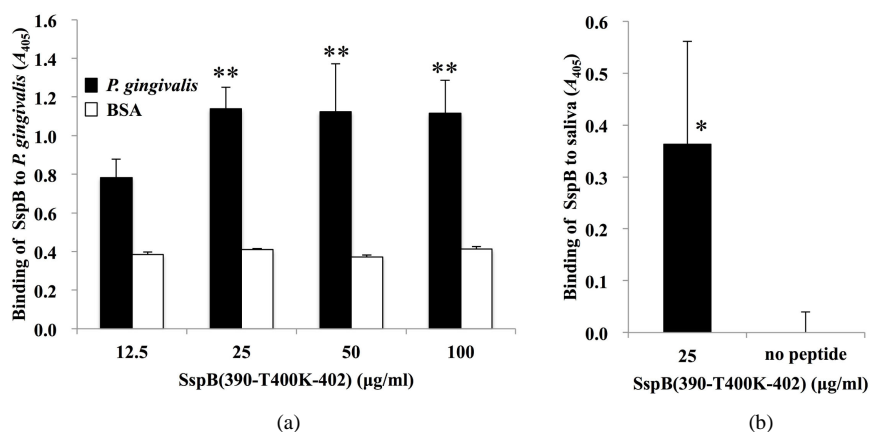


Figure 1. Binding of SspB (390-T400K-402) to *P. gingivalis*. (a) Binding response of biotinylated SspB (390-T400K-402) to *P. gingivalis* strain 33277 at various concentrations (12.5, 25, 50 and 100 µg/ml); (b) Binding response of biotinylated SspB (390-T400K-402) to saliva at a peptide concentration of 25 µg/ml. Binding is expressed as A_{405} values obtained from three independent experiments. Values are expressed as means \pm standard deviation (SD) of triplicate assays. Asterisks denote significant differences (vs. 12.5 µg/ml, ** $P < 0.01$; vs. no peptide, * $P < 0.05$).

3.2. Sandwich ELISA

As mentioned above, we utilized biotinylated SspB (390-T400K-402) peptide in the present methods. Therefore, to investigate whether biotinylation affects the binding activity of this peptide, sandwich ELISA with biotinylated and non-biotinylated SspB (390-T400K-402) peptide was performed (Figure 2). When the wells were pre-coated with non-biotinylated SspB peptide, binding activities of biotinylated SspB peptide to *P. gingivalis* cells increased in a dose-dependent manner, but decreased with a peptide concentration of 100 µg/ml (Figure 2(a)). On the other hand, when wells were pre-coated with biotin, binding activity of biotinylated SspB peptide to *P. gingivalis* cells remained unchanged (Figure 2(b)). When samples were sandwiched between pre-coated solution [DW, biotin or non-biotinylated SspB (390-T400K-402)] and biotinylated SspB (390-T400K-402), the strongest reactions were observed in *P. gingivalis* 33277 sandwiched between non-biotinylated and biotinylated SspB peptides (Figure 2(c)). In contrast, low reaction levels were observed in *S. gordonii* DL1 and BSA (Figure 2(c)).

3.3. SspB (390-T400K-402) Peptide Specificity to Mfa1 of *P. gingivalis*

We examined the specificity of the SspB (390-T400K-402) peptide for *P. gingivalis* Mfa1 fimbriae (Figure 3). *P. gingivalis* strains 33277 and W83, and oral commensals *S. gordonii* DL1, *S. mutans* MT8148 and *A. naeslundii* X600 were tested. *P. gingivalis* 33277 showed the highest peptide binding activity of the tested bacteria (Figure 3(a)), whereas *P. gingivalis* W83, which is deficient in Mfa1 fimbriae, exhibited poor binding activity, as did *S. gordonii* DL1 and BSA (Figure 3(b)).

3.4. Salivary Components Affect the Binding of SspB (390-T400K-402) Peptide to *P. gingivalis*

To determine whether the saliva samples used in this study contain *P. gingivalis*-specific sIgA and/or IgG, the binding of anti-IgA and anti-IgG antibodies to saliva-coated bacteria was compared (Figure 4(a)). The results showed that salivary immunoglobulins bound significantly better to *S. gordonii* when compared with *P. gingivalis* strains 33277 and W83. The binding of salivary immunoglobulins to the *P. gingivalis* strains was comparable to BSA, which was used to demonstrate the background level of non-specific antibodies.

