Analytica Chimica Acta 722 (2012) 63–69

Contents lists available at SciVerse ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Method development for proteome stabilization in human saliva

Hua Xiao[a,*], David T.W. Wong[a,b,c,d,e,*]

[a] Dental Research Institute, University of California, Los Angeles, CA 90095, United States
[b] School of Dentistry, University of California, Los Angeles, CA 90095, United States
[c] Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA 90095, United States
[d] Division of Head and Neck Surgery/Otolaryngology, University of California, Los Angeles, CA 90095, United States
[e] Henry Samueli School of Engineering, University of California, Los Angeles, CA 90095, United States

ARTICLE INFO

Article history:
Received 30 November 2011
Received in revised form 18 January 2012
Accepted 12 February 2012
Available online 19 February 2012

Keywords:
Human saliva
Proteome
Protein stabilization
Immunoassay
Clinical application

ABSTRACT

Human saliva is a biological fluid with emerging early detection and diagnostic potentials. However, the salivary proteome suffers from rapid degradation and thus compromises its translational and clinical utilities. Therefore, easy, reliable and practical methods are urgently required for the storage of human saliva samples. In this study, saliva samples from healthy subjects were collected and stored at room temperature (RT) and 4 °C for different lengths of time with and without specific protein stabilization treatments. SDS-PAGE was run to compare the protein profiling between samples. Reference proteins, β-actin and interleukin-1 β (IL1β), were chosen to evaluate salivary protein stability. Immunoassay was used for the detection of these target proteins. All data was compared with the positive control that had been kept at −80 °C. The results show that the salivary proteome that has been stored at 4 °C with added protease inhibitors was stable for approximately two weeks without significant degradation. By adding ethanol to the samples, the salivary proteome was stabilized at RT. After optimization, a simple, robust and convenient method is developed for the stabilization of proteins in human saliva that does not affect the downstream translational and clinical applications. The salivary proteome could be stabilized without significant degradation by adding ethanol at RT for about two weeks. This optimized method could greatly accelerate the clinical usage of saliva for future diagnosis.

1. Introduction

Human saliva is a biological fluid with translational and clinical potentials. It has several key advantages for disease diagnosis and prognosis, including non-invasiveness, minimum cost, easy sample collection and processing, and thus provides an attractive alternative for blood, serum or plasma. In addition, it is known that saliva is the mirror of human body, and many blood constituents are reflected in saliva [1].

Human saliva is secreted from three pairs of major salivary glands: parotid gland, submandibular gland and sublingual gland. It harbors a wide array of proteins, which can be informative for the detection of oral diseases (e.g. oral cancer [2] and Sjögren's syndrome [3]) and systematic diseases (e.g. breast cancer [4,5] and lung cancer [6]). Profiling the proteins in saliva over the course of disease progression could reveal potential biomarkers indicative of different stages of diseases, which may be useful in early detection and/or medical diagnosis [7]. With advanced instrumentation and developed analytical techniques, proteomics is widely envisioned as a unique and powerful approach for biomarker development. As proteomic technologies continue to mature, proteomics has the great potential for salivary proteomic biomarker development and its further clinical applications [8,9].

Centrifuged saliva samples are routinely stored at −80 °C. Such methodology is impractical for field applications or in daily clinic operation. In the extensive utilization of human saliva for clinical applications, a major challenge is to stabilize and maintain the integrity of the salivary proteome for diagnostics [10,11]. This is because salivary proteome suffers from rapid degradation and thus compromises its translational and clinical utilities. Salivary protein profiles differ significantly from those of glandular salivary secretions [12]. A major cause appears to be the rapid proteolysis of the prominent members of the salivary protein families by resident endogenous proteases [12,13]. Different protease inhibitors are usually added during sample collection to prevent proteolysis. When discovering a simple and convenient method for protein stabilization in human saliva, different temperature and pH conditions were also included since it will largely affect the proteolysis [14,15]. A recent report has found that even inhibitor's cocktail could not

* Corresponding authors at: Dental Research Institute, School of Dentistry, University of California-Los Angeles, 10833 Le Conte Avenue, 73-017 CHS, Los Angeles, CA 90095, United States. Tel.: +1 310 206 3048; fax: +1 310 825 7609.
E-mail addresses: xiaohuacn@gmail.com (H. Xiao), dtww@ucla.edu (D.T.W. Wong).

0003-2670/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2012.02.017

prevent histatin 5, statherin, or PRP1 degradation in whole saliva [15].

A previous report showed that glycerol could stabilize protein and prevent protein aggregation [16]. This method is commonly used for the preservation of protein and antibody. However, the added glycerol would cause complications in the downstream analysis. In our previous work, we have found that RNAprotect® Saliva Reagent (QIAGEN Inc., Valencia, CA) provided effective concurrent stabilization to salivary protein, DNA and RNA at RT [10]. Even though, this method requires saliva samples mix with five-time reagent, which introduced more extra work to the downstream analysis. Ethanol could replace ordered water molecules around exposed hydrophobic groups, which surround the non-polar side chains of the proteins and thus increase the stability of proteins [17]. Amylase contributed to almost 60% of total salivary proteome [18], which may largely affect the stability of salivary proteins. Besides, amylase removal may increase the stability other salivary proteins and eases the characterization of low abundant proteins [7].

