Proteomic Analysis of Two Types of Exosomes in Human Whole Saliva

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Saliva contains a large number of proteins that participate in the protection of oral tissue. Exosomes are small vesicles (30—100 nm in diameter) with an endosome-derived limiting membrane that are secreted by a diverse range of cell types. We have recently demonstrated that exosomes are present in human whole saliva. In this study, we found that whole saliva contained at least two types of exosomes (exosome I and exosome II) that are different in size and protein composition. Proteomic analysis revealed that both types of exosomes contained Alix, Tsg101 and Hsp70, all exosomal markers, immunoglobulin A and polymeric immunoglobulin receptor, whereas they had different protein compositions. Most of dipeptidyl peptidase IV known as CD26 in whole saliva, was present on the exosome II and metabolically active in cleaving chemokines (CXCL11 and CXCL12). Human whole saliva exosomes might participate in the catabolism of bioactive peptides and play a regulatory role in local immune defense in the oral cavity.

Key words human whole saliva; exosome; proteome; dipeptidyl peptidase IV

Human whole saliva is an aqueous complex mixture of proteins and minerals.[1)] It contributes to maintaining the oral cavity integrity through its lubricating, antibacterial, antiviral and buffering actions, and facilitates chewing and swallowing food. It is derived from major and minor salivary glands and also from gingival crevicular fluid. The three pairs of major salivary glands are the parotid, submandibular and sublingual glands. Human whole saliva contains a number of proteins, including alpha-amylase, mucins, histatins, cystatins and proline-rich proteins. The protein composition of saliva has been of interest for many years, and knowledge has advanced, especially during the past decade, with the development of new and powerful proteomics techniques for protein separation and identification.[2,3)]

Exosomes are small (30—100 nm) membrane vesicles of endocytic origin that are released into the extracellular environment on fusion of multivesicular bodies with the plasma membrane.[4—6)] During this process, certain cytosolic proteins are incorporated into the invaginating membranes, engulfed and enclosed in these vesicles, thereby maintaining the same topological orientation as at the plasma membrane.[7)] It has been recognized that exosomes are involved in a novel mechanism of protein release. Exosome production has been observed in a variety of cell types *in vitro*, including reticulocytes,[8)] cytotoxic T lymphocytes,[9)] B lymphocytes,[10)] dendritic cells,[11)] and neoplastic intestinal epithelial cells.[12)] Recent studies have reported that such vesicles are present in some physiological fluids, such as bronchoalveolar lavage[13)] and urine.[14)] More recently, it has been demonstrated that exosomes are released into saliva.[15,16)] While the biological functions of exosomes are still unclear, they can mediate expulsion of obsolete membrane constituents, communication between cells, facilitating processes such as antigen presentation and in *trans* signaling to neighboring cells. Recently, it was reported that exosomes contain both mRNA and microRNA (miRNA), which can be transferred to another cell, and be functional in that new environment.[17)] There is growing interest in using whole saliva as a diagnostic fluid because of the relatively simple and non-invasive collection procedures used to harvest it. Recent studies show that exosomes are attractive biomarker candidates for diseases such as cancer.[18—20)]

In a previous study, we have isolated exosomes from human whole saliva through gel-filtration, and found that dipeptidyl peptidase IV (DPP IV), actin, polymeric immunoglobulin receptor (pIgR), immunoglobulin A (IgA) and galectin-3 were associated with exosomes.[15)] However, detailed analysis of the protein composition was not performed. In addition, exosomes isolated from human whole saliva were heterogeneous in size. It prompted us to study whether they are simple mixtures of different sizes of vesicles that have the same characteristics, or a mixture of vesicles of different characteristics. In the present study, we have isolated two types of exosomes according to size from human whole saliva and performed proteomic analysis of the isolated exosomes.

MATERIALS AND METHODS

Materials Gly-Pro-4-methyl-coumaryl-7-amide (Gly-Pro-MCA) was from Peptide Institute Inc. (Osaka, Japan). Sephacryl S-500 HR, Immobiline DryStrip gel and Pharmalyte were purchased from GE Healthcare U.K. Ltd. (Buckinghamshire, U.K.). α-Cyano-4-hydroxycinnamic acid (4-CHCA) was purchased from Shimadzu GLC (Tokyo, Japan). CXCL11 and CXCL12 were from R&D Systems, Inc. (Minnesota, U.S.A.). All other reagents were of the highest quality available. DPP IV inhibitor ER319711-15 was kindly provided from Eisai Co., Ltd. (Ibaraki, Japan).

Gel Filtration of Human Whole Saliva Whole saliva

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exosomes were purified as previously described with a slight modification.[15] Briefly, human whole saliva was collected from a single healthy female volunteer (37 years old) in our laboratory with informed consent. A volume of 35 ml of whole saliva was added to an equal volume of Tris-buffered saline (20 mM Tris–HCl, pH 7.4 and 150 mM NaCl). Then, the sample was centrifuged at 10000×*g* at room temperature for 5 min to precipitate bacteria and food debris. The supernatant was filtered through a 5.0 μm cellulose acetate filter and the filtrate was concentrated to approximately 1 ml using an Amicon Ultra-15 centrifugal filter device (Millipore Corporation, Massachusetts, U.S.A.) with a 100-kDa exclusion. The concentrated filtrate was subjected to gel-filtration on a Sephacryl S-500 column (1.5×50 cm) equilibrated with Tris-buffered saline. Void fractions (Fr. I) and the following fractions displaying DPP IV activity (Fr. II) were collected, and concentrated approximately 100 fold using a Amicon Ultra-4 with a 100-kDa exclusion. These concentrated solutions were used for further characterization.

DPP IV Activity Assay DPP IV activity was assayed as previously described.[15] In brief, the assay mixture contained 50 μl of 0.4 mM Gly-Pro-MCA, 100 μl of 100 mM Tris–HCl buffer (pH 8.5) and 50 μl of enzyme solution. After incubation for 20 min at 37 °C, 2.8 ml of 1 M sodium acetate (pH 4.2) was added to terminate the reaction. Fluorescence intensity of liberated 7-amino-4-methyl-coumarin was measured at 460 nm with excitation at 380 nm. Protein concentration was determined using the BCA reagent (Pierce, U.S.A.).

Electron Microscopy Immunoelectron microscopy was performed as described previously.[15] The concentrated solution of exosome fractions prepared as described above was mixed 1 : 1 with 4% paraformaldehyde in phosphate-buffer (pH 7.2) and then applied to 200-mesh Formvar-carbon-coated nickel grids. The grid was stained with 2% uranyl acetate, pH 7, and embedded with 2% methylcellulose/0.4% uranyl acetate, pH 4. After drying, the grids were examined with a transmission electron microscope (TEM-1010; JEOL, Tokyo, Japan).

Western Blot Analysis The proteins in the exosome fractions prepared as described above were separated on a SuperSep HG, 5—20% gradient gel (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and transferred to a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer method (Bio-Rad Laboratories, Inc., California, U.S.A.). Nonspecific binding sites were blocked by incubating the membrane in 100 mM Tris–HCl (pH 7.4)/150 mM NaCl, containing 3% skim milk and 1% Tween 20. Blots were incubated overnight at 4 °C with goat anti-Alix polyclonal antibody (Santa Cruz Biotechnology, Inc., California, U.S.A.), goat anti-tumor susceptibility gene 101 (Tsg101) polyclonal antibody (Santa Cruz Biotechnology, Inc.), mouse anti-heat shock protein 70 (Hsp70) monoclonal antibody (Bioscience Inc., Victoria, Canada), rabbit anti-CD63 antigen polyclonal antibody (Santa Cruz Biotechnology, Inc.), mouse anti-actin monoclonal antibody (Chemicon, Temecula, California, U.S.A.), rabbit anti-galectin-3 polyclonal antibody (Santa Cruz Biotechnology, Inc.), goat anti-DPP IV antibody (R&D Systems, Inc., Minnesota, U.S.A.), goat anti-GW182 polyclonal antibody (Santa Cruz Biotechnology, Inc.), goat anti-Ig alpha chain antibody (Bethyl Laboratories, Inc., Texas, U.S.A.), goat anti-Ig kappa chain antibody (Invitrogen Corporation), goat anti-Ig lambda chain antibody (Invitrogen Corporation), goat anti-Ig J chain antibody (Santa Cruz Biotechnology, Inc.), goat anti-Ig gamma chain antibody (Invitrogen Corporation), or goat anti-Ig mu chain antibody (Invitrogen Corporation). These antibodies were detected using horse-radish peroxidase (HRP)-conjugated secondary antibodies and visualized using enhanced chemiluminescence (ECL or ECL Plus, GE Healthcare Bio-Science Corp., U.S.A.).