Hamada *et al.* [13] previously demonstrated that SspB (390-T400K-402) peptide had the highest binding activity to salivary components and to the salivary gp340 peptide SRCRP2. Thus, we hypothesized that when *P. gingivalis* was incubated with whole saliva or with SRCRP2, the binding activity of the SspB (390-T400K-402) peptide would increase. To assess this hypothesis, *P. gingivalis* cells incubated with saliva were studied (Figure 4(b)). When *P. gingivalis* was incubated with 100 µl of whole saliva, the binding activity of SspB (390-T400K-402)

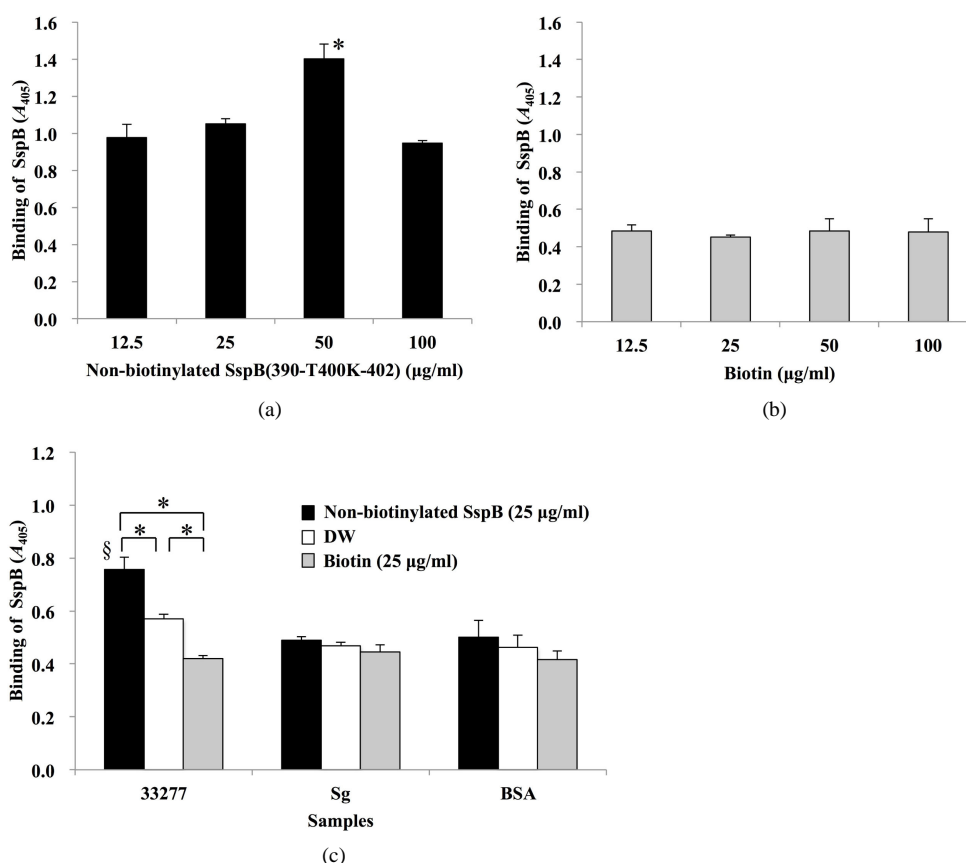


Figure 2. Sandwich assay with biotinylated and non-biotinylated SspB (390-T400K-402). (a) Microtiterplates were coated with non-biotinylated SspB (390-T400K-402) (12.5, 25, 50 and 100 $\mu\text{g/ml}$); (b) biotin (12.5, 25, 50 and 100 $\mu\text{g/ml}$), (c) DW, 25 $\mu\text{g/ml}$ biotin, or 25 $\mu\text{g/ml}$ of non-biotinylated SspB (390-T400K-402). *P. gingivalis* strain 33277 (33277), *S. gordonii* (Sg) or BSA were added to the wells of coated plates, and then 25 $\mu\text{g/ml}$ biotinylated SspB (390-T400K-402) was added. Data are expressed as A_{405} values obtained from three independent experiments. Values are expressed as means \pm SD of triplicate assays (* $P < 0.01$, § represents significant differences vs. Sg, vs. BSA; $P < 0.01$).

