molecular oral microbiology

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Alpha-amylase is a human salivary protein with affinity to lipopolysaccharide of *Aggregatibacter actinomycetemcomitans*

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SUMMARY

Aggregatibacter actinomycetemcomitans **lipopolysaccharide (Aa.LPS) is a major virulence factor associated with aggressive periodontitis. Although the recognition of Aa.LPS is potentially initiated by salivary proteins in the oral cavity, Aa. LPS-binding proteins (Aa.LPS-BPs) in saliva are poorly characterized. The purpose of this study was to capture and identify Aa.LPS-BPs in human saliva using a LTQ-Orbitrap hybrid Fourier transform mass spectrometry. Aa.LPS conjugated onto *N*-hydroxysuccinimidyl-Sepharose® 4 Fast Flow beads (Aa.LPS-beads) activated Toll-like receptor 4 and produced nitric oxide and Interferon gamma-inducible protein-10, implying that the conjugation process did not alter the biological properties of Aa.LPS. Aa.LPS-BPs were subsequently isolated from the nine human saliva samples from healthy individuals with the Aa.LPS-beads followed by identification with the mass spectrometry. Aa.LPS-BPs include α-amylase, serum albumin, cystatin, lysozyme C, submaxillary gland androgen-regulated protein 3B, immunoglobulin subunits, polymeric immunoglobulin receptor, deleted in malignant brain tumors 1, prolactin-inducible protein, lipocalin-1, and basic salivary proline-rich protein 2. Specific binding was validated using a pull-down assay with α-amylase which was captured at the highest frequency. Alpha-amylase demonstrated to interfere with the adherence and biofilm formation of *A. actinomycetemcomitans.* Even heat-inactivated α-amylase showed the interference to the same extent. Conclusively, we identified unique Aa.LPS-BPs that provide useful information to understand bacterial pathogenesis and host innate immunity in the oral cavity.**

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a Gram-negative, facultative anaerobe that lives in the oral cavity, including the supragingival plaque, subgingival plaque, saliva, gingivae, tongue, and tonsils (Asikainen *et al.*, 1991). This bacterium is one of the key etiological agents associated with 90% of localized

aggressive periodontitis and 30–50% of severe adult periodontitis (Slots & Ting, 1999). A large number of virulence factors, that aid survival of *A. actinomycetemcomitans* in the oral cavity and cause diseases, include lipopolysaccharide (LPS), bacteriocins, cytotoxins, extracellular membranous vesicles, and leukotoxin (Fives-Taylor *et al.*, 1999). Among them, LPS is considered a major cell-wall etiologic factor causing inflammation followed by tissue destruction.

Initial recognition of microbial virulence factors is crucial for immune responses against infection in the host. For example, LPS is first recognized by LPS-binding protein (LBP) and then interacts with CD14 and the Toll-like receptor 4 (TLR4)/MD2 complex on the immune cells where inflammatory responses take place. LBP and CD14 enhance the response to LPS by 100- to 300-fold and 10,000-fold, respectively (Lee *et al.*, 1992; Wurfel & Wright, 1997). Conversely, mice deficient in LBP or CD14 are hyporesponsive to LPS (Haziot *et al.*, 1996; Jack *et al.*, 1997). However, LBP does not always enhance the responsiveness to LPS since LBP expression is highly up-regulated during infections by about 100-fold to prevent over-activation of inflammation (Hamann *et al.*, 2005). Various LPS-binding proteins (LPS-BPs) besides LBP have been identified including albumin, lysozyme, complements, and deleted in malignant brain tumors 1 (DMBT1) (Freudenberg & Galanos, 1978; Ohno & Morrison, 1989; Gioannini *et al.*, 2002; End *et al.*, 2009).

Saliva contains a number of anti-microbial effector molecules and flushes microorganisms from oral mucosa and tooth surfaces. In fact, xerostomia patients, who suffer from dry mouth due to a lack of saliva, are more prone to oral infections (Walker, 2004). On the other hand, pathogens often use certain saliva components to adhere, colonize, and invade the host (Scannapieco, 1994). Thus, it is crucial to identify salivary proteins with high affinity to bacterial virulence components such as LPS, for understanding bacterial pathogenesis and host innate immunity in the oral cavity. Despite the fact that *A. actinomycetemcomitans* is a pathogen associated with periodontitis and the initial recognition of Aa.LPS is expected to take place in the oral cavity, little is known about Aa.LPS-BPs in saliva. The aims of the present study were to isolate Aa.LPS-BPs from saliva using Aa.LPS-immobilized beads (Aa.LPS-beads) and to identify Aa.LPS-BPs by LTQ-Orbitrap hybrid Fourier transform mass spectrometry (MS).

METHODS

Reagents and chemicals

A. actinomycetemcomitans ATCC 43718 was obtained from the American Type Culture Collection (Manassas, VA, USA). Pam_2CSK_4, a synthetic TLR2-stimulating lipopeptide, was purchased from InvivoGen (San Diego, CA, USA). Brain heart infusion (BHI) media and tryptic soy broth (TSB) media were purchased from BD Biosciences (Franklin, NJ, USA). All other reagents including *N*-hydroxysuccinimidyl-Sepharose® 4 Fast Flow beads and human α-amylase from saliva were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted.