The hypothesis of this study is that the salivary proteome could be stabilized at RT and further used in translational and clinical applications. The aim of this work is to discover an easy, reliable and practical method for the stabilization of salivary proteome. It is expected that this method will accelerate the extensive clinical application of human saliva. Different methods of protein stabilization, including adding protease inhibitors or ethanol, denaturing and removing amylase, were evaluated. A robust method was optimized to maximally slow down the protein degradation in saliva by adding ethanol at RT. This method will be a great impetus for salivary diagnostics in the early detection of many diseases.

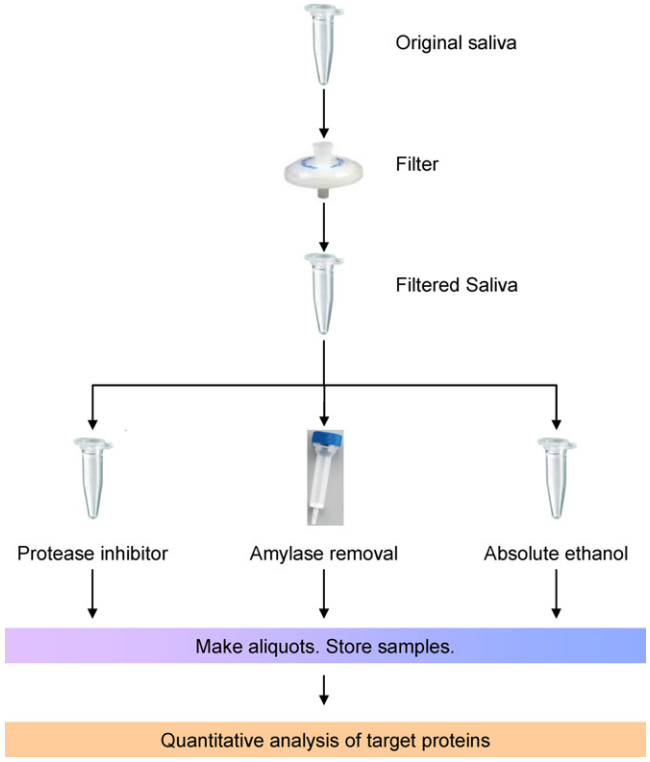

Fig. 1. Schematic diagram of the saliva sample collection and experimental design.

2. Materials and methods

2.1. Sample collection and processing

Saliva samples were collected from 10 healthy subjects under the approved institutional review board protocols (IRB#10-000505) [5,6]. Written informed consent forms and questionnaire data sheets were obtained from all participants. None of the subjects had any history of malignancy, immunodeficiencies, autoimmune disorders, hepatitis, and/or HIV infection, and had a mean age of 35 years. Subjects were asked to refrain from eating, drinking or using oral hygiene products for at least 1 h prior to collection. After rinsing their mouths with water, 5 mL saliva was collected from each subject into a 50 mL Falcon tube. These saliva samples were filtered with a 0.45 μm PVDF membrane (Millipore, Billerica, MA, USA) to remove cells and any debris. The flow through was collected. During the sample preparation, saliva samples were always kept on ice. Fig. 1 is the schematic diagram for the sample preparation.

Filtered saliva samples were then aliquoted into microcentrifuge tubes and stored at RT, 4 °C and −80 °C, respectively, after the four different treatments described as following: (I) saliva samples with protease inhibitors were prepared, aliquoted and placed at RT and 4 °C for storage. All the samples were made up with distilled water to keep the same volume. An aliquot saliva sample that had been stored at −80 °C with added protease inhibitors was used as positive control in all the experiments. Protease inhibitor stock solution was prepared by adding 1 Roche complete tablet (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) into 1 mL distilled water. For every 1 mL saliva, 20 μL stock solution was added and briefly mixed by vortex. (II) Saliva amylase depletion was conducted according to a previous report [18]. Briefly, saliva samples were eluted from starch column to deplete amylase specifically. (III) For protein denaturing experiments, saliva samples were either boiled at 95 °C for 10 min or by adding 20-time volumes absolute ethanol (Fisher Scientific, NJ, USA). Denatured samples were kept at RT for two weeks. The saliva proteins were then precipitated by centrifugation at 20,000 g for 20 min. (IV) For the non-denaturing method, every 20 μL absolute ethanol was added to 100 μL saliva. All the samples were made up to equal volume with distilled water. At different time points, 1 aliquot of saliva sample that has been kept at RT or 4 °C was moved into a −80 °C freezer and stored until further analysis.

2.2. Protein concentration measurement

The protein concentration of each saliva sample was measured by using the BCA Protein Assay Kit (Thermo Scientific Pierce, IL, USA). Equal volume of each sample was loaded into a 96 well plate in duplicates. The experiment was performed according to the manufacturer's instruction and the plate was read at 562 nm.

2.3. SDS-PAGE and western blot

Equal volume of each saliva sample was used for SDS-PAGE and western blot. For SDS-PAGE, the 10% Bis-Tris gel was run at 150 V in MES SDS Running Buffer for 1 h. Pre-stained protein standard (Invitrogen, CA, USA) was used to track protein migration. The gel was then stained with simple blue (Invitrogen, CA, USA). For western blot [5], saliva proteins were run and transferred to a PVDF membrane using the iBlot (Invitrogen, CA, USA). The membrane was incubated with the primary antibody (mouse monoclonal antibody to actin, Sigma–Aldrich, St. Louis, MI, USA) and then incubated with the secondary antibody (anti-mouse IgG, peroxidase-linked species-specific whole antibody from sheep) according to manufacturer's instructions, for 1 h at RT. Finally, the membrane was washed and visualized using ECL Plus detection kit (GE Healthcare, WI, USA).