Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Issue 3, 2012

Research Article

ESTABLISHMENT OF 2-D PROTEOMIC MAP FROM HUMAN SALIVA

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Received: 13 Dec 2012, Revised and Accepted: 19 Jan 2012

ABSTRACT

Saliva is increasingly used as a diagnostic fluid due to its relatively simple and minimal invasive collection. In order to create a reference map for comparative studies on normal and pathological condition of human diseases, globally expressed salivary proteins were studied using two-dimensional electrophoresis. To investigate the effect of meal taking on the proteome profile, the whole saliva samples were collected from an individual before and after meal. In addition, the variation among different individuals was also investigated. Two-dimensional electrophoresis results had separated around 400 protein spots. Comparative proteomic analysis of human salivary proteins before and after meal showed that 17 protein spots were altered. Out of these, two spots were found to decrease and 15 spots were found to increase. Whereas from the comparative proteomic profile among four different individuals revealed only three differentially expressed proteins. These findings suggested that the variation of 2-D proteome map among different individuals may not be significant. However our observation had shed light on important aspects that have to be considered before commencing salivary proteomic studies especially related to the timing of saliva sampling, ethnicity, individuality, oral hygiene, eating and dietary habits and even environmental factors.

Keywords: Saliva, Proteomics.

INTRODUCTION

Human saliva contains proteins exhibiting various biological activities, for example food digestion (amylase, peptidase, maltase etc.), protection functions in oral cavity and gastrointestinal tract (immunoglobulins, cystatins, lysozymes etc.). Some of them involve in the regulation of cardiovascular system (histamine, kallikrein etc.), hemopoiesis (erythropoietin, thymocyte transforming factors etc.) and nervous system (nerve growth factors etc.)[1]. Therefore, human saliva has great potential for clinical disease diagnosis[2]. In addition, oral cavity also plays an important role in drug delivery[3-4]. Saliva is relatively easy to collect and the costs of storage and shipping are lower than serum and urine[5]. This is particular crucial for collecting blood or urine samples from children, elderly, mentally handicapped and intravenous drug abuser[6].

Saliva varies greatly both intra- and inter-individual may be due to genetic polymorphisms, environmental factors, health conditions, oral hygiene and others. The presence of bacteria, epithelial cells and leukocytes further complicates the analyses of whole saliva[7]. Because of the involvement of various factors, variation among individuals is expected to be high. To our knowledge, there are no previous reports on how many proteins are common and how many are differentially expressed among individuals. In this study, our intention was to establish a preliminary normal human salivary 2-D reference map through two-dimensional electrophoresis. Besides that, we also investigated whether gender or food intake would lead to variation in this proteomic profile. This reference map will be significantly important for salivary biomarker discovery and diagnosis of human diseases in the future.

MATERIAL AND METHODS

Saliva collection

Saliva was collected from four volunteers, two females (Korean & Chinese) and two males (Korean & Indian) between the ages 24 and 31 (mean age 26.5). After tooth brushing, the whole saliva samples were collected by spitting into a vessel then kept on ice. Samples were centrifuged at 15,000 rpm for 15 minutes in a refrigerated centrifuge. The supernatants were transferred into a new tube without disturbing the undissolved materials. To compare the effect of food intake on the proteomic map, saliva was collected from one female volunteer then the sample was processed as above.

Sample preparation

Saliva proteins were concentrated by precipitation at -20°C overnight in 10% trichloroacetic acid and 0.1% dithiothreitol (DTT). The concentrated proteins were then centrifuged at 15,000 rpm for 15 minutes at 4°C. The pellet was washed with 800 µl ice-cold acetone and followed by centrifugation at 10,000 rpm for 5 minutes. This washing step was repeated once. The pellet was air-dried for 5 minutes and then resuspended in 350 µl of lysis buffer (8 M urea, 2% CHAPS, 1% DTT, 2% Pharmalyte 3-10). Protein concentration was determined using Bradford method.

Two-dimensional gel electrophoresis

For the first dimension, 50-60 µg proteins in 350 µl of rehydration buffer (8 M urea, 2% CHAPS, 0.4% DTT, 0.5% Pharmalyte 3-10, 0.1% bromophenol blue) were subjected to isoelectric focusing (IEF) on Pharmacia IPG strips (pH 3-7, 18 cm). The IPG gel strips were rehydrated with sample-containing rehydration buffer on IPGphor strip holder and covered by DryStrip cover fluid. IEF was carried out at 20°C on IPGphor using the following program: 10 V for 12 hours, 100 V for 1 hour, 500 V for 1 hour, 1000 V for 1 hour, 8000 V for 3 hours and final extension at 8000 V.