Purification of Aa.LPS

A. actinomycetemcomitans was aerobically cultured in BHI media at 37°C for 2 days. Bacteria were pelleted by centrifugation at 11,068 × *g* at 4°C for 10 min and the pellet was washed three times with phosphate-buffered saline (PBS). Aa.LPS was purified using the LPS Extraction Kit (iNtRON Biotechnology, Seongnam, Gyeonggi-do, Korea) according to the manufacturer's instructions. The amount of LPS was measured by weighing after lyophilization.

Immobilization of Aa.LPS onto N-Hydroxysuccinimidyl-Sepharose beads

N-Hydroxysuccinimidyl-Sepharose® 4 Fast Flow beads were washed with non-pyrogenic water, and 1000 mg of the beads were incubated with 6 mg of Aa.LPS with gentle agitation at 4°C for 4 h. The beads were then incubated with 0.5 M ethanolamine (pH 8.0) with gentle agitation at 4°C for 1 h to block the remaining conjugation sites. Finally, the beads were washed five times with non-pyrogenic water to remove unconjugated LPS. A *Limulus* amebocyte lysate (LAL) test kit (Cambrex Bio Science, Walkersville, MD, USA) was used to evaluate the conjugation of Aa.LPS to *N*-hydroxysuccinimidyl-Sepharose beads.

Measurement of TLR4-stimulating activity

The capability of Aa.LPS-beads to activate TLR4 was determined using an NF-κB reporter cell line CHO/CD14/TLR4. Notably, CD25 is expressed on the

surfaces of these cells in a TLR4 activation-dependent manner (Medvedev *et al.*, 2001). CHO/CD14/TLR4 cells were treated with Aa.LPS-beads (0, 0.01, 0.1, 1, or 10 mg ml^{-1}), native beads (10 mg ml^{-1}), Aa.LPS (0.1 or 1 μg ml^{-1}), or Pam_2CSK_4 (0.1 μg ml^{-1}) for 18 h. Then, CD25 expression was analyzed by flow cytometry using FACSCalibur with CellQuest software (BD Biosciences).

Measurement of the production of inflammatory mediators

The mouse macrophage cell line RAW 264.7 (TIB-71) was purchased from the American Type Culture Collection. The cells were grown in Dulbecco's Modified Eagle's Medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 37°C in a humidified incubator with 5% CO_2. RAW 264.7 cells at 1×10^6 cells ml^{-1} were incubated with various concentrations of Aa.LPS-beads (0, 0.001, 0.01, 0.1, 1 or 10 mg ml^{-1}), native beads (10 mg ml^{-1}), or Aa.LPS (0.01 or 0.1 μg ml^{-1}) for 24 h. After incubation, culture media were used to determine the production of nitric oxide (NO) and interferon gamma-inducible protein-10 (IP-10). Nitrite accumulation was determined as an indication of NO production in the culture media by mixing with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) followed by incubation for 5 min. The optical density was then measured at 540 nm using $NaNO_2$ as a standard with VersaMax microtiter-plate reader (Molecular Devices, Sunnyvale, CA, USA). IP-10 production was determined in the culture media using a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Collection of saliva samples

Use of human saliva samples was approved by the Institutional Review Board of the Seoul National University Dental Hospital (IRB No. CRI11008). To collect saliva, nine healthy subjects, who had fasted for at least 2 h, brushed their teeth without toothpaste. Then, 10 ml of saliva was obtained in a 50 ml conical tube that contained a Complete, Mini Protease Inhibitor Cocktail EDTA-free tablet (Roche, Mannheim, Germany). The saliva samples were centrifuged at 7000 × *g* at 4°C for 15 min to remove cells, debris, and insoluble materials, and the supernatant was stored at −80°C until further use.

Isolation of Aa.LPS-BPs

To eliminate non-specific binding proteins, 200 μl of each saliva sample was incubated with the native beads (100 mg) for 1 h at 4°C with gentle agitation. Then, the pre-cleared saliva was incubated with Aa.LPS-beads (100 mg) at 4°C with gentle agitation for 3 h, after which the beads were washed three times with PBS. Beads were incubated with 200 μl elution buffer (0.2 M glycine-HCl, pH 2.2) at 4°C for 10 min followed by addition of 30 μl of neutralizing buffer containing 1 M Tris(hydroxylmethyl)aminomethane-HCl, pH 9.1, to adjust the pH to 7.0. To remove salt for optimal peptide sequencing, each of the nine eluent samples was pooled and dialyzed (Spectra/Por6; Spectrum Laboratories, Inc., Rancho Dominquez, CA, USA) against 50 mM ammonium bicarbonate (pH 7.8). The dialyzed eluent was lyophilized and kept at −80°C until MS analysis.