Two Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE) Protein extraction and 2-D PAGE were performed as previously reported[21] (see also http://proteome.tmig.or.jp/2D/2DE_method.html), with slight modifications. Briefly, the concentrated fractions prepared as described above were supplemented with three volumes of the extraction buffer (5 M urea, 2 M thiourea, 0.2% CHAPS, 2% sulfobetaine10, 65 mM DL-dithiothreltol, and 2% Pharmalyte 3—10). The fractions were subsequently disrupted by sonication (20 pulses), and debris was removed by centrifugation (10000×*g*, 5 min). The supernatants were concentrated to approximately 50 μl using a Microcon Ultracel YM-3 centrifugal filter device (Millipore Corporation, Massachusetts, U.S.A.) with a 30-kDa exclusion. The protein in each of the 40 μl aliquots of the extract (*ca.* 40—80 μg protein) was loaded onto reswollen gel strips with immobilized pH gradient (IPG) (pH 3—10, 3—5.6 and 7—11, 18 cm long), and then isoelectronic focusing (IEF) was performed using a CoolPhoreStar IPG-IEF Type P (Anatech, Tokyo, Japan). After completion of IEF, the strips were sodium dodecyl sulfate (SDS) equilibrated, reduced and alkylated with dithiothreitol (DTT) and iodoacetamide. Next, SDS-PAGE (7.5% acrylamide concentration) was performed using a CoolPhoreStar SDS-PAGE Dual-200 (Anatech). After separation on 2-D PAGE, the protein spots on the gel were fixed in 50% methanol and 10% acetic acid for 30 min and stained with SYPRO Ruby protein gel stain (Invitrogen Corporation) for detection of proteins. The gel images were obtained by scanning with a Molecular Imager FX Pro (Bio-Rad Laboratories, California, U.S.A.). Quantitative analysis of proteins on gel images was performed by JustTLC software (Sweday, Lund, Sweden).

In-Gel Protein Digestion and Mass Spectrometry In-gel digestion on selected gel spots of 2-D PAGE was also performed according to the protocol listed on the URL described above. Each protein spot was subsequently excised using a FluoroPhoreStar 3000 (Anatech, Tokyo, Japan). A piece of gel from each protein spot was decolored, and decomposed into several peptides by the addition of a digestion reagent containing 10 μg/ml sequencing-grade modified trypsin (Promega, Wisconsin, U.S.A.), 50 mM ammonium bicarbonate, and 30% acetonitrile (ACN) and incubated at 30 °C, overnight. The peptides produced from in-gel digestion were directly mixed with matrix solution (5 mg/ml CHCA in 50% ACN/0.1% trifluoroacetic acid (TFA)) and applied to the target (Sample Plate 384; Shimadzu Biotech, Kyoto, Japan). The samples were analyzed by matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF-MS) using an Axima CFRplus instrument (SHIMADZU, Corp., Kyoto, Japan) in positive linear mode with 20 kV acceleration voltage. Proteins were identified with the Mascot search engine (Matrix Science, London,

U.K.; see the URL, http://www.matrixscience.com/) searching algorithms by using the Swiss-Prot protein database or NCBInr database. Mascot search parameters are shown as follows: type of search, peptide mass fingerprinting (PMF); enzyme, trypsin; max missed cleavage, 2; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); peptide mass tolerance, ±0.4 Da; mass values, MH^+; monoisotopic. When the same protein was detected from at least two gels, the Mascot search result was considered to be correct. Alternatively, digested peptides were analyzed by LC/MS/MS.

LC/MS/MS Analysis The tryptic digests were loaded onto a L-column ODS trapping column (Chemicals Evaluation and Research Institute, Tokyo, Japan). After a wash with 15 μl of 0.1% TFA, the trapping column was switched into line with the column. HPLC was performed on a Paradigm MS 4 (Michrome Biosources, Auburn, CA, U.S.A.) equipped with a C18 L-column (0.3×150 mm) at a flow rate 0.5 μl/min. The eluents consisted of water containing 2% (v/v) ACN and 0.1% (v/v) formic acid (pump A) and 90% ACN and 0.1% formic acid (pump B). Samples were eluted with 5% of B for 2 min followed by a linear gradient from 5 to 80% of pump B in 20 min.

Mass spectrometric analyses were performed using a quadrupole time-of-flight (Q-TOF) mass spectrometer QSTAR elite (Applied Biosystems, California, U.S.A.) equipped with a nano-electrospray ion source. The mass spectrometer was operated in the positive ion mode. The nanospray voltage was set at 2400 V. Mass spectra were acquired at *m*/*z* 400—2100 for MS analysis and at *m*/*z* 100—2100 for MS/MS analysis. After every regular MS acquisition, two MS/MS acquisitions against the two top multiply charged molecular ions were performed (data-dependent acquisition). The precursor ions with the same *m*/*z* as acquired previously were excluded for 60 s. The collision energy was varied depending on the size and charge of the molecular ion. All peaks were resolved monoisotopically. Tandem MS/MS data from LC/MS/MS runs were submitted to the Mascot search engine (Matrix Science, London, U.K.; see the URL, http://www.matrixscience.com/) searching algorithms by using the Swiss-Prot protein database or NCBInr database. One missed cleavage was allowed, and tolerances of 2.0 and 0.8 u mass were used for precursor and product ions, respectively. When the same protein was detected from at least two gels, the Mascot search result was considered to be correct.

Shotgun Analysis Shotgun analysis of the each exosome was performed as previously reported[22] with slight modifications. Proteins (100 μg) of the concentrated solutions from Fr. I or Fr. II were dissolved in 50 μl of 0.5 M Tris–HCl buffer (pH 8.6) that contained 8 M guanidine hydrochloride and 5 mM ethylenediaminetetraacetic acid (EDTA). After the addition of 2 μl of 1 M DTT, the mixture was incubated for 30 min at 65 °C. Then, 4.8 μl of 1 M sodium iodoacetate was added, and the resulting mixture was incubated for 40 min at room temperature in the dark. The reaction mixture was applied to a PD-10 column (GE Healthcare U.K. Ltd., Buckinghamshire, U.K.) to remove the reagents, and the eluate was lyophilized. The reduced and carboxymethylated samples were redissolved in 100 μl of 0.1 M Tris–HCl buffer (pH 8.0). An aliquot of 2 μl of 1 μg/μl sequencing-grade modified trypsin was added, and then the mixtures were incubated for 16 h at 37 °C. The enzyme digestion was stopped by boiling for 10 min and stored at −20 °C before analysis. Tryptic digests of the samples were analyzed by LC/MS/MS as described above except that a gradient condition of 5 to 80% of solvent from pump B over 60 min was employed. The MS/MS spectra were searched against the Swiss-Prot protein database using the Paragon algorithm in Protein Pilot software 2.0 (Applied Biosystems, California, U.S.A.). The experiment was repeated at least four times. Protein with a probability of >0.99 were reported.