Prior to second-dimensional electrophoresis, IPG strips were immersed in equilibration buffer (50 mM Tris-HCl, pH8.8, 6 M urea, 30% glycerol, 2% SDS) containing 20 mM DTT for 20 minutes and 4% iodoacetamide in the same equilibration buffer for another 20 minutes. The equilibrated strips were placed on 12% polyacrylamide gel and the separation was performed overnight at 80 V in the running buffer (25 mM Tris, pH8.8, 192 mM glycine, 0.1% SDS).

Gel staining and image analysis for 2D analysis

Firstly, the gel was washed in deionized water for 5 minutes. Gels were incubated in fixative solution for 1 hour, then in wash solution for 2 hours. Sensitization solution was added after washing the gel in deionized water for 5 minutes. After 30 minutes, the gels were washed three times in deionized water for a few minutes. Silver impregnation was performed in silver nitrate-containing solution for 30 minutes, followed by washing in deionized water for 1 minute each. The image was developed by immersing the gel in developer solution for around 1 minute and finally in stop solution for at least 30 minutes. The stained gels were scanned using GS-710 densitometer (Bio-Rad, CA). The digitalized gel images were normalized and comparatively analyzed using PDQuest program (Version 6.1).

Protein Identification

For protein identification, gels were stained using SeePico CBB kit (Genebiosis, Korea). After electrophoresis, gels were processed according to the manufacturer's protocol. Proteins of interest were

excised and washed with 100 µl deionized water for 15 minutes. After removing the water, the gels were incubated in 40 µl of acetonitrile/water (50:50), followed by 40 µl of acetonitrile until the gel pieces were white and sticky. The gels were rehydrated with 40 µl of 100 mM ammonium bicarbonate for 5 minutes and acetonitrile for 15 minutes. The solution was discarded and samples were dried in speed-vac for 2 hours. Reduction and alkylation were carried out by incubating the gels in 40 µl of 10 mM dithiothreitol/100 mM ammonium bicarbonate at 56°C for 45 minutes. This was followed by 40 µl of 10 mM iodoacetamide/100 mM ammonium bicarbonate. This step was performed in dark for 30 minutes. Then the gels were dehydrated and rehydrated in 40 µl of 100 mM ammonium bicarbonate and 40 µl of acetonitrile respectively. After drying the gels, proteins were digested with 2 µl trypsin (sequencing grade, Promega, USA) for 45 minutes on ice. Samples were incubated at 37°C overnight after adding of 10 µl of 50 mM ammonium bicarbonate/5 mM calcium chloride. Supernatant was transferred into a new 1.5 ml tube. Proteins were extracted with 50 µl of 60% acetonitrile/40% of 0.1% trifluoroacetic acid. The extraction step was repeated twice. The pooled samples were dried using speed-vac. The peptides were dissolved with 0.1% trifluoroacetic acetic and ready subjected to liquid chromatography mass spectrometry.

RESULTS AND DISCUSSIONS

2-D proteome map of human saliva

This study was carried out to establish a 2-D proteome map from health human saliva by taking into consideration two factors, the variation before and after meals and also the variation due to different individual. We understand that different time of samples collection may contribute to the variation of this 2-D profile because the foods that we are taking daily consist of various microorganisms. In addition, different health condition among individuals will also give different 2-D maps. Therefore, before constructing a standard 2-D proteome map for normal person, this study was initiated to see how these two factors could possibly contribute to the variation of saliva 2-D profile.

After two-dimensional separation, around 400 protein spots were reproducibly detected using PDQuest software (Fig. 1). Our 2-D gel showed similar pattern of spot distribution compared to the 2-D proteomic profiling reported by Huang[8]. By referring to this, we had managed to identity roughly some spots without doing protein identification. In Table 1, the protein spots observed in this project had been grouped according to the group of proteins identified by Huang. There were ten groups of protein, such as albumin, α-amylase, enolase, catalase, Ig gamma-1 chain c region, glyceraldehyde-3-phosphate dehydrogenase, Ig kappa chain, Ig J chain, prolactin-inducible protein, and cystatin. The resolution of our 2-D gel was higher because some of the single spots in Huang's had been clearly separated in this study. Besides that, many spots that had not been reported by Huang were also observed in the range of 31-45 kDa.