Identification of Aa.LPS-BPs

The lyophilized eluents were solubilized in 50 mM NH_4HCO_3 with 10 mM dithiothreitol (pH 7.8) and incubated at 55°C for 30 min. Following incubation, free thiol groups were subjected to alkylation using 40 mM iodoacetamide in the dark at room temperature for 25 min. Tryptic digestion was performed by treating the samples with a buffer containing 50 mM ammonium bicarbonate, 5 mM $CaCl_2$, and 10 μg ml^{-1} trypsin at 37°C for 12–16 h, followed by lyophilization. After solubilization in 0.1% formic acid, the samples were loaded onto a C_{18} RP-packed microcapillary column (75 μm silica tubing with 8 μm inner diameter of the orifice, 10 cm in length). Buffers A (0.1% formic acid) and B (80% acetonitrile containing 0.1% formic acid) were prepared and peptides were eluted with 5% of buffer B for 25 min, 20% for 5 min, 60% for 50 min, and 100% for 5 min at a flow rate of 300 nl min^{-1}. Eluted peptides were identified by MS using a 7-Tesla Finnigan LTQ-FT MS spectrometer (Thermo Electron, Bremen, Germany) equipped with

a nano-ESI source (positive ion mode, 2.5 kV spray voltage). MS and tandem MS (MS/MS) spectra were obtained with the capillary at 220°C, 2.5 kV, 35% of collision energy, and 1 Da of isolation width. A full scan was performed at a resolution of a 100,000 full width at half maximum (FWHM) intensity, after which data-dependent MS/MS analyses were performed from the three most abundant MS ions. Each spectrum was analyzed with Mascot Daemon (Matrix Science, London, UK) using the International Protein Index (IPI) human database (IPI.HUMAN.v.3.72). Peptides were identified at the peptide tolerance of $\pm$ 50 ppm, fragment mass tolerance of $\pm$ 0.8 Da, two missed trypsin cleavage, oxidation of methionine, and fixed modification of carbamidomethyl cysteine. Peptide score was calculated by $-10\times$ Log(P), where P signified the probability of a random event. Proteins were identified as Aa.LPS-BPs only when more than two peptides were present at a score of 35 or higher, implying $P < 0.05$.

Pull-down assay

Native beads (40 mg) and Aa.LPS-beads (40 mg) were equilibrated with PBS and incubated with human salivary α-amylase (10 or 100 μg) for 3 h at 4°C with gentle agitation. After washing the beads three times with PBS, the bound materials on the bead were dissociated by boiling at 100°C for 15 min in the presence of 2% SDS, 10% glycerol, 0.1% bromophenol blue, 300 mM 2-mercaptoethanol, and 62.5 mM Tris-HCl (pH 6.8) followed by electrophoresis on 12% SDS-PAGE. Then, the gel was electro-transferred to a PVDF membrane (Milipore, Bedford, MA, USA) and the membrane was blocked with 5% skim milk in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.6) at room temperature for 1 h. After washing three times with TBST (50 mM Tris-HCl, 150 mM NaCl, 0.05% tween 20, pH 7.6) for 10 min at room temperature, the membrane was kept at 4°C for overnight with mouse anti-human α-amylase antibody (Abcam, Cambridge, MA, USA) in the blocking buffer. Subsequently, the membrane was washed three times with TBST for 10 min at room temperature, and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG polyclonal antibody (West Grove, PA, USA) in the blocking buffer at room temperature for 1 h. The immunoreactive bands were detected with ECL Western blotting reagents (Neuronex Co., Pohang, Korea).

Measurement of bacterial adherence and biofilm formation

For examination of bacterial adherence, *A. actinomycetemcomitans* was cultured in the presence of α-amylase (0, 125, or 500 μg ml^{-1}) or the inactivated α-amylase (500 μg ml^{-1}) on the Fine-View Dish (KYA Technologies Co., Tokyo, Japan) for 3 h at 37°C. Non-adherent bacteria were removed by washing with PBS and adherent bacteria were stained with SYTO-9 (green) and propidium iodide (red) to stain viable and dead bacteria, respectively. Then, the samples were subjected to confocal microscopy using LSM 510 confocal laser microscope (Carl Zeiss, Oberkochen, Germany). The ability of *A. actinomycetemcomitans* to form biofilm was measured with minor modification of microtiter plate assay as previously described (Fletcher, 1977). Briefly, *A. actinomycetemcomitans* was cultured to mid-log phase in TSB media supplemented with 0.5% yeast extract and 1% glucose and cultured for 8 h at 37°C. Then, the cultured *A. actinomycetemcomitans* was adjusted to 0.1 O.D. at 600 nm, mixed with various concentration of α-amylase (0, 100, 200, or 400 μg ml^{-1}) or inactivated α-amylase (400 μg ml^{-1}) in a 96 well plate, and cultured for additional 24 h at 37°C without agitation. Inactivated α-amylase were prepared by heating at 70°C for 1 h to inactivate its enzymatic activity. Non-adherent bacteria were removed by washing with PBS twice and adherent bacteria were stained with 0.1% crystal violet for 30 min. Then, the stained crystal violet was solubilized with 95% ethanol containing 0.1% acetic acid (v/v) for measurement of the absorbance at 595 nm.

Statistics

The mean values $\pm$ standard deviations were determined from triplicate samples. One-way ANOVA with Bonferroni's *post hoc* test was used to determine statistical significance. Differences between the experimental groups and non-treatment control group were considered statistically significant when $P < 0.05$. All experiments, except the mass spectrometric analysis, were performed at least three times. The mass

spectrometric analysis was independently performed twice under similar conditions.