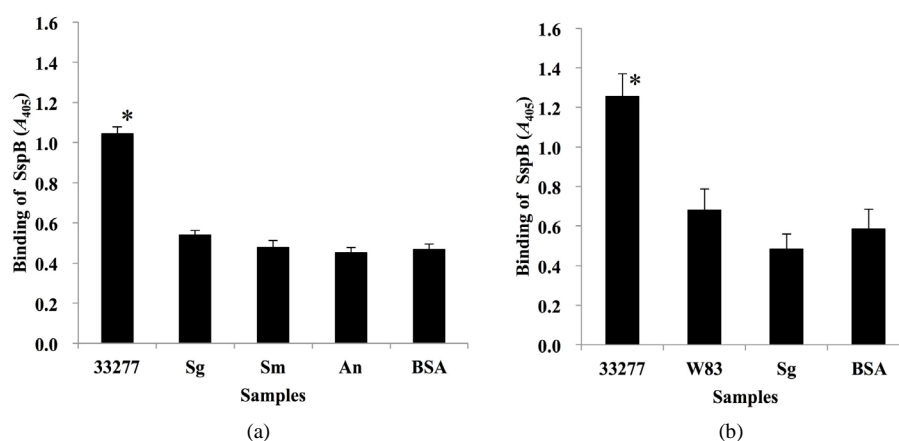


Figure 3. Binding of SspB (390-T400K-402) to *P. gingivalis* strain 33277. (a) Binding response of biotinylated SspB (390-T400K-402) peptide (25 $\mu\text{g/ml}$) to *P. gingivalis* strain 33277 (33277), *S. gordonii* DL1 (Sg), *S. mutans* MT8148 (Sm) and *A. naeslundii* X600 (An); (b) Comparison of SspB (390-T400K-402) peptide (25 $\mu\text{g/ml}$) binding to *P. gingivalis* strains between 33277 and *P. gingivalis* W83 (W83). Data are expressed as A_{405} values obtained from three independent experiments. Values are expressed as means \pm SD of triplicate assays (* $P < 0.01$).

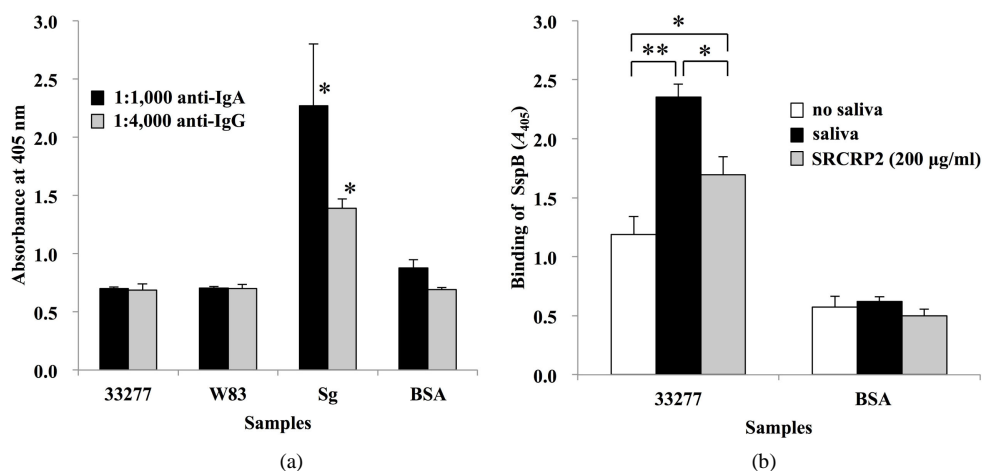


Figure 4. Analysis of salivary effects on SspB (390-T400K-402) binding to *P. gingivalis*. (a) Response of salivary immunoglobulins to bacterial samples with anti-human IgA or IgG (vs. 33277, vs. W83, vs. BSA; $P < 0.01$); (b) Effects of SRCRP2 (200 µg/ml) on SspB (390-T400K-402) binding ($*P < 0.05$, $**P < 0.01$). Data are expressed as A_{405} values obtained from three independent experiments. Values are expressed as means \pm SD of triplicate assays.

peptide increased markedly when compared to the cells without saliva incubation. When cells were incubated with 100 µl of SRCRP2 peptide (200 µg/ml), the binding activity of SspB peptide was significantly higher than in non-treated cells; however, it was lower than in saliva-treated cells (Figure 4(b)).

4. Discussion

At present, few assays are available to assess the relationships among streptococcal adhesin SspB, *P. gingivalis* minor fimbrial antigen Mfa1 and salivary agglutinin gp340. In the present study, we evaluated the binding activity of the analogous peptide SspB (390-T400K-402) to *P. gingivalis* in the presence of saliva or salivary gp340 peptide SRCRP2 in order to establish an assay for investigating the three-way interaction among *S. gordonii*, *P. gingivalis* and salivary gp340 as a unit, and to examine whether *P. gingivalis* binds to both salivary gp340 and SRCRP2.

SspB (390-T400K-402) peptide has the highest response for binding to salivary components and to SRCRP2 when compared with other SspB and streptococcal adhesin-derived peptides. Consistent with previous reports [13] [14], our study found that SspB (390-T400K-402) showed binding activity with saliva (Figure 1(b)). Furthermore, the peptide also showed binding activity with *P. gingivalis* 33277 (Figure 1(a), Figure 3(a)). The present results therefore suggest that there are critical residues for binding to *P. gingivalis* within the synthetic peptide SspB (390-T400K-402) adhesion epitope. We believe that the SspB (390-T400K-402) is markedly superior to other SspB-derived peptides with regard to binding characteristics.

