Vol. 8, No. 4, 2017
ISSN 2233-4203/ e-ISSN 2093-8950
LETTER www.msletters.org | Mass Spectrometry Letters

Global Absolute Quantitation of Proteins in Human Whole Saliva by nLC-Q-IMS-TOF Employing MS^E

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Received November 30, 2017; Accepted December 26, 2017
First published on the web December 31, 2017; DOI: 10.5478/MSL.2017.8.4.114

Abstract : While saliva can be considered as good biological fluid for monitoring biomarkers due to many advantages including its communication with blood and the non-invasive nature during its sampling, its applications to that purpose is still limited. As a part of efforts to expand the applications of saliva to the protein biomarker research, we carried out global absolute quantitation of proteins in human whole saliva (WS) by bottom-up proteomics techniques mainly based on nLC-Q-IMS-TOF employing MS^E. From the analyses of a pooled WS sample collected from 22 healthy Korean volunteers, 93 proteins ranging from 5.89×10^1 ng/mL (immunoglobulin heavy chain) to 1.59×10^4 ng/mL (α-amylase 1) were confirmed. For the validation of the present results, human serum albumin in the same sample was quantitated by ELISA and its result was compared with that from the nLC-Q-IMS-TOF study. As a result, there was no significant difference between two results from individual approaches ($1.18\times10^4 \pm 0.03\times10^4$ ng/mL from nLC-Q-IMS-TOF experiments *vs.* $1.23\times10^4 \pm 0.07\times10^4$ ng/mL from ELISA experiments, n=3, p=0.309). To our knowledge, this is the first global absolute quantitation of proteins in human whole saliva and information from the present study can be widely used as the first level reference for the discovery of new protein biomarkers from human whole saliva as well as for quantitative applications of human whole saliva proteins.

Keywords : Human whole saliva, global absolute protein quantitation, MS^E, proteome

Introduction

Saliva can be considered as good biological fluid for monitoring biomarkers due to its communication with blood, the non-invasive nature during its sampling, the easiness of sample collection, the relatively large volume of sample which can be taken, no concern of coagulation, and the low risk of infection to researchers during its sampling/preparation.[1] However, in spite of these various "virtues", the applications of saliva to that purpose were limited and its major reason is believed to be low levels of compounds of interests in saliva. Recently, the enhancement of sensitivity as well as specificity in analytical techniques has lowered the barrier and the types of components of interests in saliva are being expanded. Especially, protein is the salivary component which receives the most attention from researchers and more than 3000 proteins including many disease biomarker candidates have been reported from human saliva.[2-9] Among various types of saliva, whole saliva (WS), the final mixture of all types of gland-specific saliva in oral cavity, has been widely used as samples for saliva protein studies due to its very simple collection method compared to those for various types of gland-specific saliva.[5, 6] However, the information for individual protein levels in normal WS, which can be very useful as the first level reference for biomarker research on WS is almost absent. While normal levels of some high abundance proteins in gland-specific saliva were reported, they must be totally different from those in normal WS.[10] For example, the individual normal levels of IgG, haptoglobin, proline-rich proteins, MUC5B, amylase, SIgA, cystatins, albumin, MUC7, lactoferrin, histatins, statherin, transferrin, lysozyme, C3 complement, carbonic anhydrase V1, C4 complement, and HNP 1-3 in gland-specific saliva are known to be higher than 1.0 mg/mL, but even the total protein concentration in normal WS is generally measured to be about 1.0 mg/mL.[11,12] Thus, it is necessary to confirm and establish the normal levels of WS proteins for their better understanding and more efficient WS protein

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biomarker research. However, traditional methods to quantitate individual proteins require the standard for each protein and they have low throughput ways. Thus, it is difficult to collect a set of level information of even a series of major WS proteins by these methods and there are needs of new high throughput methods for this purpose. Recently, global absolute quantitation of proteins by MS^E, a data-independent acquisition method in mass spectrometry was reported.[13] This method is based on the correlation between the sum of signal intensity values of the three most intense proteolytic peptide ions from a certain protein and the amount of the protein in an MS^E experiment.[13] Additionally, since response factors for all proteins were reported to be constant, global absolute protein quantitation by MS^E is available.[13]

Therefore, here, we applied a global absolute protein quantitation method based on MS^E to human WS to obtain creditable absolute levels of human WS proteins for the first time. The present results were validated by the comparison of the human serum albumin (HSA) quantitation result from the present nLC-Q-IMS-TOF method with that from an ELISA method. Finally, the global absolute quantitation information of proteins in WS obtained from the present study may be able to contribute to the development of disease biomarkers from WS.