RESULTS

Conjugation of Aa.LPS onto N-Hydroxysuccinimidyl-Sepharose beads

To confirm the Aa.LPS conjugated on *N*-hydroxysuccinimidyl-Sepharose® 4 Fast Flow beads, the quantity of Aa.LPS on the Aa.LPS-bead was measured by LAL test. The LAL test demonstrated that approximately 29.3 μg of Aa.LPS were conjugated to 100 mg of beads.

Aa.LPS-beads are able to stimulate TLR4

TLR4 is known to mediate the host innate immune response to Aa.LPS (Yoshimura *et al.*, 2002). CHO/CD14/TLR4 cells were treated with Aa.LPS-beads to confirm that Aa.LPS-beads retained the ability to stimulate TLR4. CD25 expression increased in a dose-dependent manner when the cells were stimulated with Aa. LPS-beads or Aa.LPS, but no induction was observed with native beads or Pam_2CSK_4 (a TLR2-specific ligand) (Fig. 1). These results suggest that the Aa.LPS conjugated to the beads retained the ability to activate TLR4.

Aa.LPS-beads are biologically active

Next, we examined whether Aa.LPS conjugated to beads retained the ability to induce the production of inflammatory mediators such as NO and IP-10. When RAW 264.7 cells were treated with various concentrations of Aa.LPS-beads, native beads, or Aa.LPS, Aa. LPS-beads and Aa.LPS, but not native beads, led to dose-dependent production of NO (Fig. 2A) and IP-10 (Fig. 2B). These results suggest that the Aa.LPS on Aa.LPS-beads retained the immunostimulating properties as free Aa.LPS.

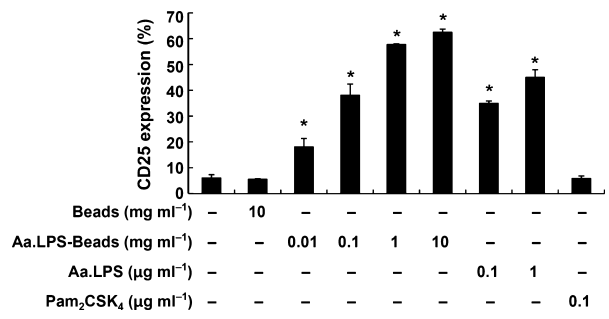

Figure 1 Aa.LPS-beads retained the TLR4-stimulating activity. CHO/CD14/TLR4 cells were treated with Aa.LPS-beads, native beads, Aa.LPS, or Pam_2CSK_4 for 18 h. Expression of CD25, a marker of TLR4-dependent NF-κB activation, was then analyzed by flow cytometry. Bars indicate the mean values ± standard deviations obtained from three independent experiments. Asterisk (*) signifies the statistical significance at $P < 0.05$ compared to the non-treatment control group.

Isolation and identification of Aa.LPS-BPs from human saliva

Prior to capturing Aa.LPS-BPs, each saliva sample from nine subjects was incubated with native beads to remove non-specific adsorption. After incubating the pre-cleared saliva samples with Aa.LPS-beads, the captured Aa.LPS-BPs were analyzed using LTQ-Orbitrap hybrid Fourier transform MS. Peptide identification revealed a total of 47 Aa.LPS-BPs (data not shown), of which 13 Aa.LPS-BPs with statistical significance ($P < 0.05$) are shown in Table 1. Among these Aa.LPS-BPs, 7 Aa.LPS-BPs are newly identified in the present experiment including α-amylase, cystatin, submaxillary gland androgen-regulated

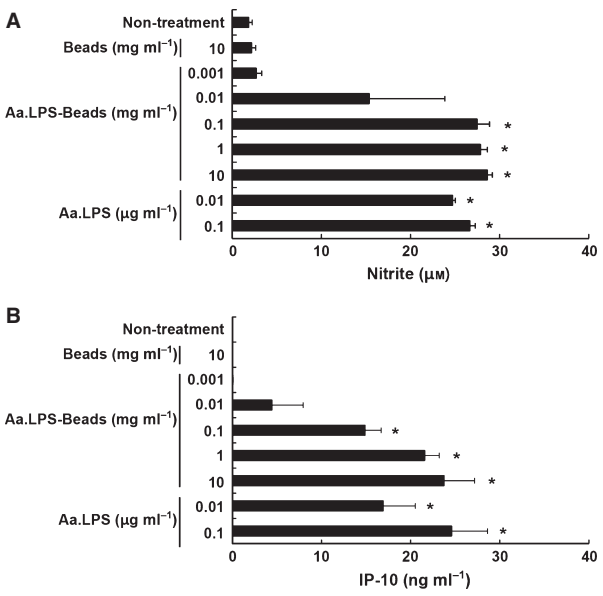

Figure 2 Aa.LPS-beads were biologically-active. Aa.LPS-beads, the native beads, or Aa.LPS were incubated with RAW 264.7 cells for 24 h. The culture media were then analyzed for (A) NO and (B) IP-10 production. Bars indicate mean values ± standard deviations. Asterisk (*) signifies the statistical significance at $P < 0.05$ compared to the non-treatment control group. The data represent one of three similar results.