Cleavage of Chemokines by Exosome CXCL11 or CXCL12 (at a final concentration of 10 μM for each peptide) were incubated with exosome II (1 μg of total protein) from human saliva for 20 or 120 min at 37 °C in a total volume of 0.2 ml of 100 mM Tris–HCl buffer (pH 7.4). The reactions were quenched by adding TFA to a final concentration of 0.1%. Using $ZipTip_{C18}$ tips (Millipore, Bedford, MA, U.S.A.), a 20 μl aliquot of the digested mixture was desalted, concentrated and then eluted with 1 μl of matrix solution (5 mg/ml CHCA in 50% ACN/0.1% TFA) and spotted onto a sample plate. Samples were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on an Axima CFRplus instrument in positive linear mode with 20 kV acceleration voltage.

RESULTS

Isolation and Characterization of Exosomes from Whole Saliva Salivary protein isoforms, which are linked to gene duplication, differential mRNA splicing, and post-translational modifications, such as proteolysis, phosphorylation and glycosylation, are expected to vary across the population.[23] Thus, repeated collection of salivary samples were obtained and pooled from only one donor. In a previous study, we have isolated human saliva exosome in the void fractions using Sephacryl S-300 gel column chromatography. In the present study, we fractionated human whole saliva using gel-filtration on a Sephacryl S-500 column with a mean exclusion size of 200 nm, which is suitable for fractionating macromolecules (Fig. 1). Two small protein peaks were observed in fraction number 28 and 38. The peak of DPP IV activity matched fraction number 38, so we collected fraction number 25—30 and 34—45 as pooled fraction Fr. I

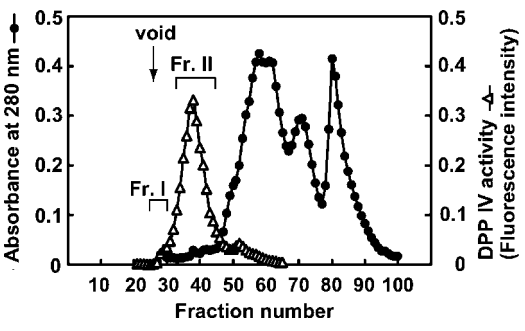

Fig. 1. Gel Filtration Chromatography of Human Whole Saliva Was Performed on a Sephacryl S-500 Column

Dipeptidyl peptidase IV activity (open triangles) was measured as described in Materials and Methods. Fraction number 25—30 and fraction number 34—45 were pooled as Fr. I and Fr. II, respectively.

and Fr. II, respectively. Fr. I corresponded to the void fraction. Then, Fr. I and Fr. II were concentrated and observed by electron microscopy (Fig. 2). Vesicles from Fr. I had a relatively large size and high electronic density. They were close to round-shape or slightly elongated. Size distribution of the vesicles varied from 30 to 250 nm with a mean diameter of 83.5 nm (SE=2.5 nm) (Fig. 3). In contrast, vesicles from Fr. II had uniform size and shape similar to those of exosomes reported from other sources.[18] Size distribution of the vesicles varied 20 to 80 nm with a mean diameter of 40.5 nm (SE=0.4 nm) (Fig. 3).

1-Dimensional (1-D) PAGE analysis revealed that the vesicular protein patterns of Fr. I and Fr. II were different from each other and from that of whole saliva (Fig. 4A). Bands higher than approximately 75 kDa were enriched in both of the vesicles. Western blot analysis showed that both vesicles were positive for exosomal markers such as Alix, Tsg101, Hsp70 and CD63, suggesting that the both vesicles are exosomes (Fig. 4B). We designated the vesicles from Fr. I and Fr. II as exosome I and exosome II, respectively. In a previous study, we have found DPP IV and galectin-3 to be present in exosomes from human whole saliva.[15] DPP IV was detected with much higher intensity in exosome II than in exosome I by western blot analysis, which was consistent with the result observed on gel-exclusion column chromatography on Sephacryl S-500 (Fig. 4B). Galectin-3 was detected in both exosome I and exosome II. In addition, GW182, which is required for miRNA function, was detected in exosome II with higher intensity than in exosome I.

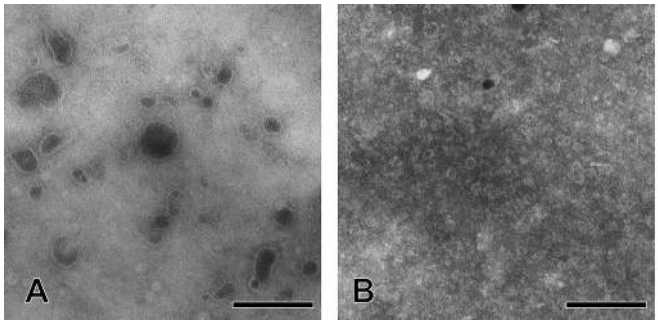

Fig. 2. Electron Microscopy of Exosome I (A) and Exosome II (B) from Human Whole Saliva

Scale bar=200 nm.

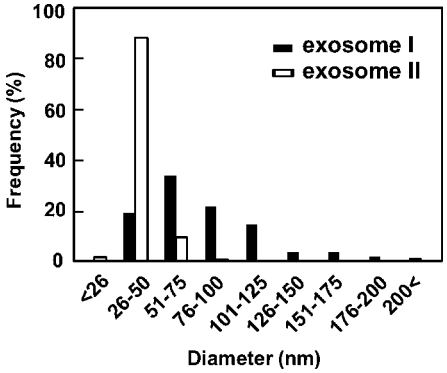

Fig. 3. Frequency Distribution of Exosome I and Exosome II by Diameter

A total of 240 exosome I and 480 exosome II were measured.

Identification of Proteins from Whole Saliva Exosomes We performed proteomic analysis of exosome I and exosome II using 2-D PAGE from pH 3 to 10. To identify acidic and basic proteins, we also used 2-D PAGE with different pH ranges (pH 3—5.6, 7—11, data not shown). Figure 5 shows representative 2-D PAGE patterns using a focusing range of pH 3—10. To identify proteins in selected gel spots, a peptide mass fingerprinting method (PMF) was carried out using MALDI-TOF-MS. When identification by MALDI-TOF-MS was not successful, further attempts were made by peptide sequencing using LC/MS/MS. The proteins successfully identified are listed in Table 1. A total of 47 and 51 proteins were identified from exosome I and exosome II, respectively, from 2-D PAGE gel spots. In both exosome I and exosome II, IgA (Ig alpha-1 chain, Ig alpha-2 chain, Ig lambda chain, Ig kappa chain, and Ig J chain) and pIgR were common major proteins. By quantitative analysis of 2-D PAGE images, Ig alpha chains and pIgR accounted for approximately 35% and 14% of total proteins in exosome I, and 20% and 9% in exosome II, respectively. Since immunoglobulins were detected from many spots in a broad area in 2-D PAGE, immunodetection was also used to identify the area containing each immunoglobulin such as Ig alpha-1 and alpha-2 chain, Ig mu chain, Ig gamma chain, Ig kappa chain, Ig lambda chain and Ig J chain (data not shown). Meanwhile, the 2-D PAGE of exosome I and exosome II showed different pat-

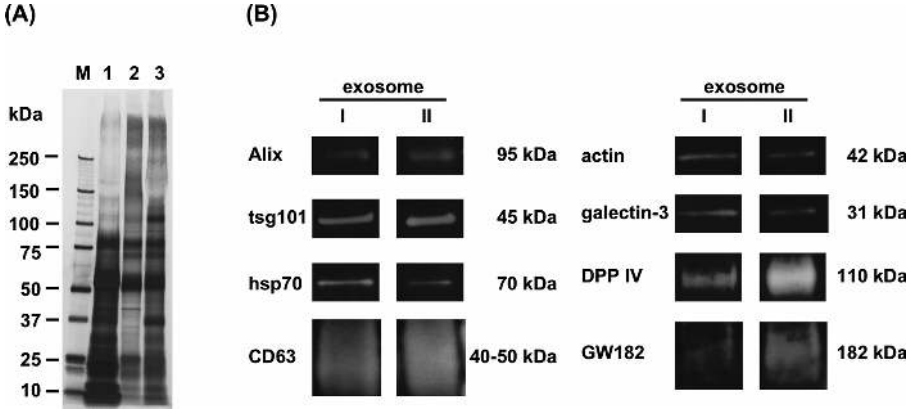

Fig. 4. 1-D PAGE and Western Blot Analysis of Exosome I and Exosome II from Human Whole Saliva

(A) SDS-PAGE patterns of whole saliva (lane 1), exosome I (lane 2) and exosome II (lane 3). Lane M, molecular weight markers. The gels were silver-stained. (B) Proteins (5 μg) from exosome I and exosome II were separated on 5—20% SDS-PAGE gel and analyzed by western blotting using antibodies against either exosomal markers (Alix, Tsg101, Hsp70, CD63), actin, galectin-3, DPP IV or GW182.