To identify the proteins, seven protein spots were excised from the gel, washed to remove the silver stain and subjected to in-gel trypsin digestion. The resulting peptides were then analyzed by LC-MS. Some of the spots (a, f, g) were selected for identification based on their abundance and had been reported by Huang. From LC-MS result (Table 2), these seven protein spots correspond to 3 different proteins, they are α-amylase, Ig kappa chain and albumin. Surprisingly, all of the other 4 spots (b, c, d and e) were found to be α-amylase 2B precursor. The theoretical M_r and p*I* for α-amylase 2B precursor were 57.7 kDa and 7.1 respectively. However, the differences between observed and theoretical M_r and p*I* may be due to the result of posttranslational modification and protein isoforms[8-9]. Similarly, this phenomenon was also observed by Hu et al.[10].

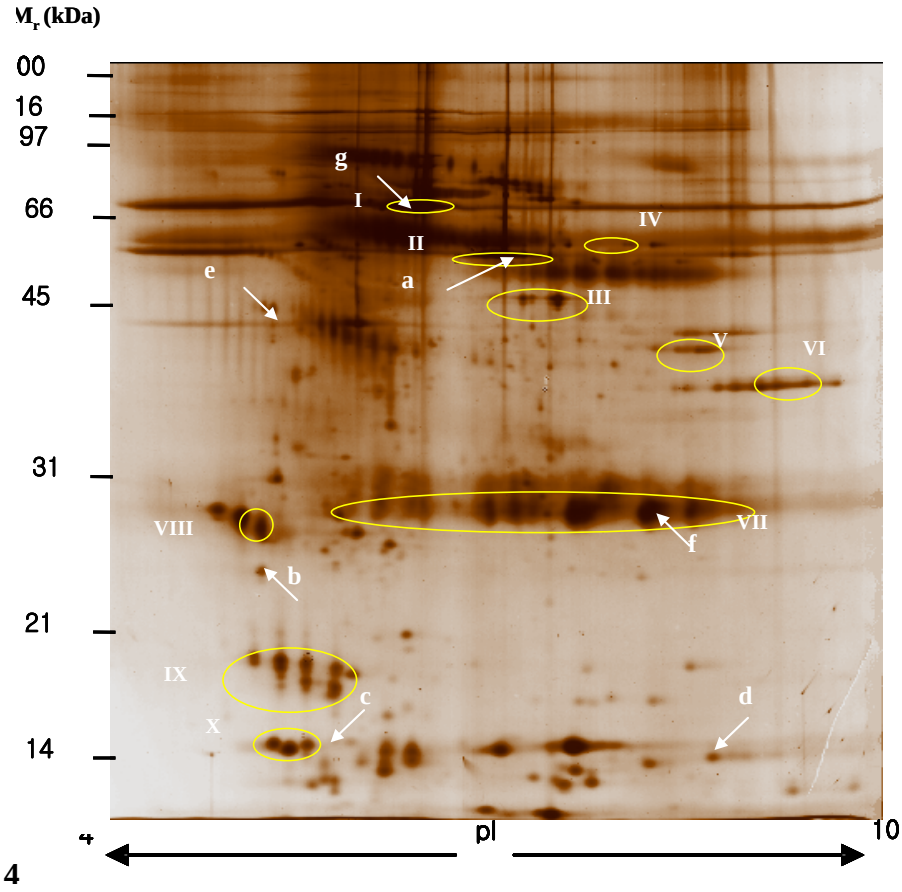

Fig. 1: Two-dimensional proteome map of human saliva. Proteins were grouped into ten groups (I-X) according to distributed pattern shown in Huang's paper. Seven protein spots (a-g) were selected for protein identification by LC-MS.