Experimental

The present study was approved by Dankook University Institutional Review Board. WS (15 mL/person) was collected from 22 healthy South Korean adult volunteers (11 male adults and 11 female adults) at 9:30 am prior to eating and after rinsing the mouth with water. A protease inhibitor cocktail solution (Sigma-Aldrich, St. Louis, MO) was spiked (the final volume ratio of 1:100) to WS samples immediately after sample collection. These protease-spiked samples were pooled and treated following Cho et al.'s method.[9] The only modification in the method is the addition of Pierce™ Digestion Indicator protein (Thermo Fisher Scientific, Waltham, MA, USA) as an internal standard (IS) to the pooled Korean WS sample (the final concentration of 24 fmol/μL) for global absolute protein quantitation. After sample treatment, 2 μL of the final form of the sample solution was subjected to nanoliquid chromatography-quadrupole-ion mobility spectroscopy-time of flight (nLC-Q-IMS-TOF) analysis employing MS^E following Cho et al.'s method.[9] Raw data from nLC-Q-IMS-TOF were analyzed with Waters ProteinLynx Global Server (PLGS) v3.0.2. First, for the identification of peptides and proteins, database search against the modified IPI human database v3.87 including the amino acid sequence of Pierce™ Digestion Indicator protein, IS, was performed. The same parameters as those employed in Cho et al.'s study were used for database search and all search results were verified manually.[9] Also, global absolute protein quantitation was carried out based on the fixed IS column-loading amount of 48 fmol. For the validation of the present quantitation results, HSA in the pooled WS sample was quantitated by an ELISA kit (Abnova, Aachen, Germany) following the manufacturer's instruction.

Results and discussion

As shown in Table 1, a total of 93 proteins were identified and quantitated from the pooled Korean WS sample by the global absolute protein quantitation method for the first time. Since the protein quantitation method by MS^E is known to have about four orders of magnitude dynamic range, proteins whose quantitation results were located out of that range were removed from Table 1.[13]

Table 1. Proteins identified and quantitated from nLC-Q-IMS-TOF analyses of a pooled whole saliva sample collected from 11 healthy Korean male adults and 11 healthy Korean female adults (n=3)

Accession number	Protein description	Protein level (ng/mL)
IPI00002745	CATHEPSIN Z	8.35E+01 ± 2.36E+01
IPI00003269	BETA-ACTIN-LIKE PROTEIN 2	1.19E+03 ± 0.42E+03
IPI00003362	78 KDA GLUCOSE-REGULATED PROTEIN	5.21E+02 ± 2.00E+02
IPI00003470	IG KAPPA CHAIN V-I REGION WES	1.07E+02 ± 0.11E+02
IPI00003815	RHO GDP-DISSOCIATION INHIBITOR 1	1.19E+02 ± 0.17E+02
IPI00003817	RHO GDP-DISSOCIATION INHIBITOR 2	1.93E+02 ± 0.27E+02
IPI00004573	POLYMERIC IMMUNOGLOBULIN RECEPTOR	1.26E+04 ± 0.11E+04
IPI00007797	FATTY ACID-BINDING PROTEIN, EPIDERMAL	2.96E+02 ± 0.80E+02
IPI00009650	LIPOCALIN-1	9.23E+02 ± 2.94E+02
IPI00009865	KERATIN, TYPE I CYTOSKELETAL 10	7.56E+02 ± 1.16E+02
IPI00010471	PLASTIN-2	6.97E+02 ± 1.00E+02
IPI00010796	PROTEIN DISULFIDE-ISOMERASE	4.92E+02 ± 0.16E+02
IPI00011229	CATHEPSIN D	3.88E+02 ± 0.07E+02
IPI00013382	CYSTATIN-SA	1.29E+03 ± 1.37E+03

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Table 1. Continued.