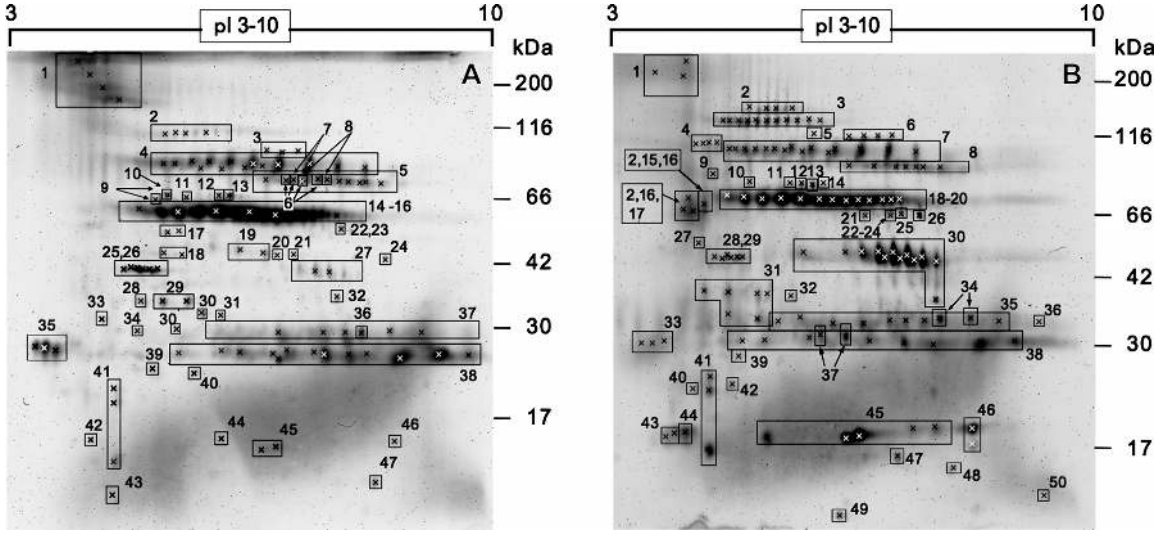

Fig. 5. Two-Dimensional PAGE Patterns of Exosome I (A) and Exosome II (B)

Proteins were separated in the first dimension on a 18-cm IPG strip (pH 3—10) followed by 7.5% SDS-PAGE. Separated proteins were stained with SYPRO Ruby. Crosses indicate spots used for protein identification. Spot numbers refer to proteins listed in Table 1. Identical proteins are shown by the surrounded area.

terns. For example, salivary proteins such as DPP IV (spot 3), carbonic anhydrase 6 (spot 30), short palate, lung and nasal epithelium carcinoma-associated protein 2 (parotid secretory protein) (spot 31), IgG Fc binding protein (spot 2), and cystatin family proteins (spot 43, 45, 46), were distinctly present in exosome II (Fig. 5B). In contrast, mucin-5B (spot 1) was present with much higher spot intensity in exosome I than in exosome II (Fig. 5A).

To increase the proteome coverage obtained by 2-D PAGE analysis, we undertook shotgun proteomics analysis. Total proteins extracted from each exosome were digested with trypsin, and resultant peptides were subjected to LC/MS/MS analysis for protein identification. A total of 76 and 135 proteins were identified from exosome I and exosome II, respectively (Table 1). Overall, 101 and 154 proteins were identified from exosome I and exosome II, respectively, by a combination of 2-D PAGE analysis, shotgun analysis and western blot analysis. Sixty-eight proteins were common between the two types of exosomes. With the information provided *via* the accession number, proteins identified in exosome I and exosome II could be grouped into categories of subcellular localization (Fig. 6). The protein composition of exosome I was similar to that from exosome II. Approximately 40% of the proteins identified were secretory or extracellular proteins, while at least 50% of the protein could be associated with the cytoplasm, plasma membrane or nucleus.

Degradation of Chemokines by Exosome II DPP IV selectively removes the N-terminal dipeptides from peptides with proline or alanine in the penultimate position. Almost all of the DPP IV activity in whole saliva was present in exosome II (Fig. 1). We next investigated whether the exosome II was active in the degradation of bioactive peptides such as chemokines. CXCL11 and CXCL12 are cleavable by DPP IV, and are known to be expressed in salivary glands.[24,25] CXCL11 was cleaved only at the Pro2-Met3 position when incubated with exosome II (data not shown). No other peptide fragments were observed. This cleavage was completely inhibited by specific DPP IV inhibitor ER319711-15.[26] By contrast, CXCL12 was first cleaved at Pro2-Val3 and subsequently hydrolyzed at Leu5-Ser6 (Fig. 7). When a specific DPP IV inhibitor was added, cleavage at Pro2-Val3 was inhibited, but CXCL12 was still cleaved at Leu4-Ser5.

DISCUSSION

In this study, human saliva exosomes were fractionated into at least two types of vesicles, exosome I and exosome II. They were different in size and protein composition. Size and shape (round-shape) of exosome II match those observed in exosomes from other sources. Exosome I was larger than exosome II, and had an electron-dense structure. Both vesicles had exosomal marker proteins such as Alix, Tsg 101, CD63 and Hsp70. Various proteins required for ESCRT (endosomal sorting complex required for transport) recruitment to endosomal membranes are associated with exosomes.[27,28] Alix and Tsg101 are components related to the ESCRT sorting mechanism. Alix is necessary for the targeting of endosomes and has been demonstrated to interact with Tsg101 which is a component of ESCRT I complex. CD63 (LAMP3, lysosomal membrane associate glycoprotein-3) which belongs to the tetraspanin family and heat shock protein Hsp 70, are often found in exosomes.[1] Recently, it has been found that exosomes contain miRNA, small noncoding RNAs, which suppress the translation of target mRNAs.[17] More recently, it was shown that exosomes also contains GW182, a component of RNA-induced silence complex, which is required for miRNA function.[29] GW182 was detected in both exosome I and exosome II. Investigations to detect miRNA in exosome I and exosome II are in progress. Apoptotic vesicles and microvesicles represent heterogeneous populations of membrane vesicles, budding directly from the plasma membrane and carrying a number of nuclear, cytosolic, and endoplasmic reticulum-derived proteins.[28,30] Sizes of those vesicles are much larger (100—1000 nm) than exosomes. Histones and integrins are enriched in apoptotic vesicles and microvesicles,[28,31] respectively, whereas those proteins were absent in exosome I and exosome II. These results support that the two types of vesicles identified in this study are exo-