Table 1 Aa.LPS-BPs identified through high-resolution LTQ-Orbitrap hybrid Fourier transform mass spectrometry

Protein description (gene symbol)	Accession Number	MASCOT Score	Peptide sequence	Peptide Score
Alpha-amylase (AMY1A; AMY1B; AMY1C; AMY2A)	IPI00300786; IPI00939512	1082	IYVSDDGK	37.6
			SGNEDEFR	60.5
			INGNCTGIK	42.4
			SSDYFGNGR	33.3
			LSGLLDLALGK	82.9
			TSIVHLFEWR	45.9
			WVDIALECER	62.9
			ALVFVDNHDNQR	78.8
			DVNDWVGPPNDNGVTK	90.1
			DFPAVPYSGWDFNDGK	78.7
			IAEYMNHLIDIGVAGFR	45.3
			AHFSISNSAEDPFIAIHAESK	66.0
			EVTINPDTTCGNDWVCEHR	84.5
			NVVDGQPFTNWYDNGSNQVAFGR	79.8
Isoform 1 of serum albumin (ALB)	IPI00745872	361	YLYEIAR	43.5
			DDNPNLPR	36.4
			FQNALLVR	45.2
			QTALVELVK	70.4
			LVAASQAALGL	92.7
			SLHTLFGDK	41.9
			KQTALVELVK	42.5
			LVNEVTEFAK	54.4
			FKDLGEENFK	51.5
			KVPQVSTPTLVEVSR	62.0
			QNCELFEQLGEYK	68.3
			VFDEFKPLVEEPQNLIK	55.2
Cystatin-S (CST4)	IPI00032294	263	QLCSFEIYEVPWEDR	45.0
			IIPGGIYDADLNDEWVQR	109.8
			SQPNLDTCAFHEQPELQK	79.0
Immunoglobulin heavy chain (IGHA2)	IPI00641229; IPI00423461; IPI00386879	258	YLTWASR	39.1
			WLQGSQELPR	61.4
			DASGVTFTWTPSSGK	93.1
			QEPSQGTTTFAVTSILR	121.2
			NFPPSQDASGDLYTTSSQLTLPATQCPDGK	37.0
Submaxillary gland androgen-regulated protein 3B (SMR3B)	IPI00023011	182	IPPPPPAPYGPGIFPPPPPQP	57.6
			GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR	57.2
Immunoglobulin light chain (IGLV2-14;IGLV2-8;IGLV1-40; IGLV3-21;IGLC3;IGLC1;IGLC6)	IPI00154742; IPI00939627; IPI00719373	165	SYSCQVTHEGSTVEK	80.5
			YAASSYLSLTPEQWK	34.3
			AAPSVTLFPPSSEELQANK	70.1
Polymeric immunoglobulin receptor (PIGR)	IPI00004573	114	ADEGWYWCGVK	58.7
			QSSGENCDVVVNTLGK	86.1
Deleted in malignant brain tumors 1 (DMBT1)	IPI00418512; IPI00900355	107	FGQGSGPIVLDDVR	97.0
			QLGCGWATSAPGNAR	41.1
Lipocalin-1 (LCN1)	IPI00009650	95	GLSTESILIPR	65.2
			NNLEALEDFEK	62.5
Prolactin-inducible protein (PIP)	IPI00022974	93	FYTIEILK	55.3
			ELGICPDDAAVIPIK	73.8
Lysozyme C (LYZ)	IPI00019038	91	STDYGIFQINSR	61.3
			TPGAVNACHLSCSALLQDNIADAVACAK	60.4
Basic salivary proline-rich protein 2 (PRB2)	IPI00552432	78	SQGPPPPGKPQGPPPQGGSK	54.8
			SPPGKPQGPPPQGGNQPQGPPPPPGKPQGPPPQGGNK	44.3
Immunoglobulin J chain (IGJ)	IPI00178926	57	FVYHLSDLCK	47.7
			CYTAVVPLVYGGETK	40.6

protein 3B, polymeric immunoglobulin receptor, prolactin-inducible protein (PIP), lipocalin-1, and basic salivary proline-rich protein 2 (Table 2).

Validation of Aa.LPS binding protein using pull-down assay

To validate whether the identified Aa.LPS-BPs interacts with Aa.LPS *in vitro*, pull-down assay using human α-amylase was performed since α-amylase was identified with the highest frequency. Human α-amylase strongly interacted with Aa.LPS-bead in a dose-dependent manner, whereas such interaction was not observed with native beads (Fig. 3).

Inhibitory effects of α-amylase on the adherence and biofilm formation of *A. actinomycetemcomitans*

Since LPS is known to be involved in the bacterial adherence and biofilm formation (Fujise *et al*., 2008), we next examined if α-amylase interfered with the bacterial adherence and biofilm formation. When *A. actinomycetemcomitans* was cultured in the presence of α-amylase (125 or 500 μg ml^{-1}) for 3 h, the bacterial adherence was substantially suppressed as determined by confocal microscopy (Fig. 4A). Similar trend was also observed at 1 h culture as quantitatively determined by a microtiter plate assay with crystal violet staining (Fig. 4B). Interestingly, heat-inactivated α-amylase also showed a suppressive effect to the same extent as intact α-amylase did, implying that the suppressive effect was not related with the enzymatic activity of α-amylase. In addition, when *A. actinomycetemcomitans* was treated with various concentration of α-amylase (0, 100, 200, or 400 μg ml^{-1}) or the inactivated α-amlyase (400 μg ml^{-1}) for 24 h, biofilm formation was also significantly decreased in a concentration-dependant manner (Fig. 4C). These results suggest that binding of α-amylase to LPS of *A. actinomycetemcomitans* interferes with the bacterial adherence and further biofilm formation.