Table 1: Protein spots grouped into ten groups based on protein distributed pattern reported by Huang[8]

Protein description	Group	Mr (kDa)	pI
Albumin	I	66.2	6.4
α-Amylase	II	55.0	7.0
Enolase	III	43.8-45.0	7.1-7.4
Catalase	IV	58.1	7.5
Ig gamma-1 chain c region	V	40.7	8.4-8.6
Glyceraldehyde-3-phosphate dehydrogenase	VI	37.6	9.0-9.3
Ig kappa chain	VII	29.0-29.7	6.1-8.8
Ig J chain	VIII	27.8-28.3	4.9-5.1
Prolactin-inducible protein	IX	18.3-20.0	5.1-5.9
Cystatin	X	14.0-17.0	5.3-5.5

Table 2: Salivary proteins identified by 2-DE/LCMS

Spot	Protein description	M_r (kDa)	pI
a	α-amylase 2B precursor	55.0	7.0
b	α-amylase 2B precursor	25.6	5.9
c	α-amylase 2B precursor	15.0	6.3
d	α-amylase 2B precursor	14.4	9.0
e	α-amylase 2B precursor	43.6	6.1
f	Ig kappa chain	28.6	8.6
g	Serum albumin precursor	66.2	6.4

Proteomic analysis of human saliva before and after meal

In an attempt to study whether proteins present in saliva would be altered after meal, saliva samples were collected from a volunteer before and after meal. 2-D electrophoresis had been repeated twice for both samples collected before and after meal. By using the PDQuest software, the correlation coefficiency for sample collected before and after meal was 0.73 (data not shown). Therefore, the variations caused by samples taken before-after meal are not significant. However, 2-D gels revealed that 17 protein spots with altered levels were shown in after meal saliva samples (Fig. 2). Out of 17 spots, the protein level of 2 spots (spot 1 & 2) were found to decrease and spot 3-17 were found to increase compare to before meal. We do not know the identity of these 17 spots; based on our literature search they have not been reported. However, the protein identification of these spots will need to be performed in order to know their potential or significant contributions towards the establishment of normal human 2-D proteome map.

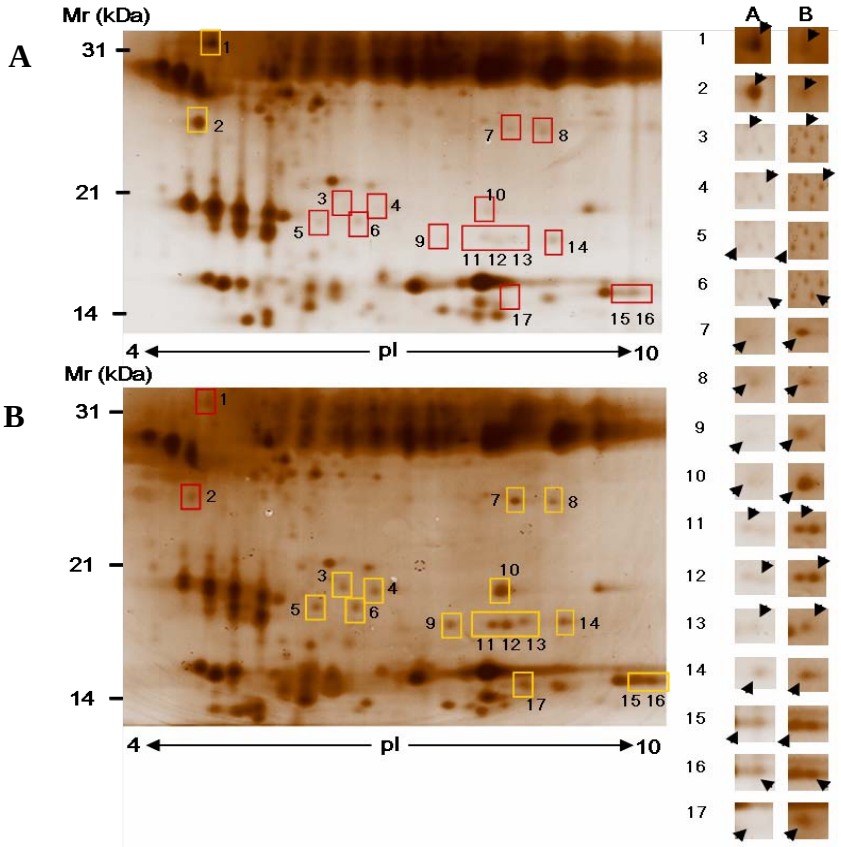

Fig. 2: Comparative proteome analysis between saliva samples collected before (A) and after (B) meal.