Table 1. Identified Proteins in Exosome I and Exosome II

Entry number	Accession number[a)]	Protein name[a)]	Molecular function[b)]	Biological processes[b)]	Identification method[c)]	Presence in exosome types[d)]
1	Q9HC84	Mucin-5B	Cell adhesion	Extracellular	PMF, LC, S	I(1), II(1)
2	Q9Y6R7	IgG Fc binding protein	Cell adhesion	Extracellular	PMF, LC, S	II(2)
3	P27487	Dipeptidyl peptidase 4 (CD26)	Catalytic activity	Integral to plasma membrane	PMF, LC, S, W	I(2), II(3)
4	Q08380	Galectin-3-binding protein	Cell adhesion	Extracellular	PMF, LC, S	I, II(4)
5	P12814	Alpha-actinin-1	Actin binding	Cytoskeleton	PMF, S	II(5)
6	Q8WUM4	Programmed cell death 6-interacting protein (ALG-2-interacting protein 1, Alix)	Protein binding	Intracellular	LC, S, W	I(3), II(6)
7	P01833	Polymeric immunoglobulin receptor	Receptor	Integral to plasma membrane	PMF, LC, S, W	I(4), II(7)
8	P01871	Ig mu chain C region	Immune response	Extracellular	LC, S, W	I(5), II(8)
9	P15311	Ezrin	Actin binding	Cytoskeleton	LC, S	I(6)
10	P26038	Moesin	Protein binding	Cytoskeleton	LC, S	I(7)
11	P35241	Radixin	Actin binding	Cytoskeleton	LC	I(8)
12	P38606	V-type proton ATPase catalytic subunit A	Catalytic activity	Intracellular	LC	I(9)
13	Q7L9B9	Endonuclease/exonuclease/phosphatase family domain-containing protein 1	DNA binding	Intracellular	PMF	II(9)
14	P11142	Heat shock cognate 71 kDa protein (hsc70)	Protein binding	Intracellular	LC	I(10), II(10)
15	P08107	Heat shock 70 kDa protein 1	Protein binding	Intracellular	LC, W	I(11), II
16	P04259	Keratin, type II cytoskeletal 6B	Structural molecule activity	Intracellular	LC	I(12), II(12)
17	P02538	Keratin, type II cytoskeletal 6A	Structural molecule activity	Intracellular	LC	II(11)
18	P02768	Serum albumin	Fatty acid binding	Extracellular	LC, S	I(13), II(13)
19	P19013	Keratin, type II cytoskeletal 4	Structural molecule activity	Intracellular	LC	II(14)
20	P48723	Heat shock 70 kDa protein 13 (Stress 70 protein chaperone microsome-associated 60 kDa protein)	ATP binding	Intracellular	LC, W	II(15)
21	Q92485	Acid sphingomyelinase-like phosphodiesterase 3b	Catalytic activity	Extracellular	LC, S	II(16)
22	P01009	Alpha-1-antitrypsin	Catalytic activity	Extracellular	LC	II(17)
23	P01876	Ig alpha-1 chain C region	Immune response	Extracellular	PMF, LC, S,W	I(14), II(18)
24	P01877	Ig alpha-2 chain C region	Immune response	Extracellular	PMF, LC, S,W	I(15), II(19)
25	P01857	Ig gamma-1 chain C region	Immune response	Extracellular	LC, S, W	I(16), II(20)
26	P15313	V-type proton ATPase subunit B, kidney isoform	Catalytic activity	Intracellular	LC	I(17)
27	O14745	Na^+/H^+ exchange regulatory cofactor NHE-RF1 (Ezrin-radixin-moesin-binding phosphoprotein 50)	PDZ domain binding	Cytoskeleton	LC, S	I(18)
28	P08779	Keratin, type I cytoskeletal 16	Structural molecule activity	Intracellular	LC	I(19)
29	Q99816	Tumor susceptibility gene 101 protein	DNA binding	Intracellular	LC, W	I(20), II
30	P50395	Rab GDP dissociation inhibitor beta	GTP-binding	Membrane	LC	I(21)
31	Q13217	DnaJ homolog subfamily C member 3	Enzyme regulator activity	Intracellular	LC, S	II(21)
32	P02533	Keratin, type I cytoskeletal 14	Structural molecule activity	Intracellular	LC	II(22)
33	P19012	Keratin, type I cytoskeletal 15	Structural molecule activity	Intracellular	LC	I(22), II(23)
34	P08727	Keratin, type I cytoskeletal 19	Structural molecule activity	Intracellular	LC	I(23), II(24)
35	P06733	Alpha-enolase	Catalytic activity	Intracellular	LC	I(24)
36	P04745	Alpha-amylase 1	Catalytic activity	Secreted	PMF, LC, S	I, II(25)
37	Q8TDL5	Long palate, lung and nasal epithelium carcinoma-associated protein 1	Lipid binding	Secreted	LC, S	I, II(26)
38	P10909	Clusterin	Immune response	Extracellular	LC, S	II(27)
39	P60709	Actin, cytoplasmic 1	Structural constituent of cytoskeleton	Cytoskeleton	PMF, LC, S	I(25), II(28)
40	P63261	Actin, cytoplasmic 2	Structural constituent of cytoskeleton	Cytoskeleton	PMF, LC, S	I(26), II(29)
41	P23280	Carbonic anhydrase 6	Catalytic activity	Secreted	PMF, LC, S	I(27), II(30)
42	Q96DR5	Short palate, lung and nasal epithelium carcinoma-associated protein 2 (parotid secretory protein)	Lipid binding	Secreted	PMF, LC, S	I(28), II(31)
43	P62873	Guanine nucleotide-binding protein Gi/Gs/Gt subunit beta-1	GTP-binding	Membrane	LC	I(29)
44	P12429	Annexin A3	Calcium ion binding	Intracellular	LC	I(30)
45	P09525	Annexin A4	Calcium ion binding	Intracellular	LC	I(31)
46	P04083	Annexin A1	Calcium ion binding	Intracellular	LC, S	I(32)
47	P08758	Annexin A5	Calcium ion binding	Intracellular	PMF, S	I(33)
48	O00299	Chloride intracellular channel protein 1 (Nuclear chloride ion channel 27)	Chloride channel	Intracellular	LC	I(34)
49	Q9Y604	Transcription factor ETV7	Transcription factor activity	Intracellular	PMF	II(32)
50	P01591	Immunoglobulin J chain	Immune response	Extracellular	PMF, LC, S, W	I(35), II(33)
51	O00560	Syntenin-1 (Syndecan-binding protein)	Cytoskeletal adaptor activity	Cytoskeleton	LC, S	I(36), II(34)
52	P01842	Ig lambda chain C region	Immune response	Extracellular	LC, S, W	I(37), II(35)
53	Q53RT3	Retroviral-like aspartic protease 1	Catalytic activity	Intracellular	PMF	II(36)
54	Q9BXJ4	Complement C1q tumor necrosis factor-related protein 3	Unknown	Extracellular	LC, S	I, II(37)
55	P01834	Ig kappa chain C region	Immune response	Extracellular	PMF, LC, S, W	I(38), II(38)
56	P02647	Apolipoprotein A—I	Lipid binding	Secreted	PMF, S	I(39), II(39)
57	P32119	Peroxiredoxin-2	Antioxidant activity	Intracellular	LC	I(40)
58	P02753	Plasma retinol-binding protein	Retinol binding	Extracellular	PMF	II(40)
59	P12273	Prolactin-inducible protein	Actin binding	Extracellular	PMF, S	I(41), II(41)
60	Q9NP55	Protein Plunc (palate lung and nasal epithelium clone protein)	Lipid binding	Extracellular	PMF, S	I, II(42)
61	P01036	Cystatin-S (Cystatin-4, Salivary acidic protein 1)	Protease inhibitor	Secreted	PMF	II(43)
62	P09228	Cystatin-SA (Cystatin-2)	Protease inhibitor	Secreted	PMF, S	I(42), II(44)
63	P06703	Protein S100-A6 (Calcyclin)	Calcium ion binding	Intracellular	LC	I(43)
64	P06702	Protein S100-A9 (Calgranulin-B)	Calcium ion binding	Intracellular	LC, S	I(44), II
65	P28325	Cystatin-D (Cystatin-5)	Protease inhibitor	Secreted	LC, S	I(45), II(45)