Table 2 Classification and expected function of the identified Aa.LPS-BPs

State	Protein Name	Functions	Reference
Known LPS-BPs	Lysozyme	Hydrolyzes peptidoglycans; Reduces immunostimulatory activity of LPS	Takada *et al.* (1994)
	Immunoglobulin	Inhibits bacterial adhesion; Promotes bacterial clearance	Pudifin *et al.* (1985)
	Serum albumin	Binds to LPS; Transfers LPS to CD14 and LBP followed by cell activation	Rietschel *et al.* (1973); Gioannini *et al.* (2002)
	Deleted in malignant brain tumors 1	Receptor for cysteine-rich proteins, mediates adhesion and aggregation of bacteria; Functions as PRR for LPS or LTA to inhibit TLR-mediated NF-κB activation	Rosenstiel *et al.* (2007); End *et al.* (2009)
Unknown LPS-BPs	Alpha-amylase	Digests starch; Promotes adhesion of oral Streptococci to hydroxyapatite; Inhibits biofilm formation of Staphyloccous, Vibrio, Pseudomonas spp.	Scannapieco *et al.* (1993); Kalpana *et al.* (2012); Lequette *et al.*; Craigen *et al.* (2011)
	Cystatin-S	Inhibits cysteine protease; Binds to hydroxyapatite	Shomers *et al.* (1982)
	Basic salivary proline-rich protein 2	Binds to hydroxyapatite	Clark *et al.* (1989); Gillece-Castro *et al.* (1991); Ligtenberg *et al.* (1992)
	Prolactin-inducible protein	Binds to oral bacteria and is thought to inhibit bacteria colonization in oral cavity	Schenkels *et al.* (1997)
	Polymeric immunoglobulin receptor	Mediates transcellular transport of polymeric Immunoglobulin; Exhibits scavenger functions against bacteria to inhibit bacterial colonization	Norderhaug *et al.* (1999); de Oliveira *et al.* (2001)
	Lipocalin-1	Acts as lipid-binding protein for fatty acid, phospholipid, glycolipid, and cholesterol; Inhibits cysteine proteinase similar to cystatin; Exhibits antimicrobial activity by scavenging microbial siderophores	Flower (1996); Fluckinger *et al.* (2004)
	Submaxillary gland androgen-regulated protein 3B	Function unknown; May be related in promoting angiogenesis and establishing microvasculature	Guo *et al.* (2006)

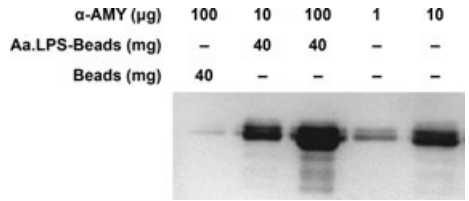

Figure 3 Interaction of α-amylase with Aa.LPS was confirmed using a pull-down analysis. The native beads (40 mg, lane 1) or Aa.LPS-beads (40 mg, lanes 2 and 3) were incubated with human α-amylase (α-AMY; 10 or 100 μg) for 3 h at 4°C. After washing with PBS, the interaction between Aa.LPS and α-amylase was analyzed by Western blotting with anti-human α-amylase antibody. Human α-amylase (1 or 10 μg, lanes 4 and 5) were used as positive control. One of the three similar results is shown.

DISCUSSION

This study demonstrated human saliva screened for Aa.LPS-BPs by using interaction of Aa.LPS-beads and Aa.LPS-BPs followed by a high-resolution mass spectrometry. To note, we were able to identify potential Aa.LPS-BPs from human saliva samples without time-consuming or laborious conventional assays. This method offers the following advantages: (i) an easy, simple, fast, and convenient immobilization process; (ii) Aa.LPS firmly and irreversibly adheres to the beads through covalent bonds; (iii) biological properties of Aa.LPS are not altered during the conjugation process; (iv) Aa.LPS-BPs are selectively isolated using Aa.LPS-beads; and (v) high-throughput identification, even in minute quantities, is possible by high-resolution mass spectrometry. This method is therefore a potentially-useful tool for capturing and identifying proteins that bind to virulence factors such as LPS, due to its simple, convenient, sensitive, accurate, and high-throughput characteristics. Most Aa.LPS-BPs identified in this study are related with bacterial adhesion and colonization, anti-microbial function, or modulation of immune responses.

Aa.LPS-BPs that are likely to be involved in bacterial adhesion and colonization in the host include cystatins, basic proline-rich proteins and PIP. Cystatins are known to inhibit cysteine proteases, which degrade immunoglobulins and type I collagen, while salivary cystatins bind to the hydroxyapatite present in dental enamel (Shomers *et al.*, 1982). Basic proline-rich proteins comprise approximately 23% of saliva proteins and bind oral bacteria, *e.g. A. viscous* (Clark *et al.*, 1989), *F. nucleatum* (Gillece-Castro *et al.*, 1991), and *S. gordonii* (Ligtenberg *et al.*, 1992) as well as hydroxyapatite. Furthermore, the surface of various oral bacterial strains such as *Gemella haemolysans*, *Gemella morbillorium*, *Streptococcus acidominimus*, *Streptococcus oralis*, *Streptococcus salivarius*, and *Streptococcus parasanguinis* binds to salivary PIPs (Schenkels *et al.*, 1997).