Proteomic analysis of human saliva from different individuals

To aid in future studies, saliva from two females (F1 & F2) and two males (M1 & M2) were collected to study the variation of protein expression among individual. From the correlation coefficiency analysis via PDQuest software, the range of value among these four volunteers was 0.63-0.73 (data not shown). We also observed different expression levels of some protein spots (Fig. 3). These protein spots were grouped into A, B and C. Based on the protein profile reported by Huang, the identity of spot A and B are thioredoxin (Trx) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) respectively. We have yet to identify spot C. However, all these three spots need to be verified again by LC-MS in order to confirm their identities.

We found that female participants showed higher protein expression of protein B (GAPDH) as compare to male. Interestingly, F1 and M1 were found to have higher intensity of spot A (TRX) and C than F2 and M2. Both saliva samples of F1 and M1 were collected from Korean volunteers while samples F2 and M2 were contributed by a Chinese and an Indian respectively. These findings suggested that genetic polymorphisms and dietary habit may contribute to the variation of protein expression.

For the two "known" proteins, thioredoxin is a cellular redox-active protein that catalyzes dithiol/disulfide exchange reactions, thus controlling multiple biological functions, including cell growth-promoting activity, enhancement of cytokine activities, and defense against cellular damage caused by oxidative stress[11]. High levels of thioredoxin expression have been detected in biopsies of human colorectal, gastric and lung cancers[12].

GAPDH is a key glycolytic enzyme and plays pivotal role in energy production. Recently, a number of diverse non-glycolytic activities of GAPDH in mammalian cells have been reported although the physiological importance of these activities is not clear. For example in membrane fusion, microtubule bundling, nuclear tRNA transport, uracil-DNA glycosylase and phosphorylating activities[13]. This enzyme was found to involve in apoptosis also, where overexpression of GAPDH and GAPDH antisense oligonucleotides prevent apoptosis[14-15]. Although protein C is still unknown, but we speculate that protein C as well as TRX and GAPDH possessed the potential as a diagnostic properties.

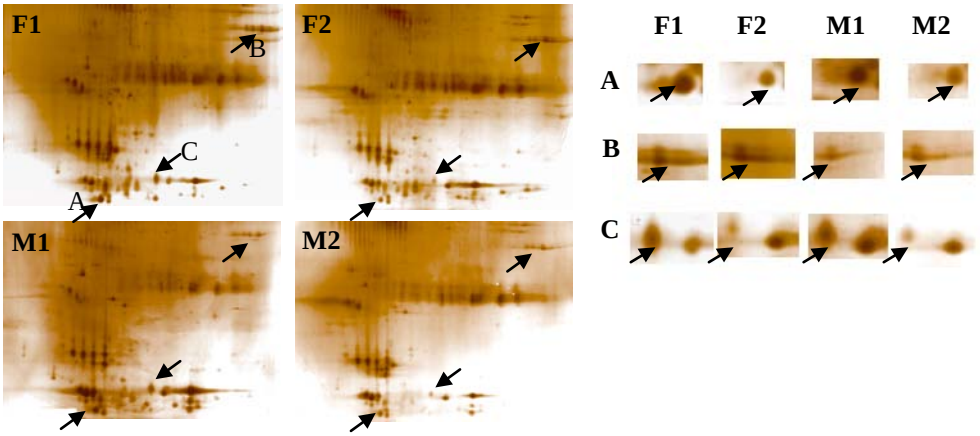

Fig. 3: Comparative proteomic analysis between saliva samples collected from four different individuals, two female (F1 & F2) and two male (M1 & M2). Proteins with differentially expression were labeled as A, B and C.

CONCLUSION

In summary, we have performed a preliminary study of proteomic analysis of human saliva. More than 400 protein spots were detected by silver staining. To establish a standard 2-D proteome map for human saliva, a thorough identification and examination of salivary proteins and factors that may cause variation such as genetic polymorphisms, environmental factors, ethnicity, individuality, health conditions, and oral hygiene need to be taken into account. Furthermore, a full understanding of the roles of salivary protein in the oral biology will warrant the reliability of using saliva for diagnosis of hereditary disorders, autoimmune diseases, malignant and infectious diseases.

ACKNOWLEDGEMENT

The author thank Medicinal Biochemistry Laboratory and Mass Spectrometry Laboratory of Gwangju Institute of Science and Technology (GIST), Korea and Universiti Malaysia Sabah for supporting this work.

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