A sequence of the C-terminal region of SspB, designated BAR (amino acid residues 1167-1193), mediates binding to *P. gingivalis* [22]. Here we demonstrate that streptococcal peptide analog SspB (390-T400K-402) is also capable of binding to *P. gingivalis* 33277, although the peptide is derived from the N-terminal A region (amino acid residues 390-402). SspB (390-T400K-402) is produced by a substitution of threonine for lysine at 400 of SspB (390-402) and consequently, the adhesion epitope of the peptide to *P. gingivalis* may be conferred by conformation changes.

P. gingivalis 33277 has two distinct fimbriae molecules, major fimbriae FimA encoded by *fimA* [23] and minor fimbriae Mfa1 encoded by *mfa1* [24]. The Mfa1 of *P. gingivalis* mediates adherence to the SspB of *S. gordonii*, so that the Mfa1-SspB interaction is necessary for biofilm development [7] [25]. Strain W83 expresses the type IV major fimbriae [23], but the minor fimbriae Mfa1 has not been observed [26]. These reports, together with our observations that *P. gingivalis* 33277 showed the highest peptide binding activity of the bacterial samples (Figure 3(a)), whereas *P. gingivalis* W83 deficient in Mfa1 fimbriae exhibited poor peptide binding (Figure 3(b)), suggest that the analogous peptide SspB (390-T400K-402) binds to Mfa1. However, some limitations are worth noting. We used *P. gingivalis* W83 as an Mfa1-deficient strain in the present study. This assay

needs to be tested further by using *mfa1*-knockout mutants derived from strain 33277 in order to compare binding reactions in a 33277 genetic background.

In this study, we used biotinylated SspB (390-T400K-402) peptide, which was synthesized with a single N-terminal biotinylation. Indeed, biotinylated SspB (390-T400K-402) peptide had a high binding activity against whole salivary components on Western blotting [13]. Thus, in the present study, we assessed whether biotinylation in the N-terminal of SspB (390-T400K-402) enhances binding activity to *P. gingivalis* (Figure 2). In Sandwich ELISA, pre-coating with biotin did not affect SspB binding to *P. gingivalis* (Figure 2(b)). In contrast, the SspB binding levels varied and peaked at a concentration of 50 µg/ml non-biotinylated SspB (Figure 2(a)), suggesting that the adhesion epitope of Mfa1 was occupied by the pre-coated non-biotinylated peptide. Moreover, the strongest reaction was observed in *P. gingivalis* 33277 sandwiched between non-biotinylated and biotinylated SspB peptides (Figure 2(c)). Overall, these results suggest that biotinylation in the N-terminal of SspB (390-T400K-402) has no promotional effect on peptide binding activity.

Salivary molecules, proline-rich protein 1 and statherin, have been reported to promote the adherence of *P. gingivalis* to saliva-coated oral surfaces through specific interactions [17]–[19]. In fact, saliva appears to have a significant impact on SspB binding to *P. gingivalis*. The increase in SspB peptide binding to saliva-incubated *P. gingivalis* (Figure 4(b)) suggests a saliva-*P. gingivalis* interaction. Moreover, the increase in SspB peptide binding to SRCRP2-incubated *P. gingivalis* (Figure 4(b)) indicates that *P. gingivalis* interacts with salivary gp340. SIgA, a salivary agglutinin, is the predominant immunoglobulin found in all mucosal secretions, including saliva. Salivary sIgA may promote colonization of certain strains of bacteria [27]. We previously demonstrated that salivary sIgA promoted initial attachment of *Streptococcus mutans* on the mouse tooth surface [21]. In contrast, decreased salivary sIgA negatively modulated *Candida albicans* populations in the oral cavity of mice [28]. In addition, IgG has been shown to be the major antibody class that mediates host immune response against *P. gingivalis* [29]. In the present study, we confirmed that the saliva did not contain either sIgA or IgG specific to *P. gingivalis* (Figure 4(a)). These findings provide evidence that the SspB peptide-*P. gingivalis* interaction via saliva was not induced by salivary antibodies, but rather by salivary gp340.

Taken together, a peptide binding assay using SspB (390-T400K-402) presented herein may provide important insights into the mechanisms of supra- and sub-gingival oral biofilm formation.

5. Conclusion

A novel assay using the analogous SspB (390-T400K-402) peptide established in this research is available to assess the relationships among SspB, Mfa1 and salivary gp340 as a unit. The assay also reveals that *P. gingivalis* binds to salivary gp340 and to SRCRP2.

Acknowledgements

We would like to thank all volunteers for their cooperation in this study. This work was supported by a Grant-in-Aid for Young Scientists (B) 25862034 and a Grant-in-Aid for Exploratory Research 19659559 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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