Table 1. Continued

Entry number	Accession number[a)]	Protein name[a)]	Molecular function[b)]	Biological processes[b)]	Identification method[c)]	Presence in exosome types[d)]
66	P01037	Cystatin-SN (Cystatin-1, Salivary cystatin-SA-1)	Protease inhibitor	Secreted	PMF, S	I(46), II(46)
67	P61769	Beta-2-microglobulin	Immune response	Secreted	LC, S	II(47)
68	P05109	Protein S100-A8 (calgranulin-A)	Calcium ion binding	Intracellular	PMF, LC, S	I(47), II(48)
69	Q96DA0	Zymogen granule protein 16 homolog B (Uncharacterized protein UNQ773/PRO1567 Precursor, Similar to common salivary protein 1)	Sugar binding	Secreted	PMF, S	I, II(49)
70	P07737	Profilin-1	Actin binding	Cytoskeleton	PMF	II(50)
71	Q9UGM3	Deleted in malignant brain tumors 1 protein (Glycoprotein 340)	Immune response	Secreted	S	I, II
72	Q8WXI7	Mucin-16	Cell adhesion	Extracellular	S	I
73	O43490	Prominin-1	Response to stimulus	Integral to plasma membrane	S	I, II
74	Q07654	Trefoil factor 3	Defense response	Extracellular	S	I, II
75	P13987	CD59 glycoprotein	Blood coagulation	Integral to plasma membrane	S	I, II
76	P15144	Aminopeptidase N	Catalytic activity	Integral to plasma membrane	S	I
77	Q9NQ84	G-protein coupled receptor family C group 5 member C	G-protein coupled receptor	Integral to plasma membrane	S	I, II
78	P15941	Mucin-1	Cell adhesion	Extracellular	S	I
79	P01764	Ig heavy chain V-III region VH26	Immune response	Extracellular	S	I, II
80	P18136	Ig kappa chain V-III region HIC	Immune response	Extracellular	S	I, II
81	P80303	Nucleobindin-2	Calcium ion binding	Extracellular	S	I, II
82	P62158	Calmodulin	Calcium ion binding	Intracellular	S	I
83	P11215	Integrin alpha-M	Cell adhesion	Membrane	S	I
84	Q8TAX7	Mucin-7	Cell adhesion	Extracellular	S	I, II
85	Q8TED4	Sugar phosphate exchanger 2	Sugar transport	Membrane	S	I, II
86	P08571	Monocyte differentiation antigen CD14	Receptor	Integral to plasma membrane	S	I, II
87	Q8N4F0	Bactericidal/permeability-increasing protein-like 1 (Long palate, lung and nasal epithelium carcinoma-associated protein 2)	Lipid binding	Secreted	S	I, II
88	P06310	Ig kappa chain V-II region RPMI 6410	Immune response	Extracellular	S	I, II
89	P01743	Ig heavy chain V-I region HG3	Immune response	Extracellular	S	I
90	P10599	Thioredoxin	Cell redox homeostasis	Cytosol	S	I
91	P55064	Aquaporin-5	Water channel activity	Integral to plasma membrane	S	I, II
92	P01766	Ig heavy chain V-III region BRO	Immune response	Extracellular	S	I, II
93	P60033	CD81 antigen	Receptor	Integral to plasma membrane	S	I, II
94	P63104	14-3-3 protein zeta/delta	Protein binding	Intracellular	S	I, II
95	P59666	Neutrophil defensin 3	Immune response	Secreted	S	I, II
96	Q5JWF2	Guanine nucleotide-binding protein Gs subunit alpha isoforms Xlas	GTP binding	Membrane	S	I
97	P13688	Carcinoembryonic antigen-related cell adhesion molecule 1	Angiogenesis	Integral to plasma membrane	S	I, II
98	P08174	Complement decay-accelerating factor (CD55 antigen)	Immune response	Integral to plasma membrane	S	I, II
99	P21926	CD9 antigen	Cell adhesion	Integral to plasma membrane	S	I, II
100	P04220	Ig mu heavy chain disease protein	Immune response	Membrane	S	I, II
101	P62988	Ubiquitin	Protein ubiquitination	Intracellular	S	I, II
102	P03973	Antileukoproteinase	Protease inhibitor	Secreted	S	I, II
103	P01772	Ig heavy chain V-III region KOL	Immune response	Extracellular	S	I,I I
104	P01780	Ig heavy chain V-III region JON	Immune response	Extracellular	S	I, II
105	P22079	Lactoperoxidase	Catalytic activity	Secreted	S	I, II
106	Q15758	Neutral amino acid transporter B0	Neutral amino acid transporter	Integral to plasma membrane	S	I
107	P01700	Ig lambda chain V-I region HA	Immune response	Extracellular	S	I
108	P01609	Ig kappa chain V-I region Scw	Immune response	Extracellular	S	I
109	Q9NZH0	G-protein coupled receptor family C group 5 member B	G-protein coupled receptor	Integral to plasma membrane	S	I, II
110	P62834	Ras-related protein Rap-1A	GTP binding	Intracellular	S	I
111	P04080	Cystatin-B	Protease inhibitor	Cytoplasm	S	I, II
112	Q9HD89	Resistin	Hormone activity	Secreted	S	I
113	P31949	Protein S100-A11	Calcium ion binding	Intracellular	S	I
114	P15515	Histatin-1	Defense response	Extracellular	S	I, II
115	O60635	Tetraspanin-1	Uncharacterized	Membrane	S	I
116	P07996	Thrombospondin-1	Cell adhesion	Extracellular	S	II
117	P08582	Melanotransferrin	Ion transport	Integral to plasma membrane	S	II
118	P14618	Pyruvate kinase isozymes M1/M2	Catalytic activity	Cytosol	S	II
119	Q6P5S2	Uncharacterized protein C6orf58	Unknown	Secreted	S	II
120	P51993	Alpha-(1,3)-fucosyltransferase	Catalytic activity	Intracellular	S	II
121	P07602	Proactivator polypeptide	Enzyme activation activity	Intracellular	S	II
122	Q02809	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	Catalytic activity	Intracellular	S	II
123	Q9H173	Nucleotide exchange factor SIL1	Protein folding	Intracellular	S	II
124	P55058	Phospholipid transfer protein	Lipid binding	Secreted	S	II
125	P07237	Protein disulfide-isomerase	Catalytic activity	Intracellular	S	II
126	Q13438	Protein OS-9	Protein binding	Intracellular	S	II
127	Q8IZP9	G-protein coupled receptor 64	G-protein coupled receptor	Integral to plasma membrane	S	II
128	P06314	Ig kappa chain V-IV region B17	Immune response	Extracellular	S	II
129	Q01469	Fatty acid-binding protein, epidermal	Fatty acid binding	Intracellular	S	II
130	Q9Y4L1	Hypoxia up-regulated protein 1	Chaperone	Intracellular	S	II
131	O95479	GDH/6PGL endoplasmic bifunctional protein	Catalytic activity	Intracellular	S	II
132	Q6MZM9	Uncharacterized protein C4orf40	Uncharacterized	Secreted	S	II
133	O95395	Beta-1,3-galactosyl-*O*-glycosyl-glycoprotein beta-1,6-*N*-acetylglucosaminyltransferase 3	Catalytic activity	Intracellular	S	II
134	P00558	Phosphoglycerate kinase 1	Catalytic activity	Intracellular	S	II