Accession number	Protein description	Protein level (ng/mL)
IPI00013808	ALPHA-ACTININ-4	5.91E+02 ± 2.69E+02
IPI00019038	LYSOZYME C	1.61E+03 ± 0.22E+03
IPI00019359	KERATIN, TYPE I CYTOSKELETAL 9	1.04E+03 ± 0.06E+03
IPI00020487	EXTRACELLULAR GLYCOPROTEIN LACRITIN	2.34E+02 ± 0.59E+02
IPI00021263	14-3-3 PROTEIN ZETA/DELTA	4.62E+02 ± 1.18E+02
IPI00021304	KERATIN, TYPE II CYTOSKELETAL 2 EPIDERMAL	2.52E+02 ± 0.92E+02
IPI00021439	ACTIN, CYTOPLASMIC 1	3.04E+03 ± 0.16E+03
IPI00021828	CYSTATIN-B	1.06E+03 ± 0.50E+03
IPI00021841	APOLIPOPROTEIN A-I	5.23E+02 ± 2.68E+02
IPI00022429	ALPHA-1-ACID GLYCOPROTEIN 1	1.89E+02 ± 0.13E+02
IPI00022463	SEROTRANSFERRIN	3.46E+03 ± 2.68E+03
IPI00022488	HEMOPEXIN	3.24E+02 ± 0.23E+02
IPI00022733	45 KDA PROTEIN	1.63E+02 ± 0.21E+02
IPI00022974	PROLACTIN-INDUCIBLE PROTEIN	2.48E+03 ± 1.11E+03
IPI00023673	GALECTIN-3-BINDING PROTEIN	1.09E+03 ± 0.29E+03
IPI00025753	DESMOGLEIN-1	1.92E+03 ± 0.07E+03
IPI00025846	ISOFORM 2A OF DESMOCOLLIN-2	1.75E+03 ± 0.11E+03
IPI00027462	PROTEIN S100-A9	4.69E+02 ± 0.46E+02
IPI00031547	DESMOGLEIN-3	1.41E+03 ± 0.11E+03
IPI00032293	CYSTATIN-C	1.28E+03 ± 0.12E+03
IPI00032294	CYSTATIN-S	1.06E+03 ± 1.08E+03
IPI00060800	ZYMOGEN GRANULE PROTEIN 16 HOMOLOG B	2.05E+03 ± 0.81E+03
IPI00152154	MUCIN-7	5.92E+03 ± 1.52E+03
IPI00166729	ZINC-ALPHA-2-GLYCOPROTEIN	2.44E+03 ± 0.29E+03
IPI00178926	IMMUNOGLOBULIN J CHAIN	7.48E+02 ± 3.60E+02
IPI00216691	PROFILIN-1	4.72E+02 ± 0.70E+02
IPI00216984	CALMODULIN-LIKE PROTEIN 3	2.26E+02 ± 0.33E+02
IPI00219365	MOESIN	2.47E+02 ± 1.03E+02
IPI00219757	GLUTATHIONE S-TRANSFERASE P	5.62E+02 ± 0.77E+02
IPI00220327	KERATIN, TYPE II CYTOSKELETAL 1	2.19E+03 ± 0.06E+03
IPI00291410	ISOFORM 1 OF LONG PALATE, LUNG AND NASAL EPITHELIUM CARCINOMA- ASSOCIATED PROTEIN 1	2.05E+03 ± 0.16E+03
IPI00295542	NUCLEOBINDIN-1	2.89E+02 ± 0.74E+02
IPI00296654	BACTERICIDAL/PERMEABILITY-INCREASING PROTEIN-LIKE 1	1.30E+03 ± 0.12E+03
IPI00297056	CORNULIN	1.32E+03 ± 0.44E+03
IPI00299729	TRANSCOBALAMIN-1	2.23E+03 ± 0.16E+03
IPI00300786	ALPHA-AMYLASE 1	1.59E+04 ± 0.53E+04
IPI00304557	SHORT PALATE, LUNG AND NASAL EPITHELIUM CARCINOMA-ASSOCIATED PROTEIN 2	3.92E+03 ± 0.47E+03
IPI00304925	HEAT SHOCK 70 KDA PROTEIN 1A/1B	6.26E+02 ± 1.43E+02
IPI00305477	CYSTATIN-SN	1.58E+03 ± 0.07E+03
IPI00374315	UPF0762 PROTEIN C6ORF58	3.01E+02 ± 0.76E+02
IPI00382476	IG HEAVY CHAIN V-III REGION WEA	6.18E+01 ± 1.49E+01
IPI00382500	IG HEAVY CHAIN V-III REGION GAL	1.17E+02 ± 0.31E+02
IPI00384404	RHEUMATOID FACTOR RF-ET9 (FRAGMENT)	2.15E+02 ± 0.72E+02
IPI00386879	CDNA FLJ14473 FIS, CLONE MAMMA1001080, HIGHLY SIMILAR TO HOMO SAPIENS SNC73 PROTEIN (SNC73) MRNA	9.84E+03 ± 0.48E+03
IPI00399007	PUTATIVE UNCHARACTERIZED PROTEIN DKFZP686I04196 (FRAGMENT)	8.70E+02 ± 7.20E+02
IPI00410714	IPI00410714 HEMOGLOBIN SUBUNIT ALPHA	2.54E+02 ± 0.33E+02
IPI00418512	ISOFORM 4 OF DELETED IN MALIGNANT BRAIN TUMORS 1 PROTEIN	1.03E+04 ± 0.13E+04

Table 1. Continued.