Anti-microbial proteins include lysozyme C, immunoglobulins, and polymeric immunoglobulin receptors. Lysozymes hydrolyze peptidoglycans and disrupt bacterial cell walls, but they can also bind to LPS (Ohno & Morrison, 1989), which results in the reduction of immunostimulatory activity (Takada *et al.*, 1994) probably due to a conformational change in LPS (Brandenburg *et al.*, 1998). LPS-binding immunoglobulins (*i.e.,* anti-LPS-antibodies) are involved in protection against infection by Gram-negative bacteria by inhibiting bacterial colonization and adhesion to host cells, and by promoting clearance of bacteria through enhanced opsonophagocytosis (Pudifin *et al.*, 1985). Mice deficient in anti-LPS antibodies are more prone to septic shock upon bacterial infection than wild type mice (Reid *et al.*, 1997). The polymeric immunoglobulin receptors contribute to innate antimicrobial defense due to its primary functions for mediating transcytosis of secretory immunoglobulin A (IgA) across the epithelial monolayer and protecting IgA from proteolytic degradation (Norderhaug *et al.*, 1999). Polymeric immunoglobulin receptors also inhibit bacterial adhesion to epithelial cells through its scavenger properties against bacterial components including fimbrial lectin from *E. coli* (de Oliveira *et al.*, 2001).

Proteins involved in the modulation of immune responses include serum albumin, DMBT1, and lipocalin-1. LPS of Gram-negative bacteria (Rietschel *et al.*, 1973) and lipooligosaccharide (LOS) of *Neisseria meningitidis* (Gioannini *et al.*, 2002) are known to bind to serum albumin. Serum albumin seems to facilitate, rather than inhibit, immune cell stimulation by assisting the transfer of LPS or LOS to CD14 and TLR4/MD2 complex (Gioannini *et al.*, 2002). DMBT1 belongs to a group of scavenger receptor cysteine-rich proteins and appears to be involved in the innate immune response due to its ability to interact with a broad range of Gram-positive and Gram-negative bacteria and its inhibitory effects on LPS-induced inflammation (Rosenstiel *et al.*, 2007). Recently, DMBT1 was reported to act as a pattern recognition