Table 1. Continued

Entry number	Accession number[a]	Protein name[a]	Molecular function[b]	Biological processes[b]	Identification method[c]	Presence in exosome types[d]
135	P54108	Cysteine-rich secretory protein 3	Immune response	Secreted	S	II
136	P13796	Plastin-2	Calcium ion binding	Intracellular	S	II
137	Q14515	SPARC-like protein 1	Calcium ion binding	Extracellular	S	II
138	P09958	Furin	Catalytic activity	Integral to plasma membrane	S	II
139	P01593	Ig kappa chain V-I region AG	Immune response	Extracellular	S	II
140	P09211	Glutathione S-transferase P	Catalytic activity	Intracellular	S	II
141	P04196	Histidine-rich glycoprotein	Protease inhibitor	Secreted	S	II
142	P23083	Ig heavy chain V-I region V35	Immune response	Extracellular	S	II
143	Q8NBJ4	Golgi membrane protein 1	Unknown	Intracellular	S	II
144	P80748	Ig lambda chain V-III region LOI	Immune response	Extracellular	S	II
145	Q14697	Neutral alpha-glucosidase AB	Catalytic activity	Intracellular	S	II
146	P04208	Ig lambda chain V-I region WAH	Immune response	Extracellular	S	II
147	P02649	Apolipoprotein E	Receptor binding	Secreted	S	II
148	P02788	Lactotransferrin	Ferric iron binding	Secreted	S	II
149	Q99935	Proline-rich protein 1	Innate immune response	Secreted	S	II
150	P06396	Gelsolin	Actin binding	Intracellular	S	II
151	Q9NRA1	Platelet-derived growth factor C	Growth factor activity	Secreted	S	II
152	Q02818	Nucleobindin-1	Calcium ion binding	Intracellular	S	II
153	P12110	Collagen alpha-2(VI) chain	Extra cellular matrix	Extracellular	S	II
154	Q9UHG3	Prenylcysteine oxidase 1	Catalytic activity	Intracellular	S	II
155	P07437	Tubulin beta chain	Microtubule	Intracellular	S	II
156	P33908	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	Catalytic activity	Intracellular	S	II
157	Q5XXA6	Anoctamin-1	Ion transport	Membrane	S	II
158	P30740	Leukocyte elastase inhibitor	Protease inhibitor	Intracellular	S	II
159	P31146	Coronin-1A	Actin binding	Cytoskeleton	S	II
160	P05164	Myeloperoxidase	Catalytic activity	Intracellular	S	II
161	O00462	Beta-mannosidase	Catalytic activity	Intracellular	S	II
162	P29508	Serpin B3	Protease inhibitor	Intracellular	S	II
163	P13639	Elongation factor 2	Elongation factor	Intracellular	S	II
164	P01859	Ig gamma-2 chain C region	Immune response	Extracellular	S	II
165	Q11201	CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase	Catalytic activity	Intracellular	S	II
166	P01703	Ig lambda chain V-I region NEWM	Immune response	Extracellular	S	II
167	Q14978	Nucleolar phosphoprotein p130	Mitosis	Intracellular	S	II
168	P01714	Ig lambda chain V-III region SH	Immune response	Extracellular	S	II
169	O00391	Sulfhydryl oxidase 1	Catalytic activity	Intracellular	S	II
170	Q96HE7	ERO1-like protein alpha	Electron carrier activity	Intracellular	S	II
171	P27348	14-3-3 protein theta	Protein binding	Intracellular	S	II
172	P25311	Zinc-alpha-2-glycoprotein	MHC class I family	Secreted	S	II
173	Q9NS98	Semaphorin-3G	Multi cellular organismal development	Secreted	S	II
174	Q9UKU9	Angiopoietin-related protein 2	Receptor binding	Secreted	S	II
175	P28289	Tropomodulin-1	Actin binding	Cytoskeleton	S	II
176	Q9P1U1	Actin-related protein 3B	Actin binding	Cytoskeleton	S	II
177	P15309	Prostatic acid phosphatase	Catalytic activity	Extracellular	S	II
178	Q9UBS3	DnaJ homolog subfamily B member 9	Protein binding	Nucleus	S	II
179	A6NIZ1	Ras-related protein Rap-1b-like protein	GTP binding	Intracellular	S	II
180	Q9NZP8	Complement C1r subcomponent-like protein	Immune response	Secreted	S	II
181	P52566	Rho GDP-dissociation inhibitor 2	GTPase activation activity	Intracellular	S	II
182	P40199	Carcinoembryonic antigen-related cell adhesion molecule 6	Signal transduction	Integral to plasma membrane	S	II
183	P45877	Peptidyl-prolyl cis-trans isomerase C	Catalytic activity	Cytoplasm	S	II
184	Q8N0Y7	Probable phosphoglycerate mutase 4	Catalytic activity	Cytoplasm	S	II
185	P17931	Galectin-3	Sugar binding	Secreted	W	I, II
186	P08962	CD63	Receptor	Integral to plasma membrane	W	I, II
187	Q8NDV7	Trinucleotide repeat-containing gene 6A protein (GW182)	RNA binding	Cytoplasm	W	I, II

a) The Mascot algorithm was used to identify the protein name and the accession number in Swiss-Prot. *b*) Protein annotation was obtained by using UniProtKB/Swiss-Prot. *c*) Identification method: PMF, 2-D PAGE followed by MALDI-TOF-MS peptide fingerprint; LC, 2-D PAGE followed by peptide sequencing using LC/MS/MS; S, Shotgun analysis; W, western blot analysis. *d*) Exosome types and spot number in 2-D PAGE (Fig. 5) (in parentheses): I, exosome I; II, exosome II.

somes. However, the morphology of exosome I was somewhat different from that of typical exosomes. Further characterization will be necessary to confirm that these vesicles are exosomes.

Exosomes are generated with transmembrane and peripheral membrane proteins incorporated into the invaginating membrane in the same topological orientation as at the plasma membrane, while cytosolic proteins are engulfed and enclosed into the vesicles. Exosome I and exosome II contain many plasma membrane proteins: single-pass transmembrane proteins such as type I membrane proteins-carcinoembryonic antigen-related cell adhesion molecule 1, and type II membrane protein-DPP IV and Golgi membrane protein 1; tetraspanins-CD63, CD81, and CD9; pentaspan membrane protein-Prominin-1 (CD133); six-pass membrane protein-aquaporin-5; seven-pass membrane protein-G-protein coupled receptor family C group 5 member C; GPI-anchored proteins-CD59, melanotransferrin, CD14 and complement decay-accelerating factor (CD55). These membrane proteins have often been identified in known exosomes. Integral plasma membrane proteins were 10—15% of identified proteins in exosome I and exosome II (Fig. 6). This ratio is simi-

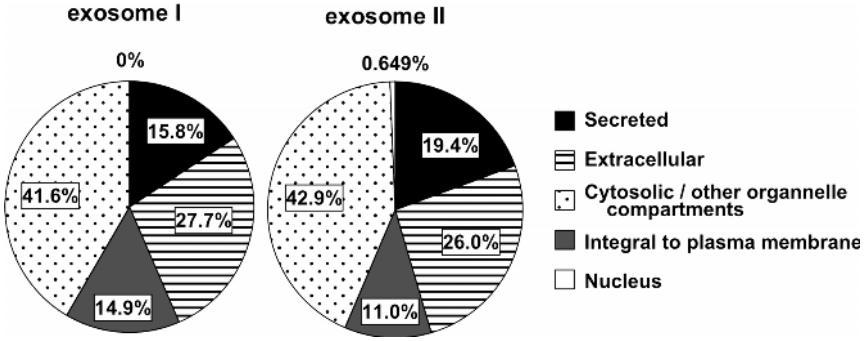

Fig. 6. Annotation of Identified Proteins from Exosome I and Exosome II by Subcellular Location

Protein classification was obtained by using UniProKB/Swiss-Prot database. A total of 101 proteins from exosome I and 154 proteins from exosome II were used for classification.