Accession number	Protein description	Protein level (ng/mL)
IPI00419215	ALPHA-2-MACROGLOBULIN-LIKE PROTEIN 1	2.36E+03 ± 0.17E+03
IPI00419585	PEPTIDYL-PROLYL CIS-TRANS ISOMERASE A	3.62E+02 ± 0.13E+02
IPI00423461	PUTATIVE UNCHARACTERIZED PROTEIN DKFZP686C02220 (FRAGMENT)	2.79E+03 ± 0.52E+03
IPI00426051	PUTATIVE UNCHARACTERIZED PROTEIN DKFZP686C15213	1.66E+03 ± 0.16E+03
IPI00451401	ISOFORM 2 OF TRIOSEPHOSPHATE ISOMERASE	7.05E+02 ± 1.04E+02
IPI00465248	ISOFORM ALPHA-ENOLASE OF ALPHA-ENOLASE	1.63E+03 ± 0.15E+03
IPI00465439	FRUCTOSE-BISPHOSPHATE ALDOLASE A	6.92E+02 ± 0.62E+02
IPI00479743	ISOFORM 1 OF POTE ANKYRIN DOMAIN FAMILY MEMBER E	5.42E+02 ± 2.55E+02
IPI00549725	PHOSPHOGLYCERATE MUTASE 1	1.28E+02 ± 0.26E+02
IPI00550363	TRANSGELIN-2	1.47E+02 ± 0.31E+02
IPI00550731	PUTATIVE UNCHARACTERIZED PROTEIN	1.42E+03 ± 1.78E+03
IPI00646304	PEPTIDYL-PROLYL CIS-TRANS ISOMERASE B	3.58E+02 ± 0.09E+02
IPI00647704	CDNA FLJ41552 FIS, CLONE COLON2004478, HIGHLY SIMILAR TO PROTEIN TRO ALPHA1 H, MYELOMA	4.44E+03 ± 0.13E+03
IPI00654755	HEMOGLOBIN SUBUNIT BETA	3.50E+02 ± 0.26E+02
IPI00719373	IGL@ PROTEIN	1.05E+03 ± 0.64E+03
IPI00735451	IMMUNOGLOBULIN HEAVY CHAIN	5.89E+01 ± 0.52E+01
IPI00745872	ISOFORM 1 OF SERUM ALBUMIN	1.18E+04 ± 0.03E+04
IPI00783024	MYOSIN-REACTIVE IMMUNOGLOBULIN HEAVY CHAIN VARIABLE REGION (FRAGMENT)	4.58E+02 ± 1.64E+02
IPI00783287	IMMUNGLOBULIN HEAVY CHAIN VARIABLE REGION (FRAGMENT)	7.86E+02 ± 6.45E+02
IPI00783625	ISOFORM 1 OF SERPIN B5	2.42E+02 ± 0.26E+02
IPI00783987	COMPLEMENT C3 (FRAGMENT)	1.70E+03 ± 0.25E+03
IPI00784985	IGK@ PROTEIN	4.40E+03 ± 1.21E+03
IPI00829827	13 KDA PROTEIN	2.55E+02 ± 1.55E+02
IPI00876888	CDNA FLJ78387	4.40E+03 ± 1.06E+03
IPI00896380	ISOFORM 2 OF IG MU CHAIN C REGION	2.62E+03 ± 0.21E+03
IPI00903112	CDNA FLJ36533 FIS, CLONE TRACH2004428, HIGHLY SIMILAR TO LACTOTRANSFERRIN (FRAGMENT)	2.99E+03 ± 0.38E+03
IPI00916434	ANTI-(ED-B) SCFV (FRAGMENT)	1.84E+03 ± 0.35E+03
IPI00930442	PUTATIVE UNCHARACTERIZED PROTEIN DKFZP686M24218	1.20E+03 ± 0.61E+03
IPI00940245	IMMUNOGLOBULIN HEAVY CHAIN VARIANT (FRAGMENT)	4.29E+03 ± 0.45E+03
IPI00947285	SUPRABASIN ISOFORM 1 PRECURSOR	7.16E+02 ± 1.58E+02
IPI00985211	SIMILAR TO VH-3 FAMILY (VH26)D/J PROTEIN	1.53E+02 ± 0.14E+02
IPI01011189	UNCHARACTERIZED PROTEIN	3.53E+03 ± 0.47E+03