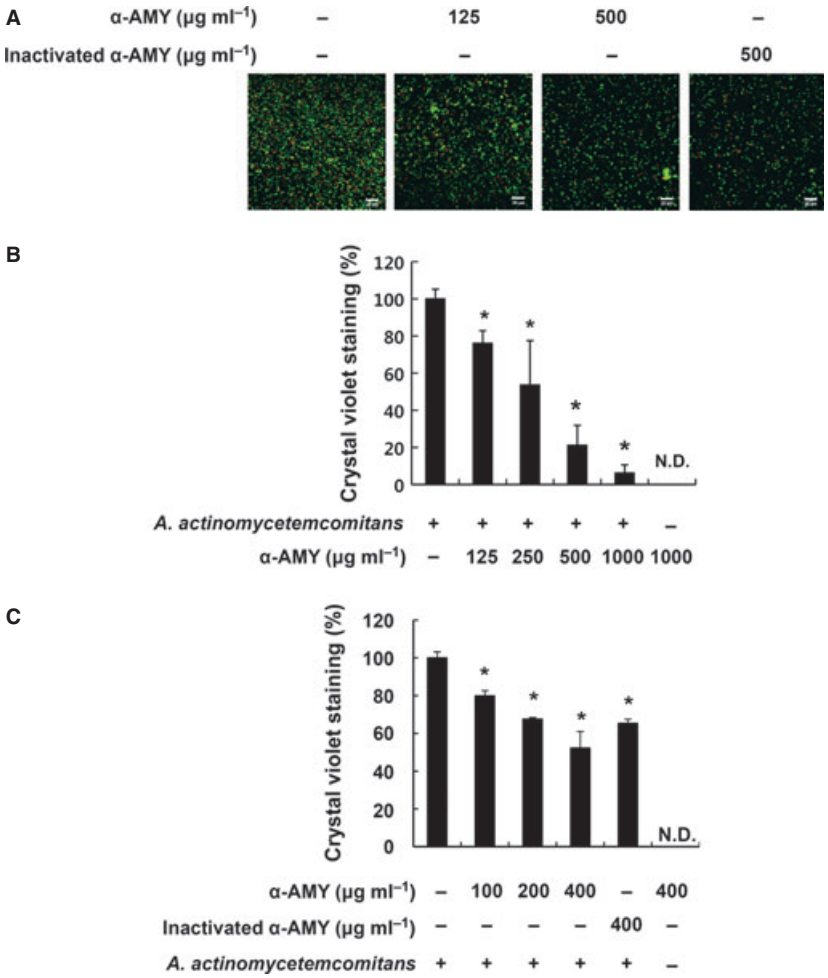

Figure 4 Alpha-amylase interfered with the adherence and biofilm formation of *Aggregatibacter actinomycetemcomitans*. (A) *A. actinomycetemcomitans* was cultured in the presence of α-amylase (α-AMY; 0, 125, or 500 μg ml^{-1}) or heat-inactivated α-amylase (Inactivated α-AMY; 500 μg ml^{-1}) on the Fine-View Dish for 3 h at 37°C without agitation. After removing non-adherence cells by washing with PBS, the adherent cells were stained with SYTO-9 (green) and propidium iodide (red) to indicate live and dead cells, respectively. The images were obtained through confocal microscopy. One of the three similar results is shown. Scale bar indicates 20 μm. (B, C) *A. actinomycetemcomitans* was cultured in the presence of α-amylase (α-AMY; 0 – 1000 μg ml^{-1}) or heat-inactivated α-amylase (Inactivated α-AMY; 400 μg ml^{-1}) onto a 96-well plate for 1 h (B) or 24 h (C) at 37°C without agitation. After washing with PBS, the adherent cells were stained with crystal violet to determine biofilm formation. Bars indicate the mean values ± standard deviations obtained from three independent experiments. Asterisk (*) signifies the statistical significance at $P < 0.05$ compared to the non-treatment control group. N.D. means not detected (under detection limit).

receptor for poly-sulfated or poly-phosphorylated ligands such as LPS and lipoteichoic acid (End *et al.*, 2009). Lipocalin possesses diverse functions including transporting hydrophobic molecules, mediating prostaglandin D synthesis, and immune modulation (Flower, 1996). Various lipophilic ligands including fatty acids, phospholipids, glycolipids, cholesterols, retinols, and arachidonic acids have been shown to bind to lipocalin-1 (Flower, 1996). Notably, lipocalin-1 is often found in various mucosal tissues including eyes, nasal mucosa, and tracheal mucosa (Redl, 2000). Despite acting as the principal lipid binding protein, lipocalin-1 is also involved in protection against bacterial infection due to its ability to inhibit bacterial growth by scavenging microbial siderophores (Fluckinger *et al.*, 2004).

Our experiments using the MS analysis and pull-down assay demonstrated that α-amylase is indeed a major Aa.LPS-BP. To the best of our knowledge, this is the first report proving the ability of α-amylase to

bind LPS. Furthermore, the functional assays showed that α-amylase interfered with bacterial adherence and biofilm formation. These results are coincident with previous studies that α-amylase could inhibit the biofilm formation by other pathogens such as *Staphylococcus aureus*, *Vibrio cholerae*, and *Pseudomonas aeruginosa* (Lequette *et al.*, 2010; Craigen *et al.*, 2011; Kalpana *et al.*, 2012). In the view of the fact that high concentration of α-amylase (0.1–2.6 mg ml^{-1}) remains in human saliva (Scannapieco *et al.*, 1993), α-amylase seems to be an important defense molecule essential for the innate immunity in the oral cavity.

The profiles of Aa.LPS-BPs identified from this study is similar to those of salivary *P. gingivalis* LPS-binding proteins (Pg.LPS-BPs), which were identified from our previous study including α-amylase, serum albumin, cystatins, immunoglobulin, PIP, lipocalin-1 and lysozyme (Choi *et al.*, 2011). This similarity between Aa.LPS-BPs and Pg.LPS-BPs might be explained with a broad spectrum of host to recognize pathogen with the molecular pattern. Besides, some salivary proteins seem to differently recognize the LPS with its structural difference between species or strains and it might make a distinct profile of Aa. LPS-BPs from that of Pg.LPS-BPs.

In this study, we used the smooth colony phenotype *A. actinomycetemcomitans* Y4 strain to examine the inhibitory effect of α-amylase on biofilm formation because the LPS immobilized onto the beads was purified from the smooth strain. Previous studies have reported that the clinical isolates of the *A. actinomycetemcomitans* have a rough colony phenotype (Scannapieco *et al.*, 1987; Rosan *et al.*, 1988) and the rough to smooth transition takes place at the culture of rough colony type strain in high nutrient media (Amarasinghe *et al.*, 2009). Notably, the rough strain produces approximately 1.2–4.8 times more biofilm than the smooth strain although both strains form tenacious biofilm (Haase *et al.*, 2006). Among the biofilm determinants of *A. actinomycetemcomitans*, fimbriae and extracellular polymeric substance seem to be important in the biofilm formation since their expression was higher in the rough strain than in the smooth strain (Amarasinghe *et al.*, 2009). No difference has been reported in the LPS expression between the strains (Amarasinghe *et al.*, 2009). Further studies using a rough colony type strain are required to examine the inhibitory effects of α-amylase on biofilm formation in clinical situations.

Despite the close association between *A. actinomycetemcomitans* and periodontitis, understanding of the bacterial pathogenesis and innate immunity has been hampered due to a lack of information on host recognition of the major etiological virulence factor, LPS, in the oral cavity. Here we identified several Aa. LPS-BPs that could provide important clues towards a more comprehensive understanding of bacterial infection and immunity in the oral cavity, and for the development of preventive medicine and advancement of improved treatments.

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