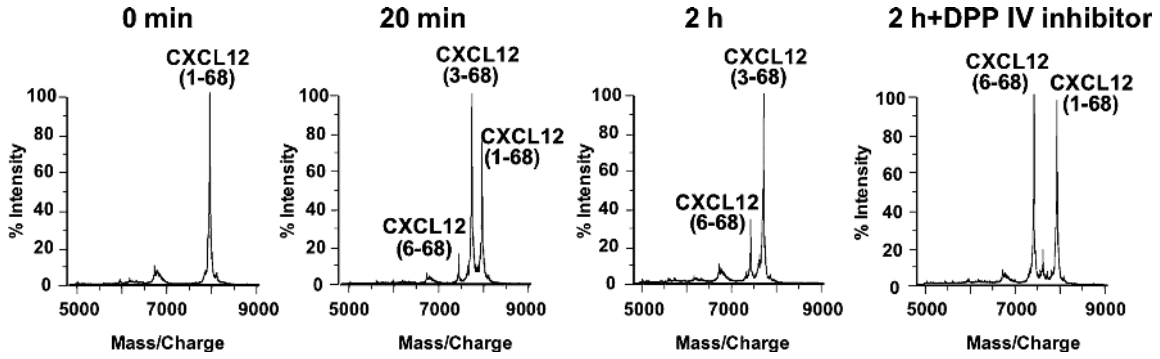

Fig. 7. MALDI-TOF Mass Spectra of Degradation of CXCL12 Induced by Exosome II from Human Whole Saliva

Exosome II was incubated with CXCL12 for 20 min or 2 h at 37 °C, or in combination with DPP IV inhibitor ER319711-15 for 2 h at 37 °C.

lar to that (13%) in parotid gland exosome.[16] The relatively high proportion of plasma membrane proteins in the exosome is supported by the fact that the exosome is a membrane vesicle of endocytotic origin.[4]

IgA and pIgR were major common components of exosome I and exosome II. Meanwhile, there were notable differences between them, even if there was any cross contamination due to limitations of the separation method. DPP IV, carbonic anhydrase 6, and cystatin family proteins are expressed more abundantly in exosome II. Moreover, exosome II contained more IgG Fc binding protein and galectin-3 binding protein than exosome I. In contrast, exosome I exclusively contained a number of proteins, including ezrin, moesin, radixin, Rab GDP dissociation inhibitor beta, alpha-enolase, guanine nucleotide-binding protein Gi/Gs/Gt subunit beta-1 and annexins. In addition, mucin-5B was abundant in exosome I. Whereas alpha-amylase (about 20% in weight of salivary proteins) and proline-rich proteins (about 40% in weight of salivary proteins), both abundant protein components in whole saliva,[32] only a small amount or none of these proteins were detected in exosome I and exosome II. In contrast, carbonic anhydrase 6 and cystatin family proteins are also extracellularly secreted proteins that are minor components in whole salivary proteins, but distinct amounts of these proteins were detected in exosome II. It is likely that salivary proteins may be associated with each exosome in a specific way during exosome formation.

Human seminal fluid contains exosome-like vesicles called prostasomes.[33] Their size generally varies from 50 to 200 nm. Recently, Poliakov *et al.* described that the prostasome could be classified into at least three distinct structural types of vesicles, one of which was named "a dark vesicle" that had high electron density.[34] Its size ranged from 100 to 200 nm, and it was nearly round or slightly elongated. These features appear similar to those of exosome I. However, it remains to be elucidated whether exosome I is the same type of vesicle as a "dark vesicle." At present, the underlying mechanism for formation of prostasome heterogeneity is unclear. However, Poliakov *et al.* postulated that the structural heterogeneity of prostasomes may reflect their origin from several organs such as prostate, testes and epididymis. The structural heterogeneity of salivary exosomes might reflect the origin from several salivary glands. Whole saliva is composed mainly of salivas from the contralateral major (parotid, submandibular, sublingual) and minor salivary glands. Primary saliva secretion is elaborated by the acinar cells in the respective salivary gland and then it is modified as it passes through a series of progressively large ducts.[35] The Human Protein Atlas (http://www.proteinatlas.org/) shows that pIgR locates acinar cells and the ductal epithelial cells in human salivary glands, and that galectin-3 binding protein, which was detected in exosome I and exosome II, is located in the surface of the ductal epithelial cell. In addition, galectin-3 has been reported to be expressed in ductal epithelium cells in salivary glands.[36] DPP IV is expressed in ductal epithelium cells and acinar cells.[37] The distributions of these salivary gland proteins indicate the possible origin of exosome I and exosome II from ductal, acinar or both cell types. Sublingual secretion contains high amounts of mucin-5B,[38] suggesting that exosome I might be derived from at least the sublingual gland. In

contrast, moesin, which was detected only in exosome I, has been reported to be present in B cell-derived exosomes.[39] Exosome I may be partially derived from B cells in salivary glands. Indeed, salivary glands are rich in B cells that produce IgA. Recently, exosomes were isolated from parotid saliva, and 491 proteins were identified by shotgun analysis using multidimensional protein identification mass spectrometry.[16] Many of the proteins were common to the proteins found in exosome I and exosome II, including exosomal markers (Alix, Tsg101, Hsp70, CD63), actin, DPP IV and galectin-3. Parotid gland exosome contains carbonic anhydrase 6, suggesting that exosome II may be derived at least in part from the parotid gland. In addition, a previous immunohistochemical study has demonstrated the cellular origins of carbonic anhydrase 6 in the parotid and submandibular glands of humans.[40] Exosome II may be derived from, at least in part, both of these salivary glands. Thus, it is considered that exosome I and exosome II are derived from several salivary glands and cell types. However, further study will be needed to clarify the origins of exosome I and exosome II.

Salivary glands express a variety of bioactive peptides such as neuropeptides, incretin and chemokines.[41,42] In a previous study, we showed that exosomes from human whole saliva cleave substance P and glucose-dependent insulinotropic polypeptide (GIP).[15] CXCL11, CXCL12 (SDF-1), CXCL10, and CCL5 (RANTES) share the X-Pro motif at the N-terminus and are released from salivary epithelial cells.[24,25,43,44] The present study revealed that exosome II was metabolically active in cleaving CXCL11 and CXCL12. Cleavage of CXCL11 was completely inhibited by DPP IV inhibitor, suggesting that DPP IV is responsible for its cleavage. Interestingly, CXCL12 was cleaved at Leu5-Ser6 even in the presence of a DPP IV-specific inhibitor, suggesting that exosome II contains another protease. Unfortunately, a candidate protease could not be found in the list of the proteins identified in the present study. Further research will be needed to elucidate a protease capable of cleaving CXCL12.

Regardless of their uncertain physiological role, exosomes have the potential to modulate the immune response through activation and perhaps also suppressing the immune system.[28] Exosome I and exosome II contained IgA at a high rate, suggesting that these exosomes might play a regulatory role in local immune defense in the oral cavity. Pathogens captured by IgA on exosome I and exosome II may be delivered to the lymphoid organs such as the tonsils and uptaken by dendritic cells. It is reported that exosomes are internalized efficiently by dendritic cells.[45] Dendritic cells are the most potent antigen-presenting cells that specialize in the initiation of immune responses by directing the activation and differentiation of naïve T lymphocytes.[46] Our preliminary experience shows that the antibody against DPP IV was produced successfully when exosome II was injected as an antigen without adjuvant intraperitoneally in mice. This antibody production was higher than that raised by a soluble recombinant DPP IV as an antigen. These results suggest that exosome II exhibits higher immunogenicity. In fact, it is reported that delivering antigens *in vivo* through exosomes is more immunogenic than the mere delivery of the soluble form in tumor models.[47] DPP IV, also known as CD26, functions not only to degrade biologically active peptides, but also to regulate the immune response.[48] DPP IV/CD26 influences proliferation in stimulated human T cells through binding to caveolin-1 on antigen-presenting cells (APC).[49,50] As exosome II contains a characteristic amount of DPP IV, exosome II may have both antigen-delivering function but also direct APC stimulating function. The present study is the first exploration into the proteome of exosomes from human whole saliva, and results indicate that the proteome from the two types of exosome differs. Future studies are needed to characterize the two types of exosomes more in detail and clarify the biological significance of these exosomes in the oral cavity. Our proteomic analysis may provide a list of candidate proteins for future structural and functional studies.

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