Interestingly, about 87.1% (81 out of 93) of the proteins indexed in Table 1 is also found in the Korean WS proteome catalogue recently reported by our group and this portion is even larger than the inner-system repeatability of the standardized protein identification system (about 70-80%).[9,14] Although it is difficult to provide an exact explanation on this observation, the failure of the identification of some low abundance proteins by the consequence of their dilution during saliva pooling and/or the removal of some identified low abundance proteins, whose levels were found to be out of the dynamic range of the method, from the list may contribute to the high overlap ratio. Additionally, Table 1 includes some of common salivary proteins frequently observed in salivary proteomics (amylase, cystatins, mucins, lactotransferrin, lysozymes, and albumin).[8,9,15] However, eight groups of common salivary proteins such as acidic proline rich proteins, basic proline rich proteins, carbonic anhydrase, peroxidases, statherines, thymosins, defensins, and histatins are missing in Table 1 and some of its reasons might be protein degradation into peptides, binding of the degraded peptides to tissues, protein loss during sample preparation, and/or the under-sampling problem induced by sample complexity.[8,9,15,16]

The range of protein levels observed in the present study was from 5.89×10^{1} ng/mL (immunoglobulin heavy chain) to 1.59×10^{4} ng/mL (α-amylase 1) and resulting levels of high abundance-expected proteins, whose levels in gland-

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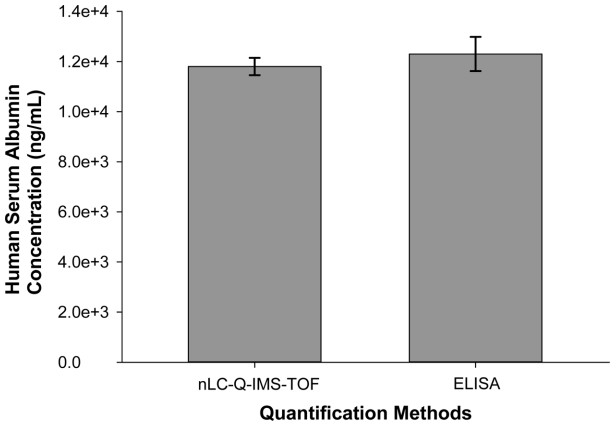

Figure 1. Comparison of human serum albumin level in a pooled whole saliva sample collected from 11 healthy Korean male adults and 11 healthy Korean female adults resulted from nLC-Q-IMS-TOF with that resulted from ELISA

specific saliva were known to be higher than 1.0 mg/mL, such as IgG, MUC5B, amylase, cystatins, albumin, MUC7, transferrin, and lysozyme, were much lower than 1.0 mg/mL in the pooled Korean WS sample (Table 1).[11] Even the total protein concentration of the pooled Korean WS sample was 0.936 ± 0.017 mg/mL (data not shown) and the dilution of individual proteins from various salivary glands by their mixing in WS can be a probable explanation for their much lower levels in WS than those in specific gland saliva.

For the validation of the present results, HSA in the pooled WS sample was quantitated by ELISA and its observed level was compared with that from the global absolute protein quantitation. As shown in Figure 1, $1.23\times10^4 \pm 0.07\times10^4$ ng/mL of HSA in the pooled Korean WS sample was confirmed from the ELISA study and no significant difference was observed from its comparison with that from the nLC-Q-IMS-TOF study ($1.18\times10^4 \pm 0.03\times10^4$ ng/mL, n=3, p=0.309).

Conclusions

The pooled Korean WS sample was analyzed by the nLC-Q-IMS-TOF system employing MS^E and 93 proteins were identified and quantitated. The resulting levels of proteins were ranging from 5.89×10^1 ng/mL (immunoglobulin heavy chain) to 1.59×10^4 ng/mL (α-amylase 1) and the present global absolute protein quantitation results were successfully validated by the comparison of its HSA quantitation result with that from the ELISA study. To our knowledge, this is the first global absolute quantitation of proteins in human WS and information from the present study can be widely used as the first level reference for the discovery of new protein biomarkers from human WS as well as for quantitative applications of human WS proteins.

Acknowledgments

The authors would like to thank Ms. Erica Oh (Waters Korea), Mr. Paul Park (Waters Korea), Mr. Hyo Chun Lee (Dankook University), and Mr. Dong Yoon Kim (Dankook University) for their technical support. This work was supported by a grant (2014R1A1A1004674 to YSC) of the National Research Foundation of Korea funded by the Korean Government.

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