ISSN (Paper) ISSN
Vol 0, No.0, 2016

RESEARCH PAPER

Journal of Al-Qasim Green University

Study of human saliva profile variations of diabetic patients by SDS polyacrylamide gel electrophoresis

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ABSTRACT

The polyacrylamide gel electrophoresis technique applied in this study was a rapid and simplified method, requiring only minute amounts of saliva for the analysis. Saliva samples were collected from patients suffering from diabetes mellitus and a control group represented by normal individuals. Samples were submitted to SDS-PAGE without previous concentration. The protein profiles of whole saliva of diabetic and non-diabetic were compared. Considerable variations between individuals in the protein profiles were observed. The saliva from diabetic patients appeared to have distinct and unique profile of proteins compared with normal counterparts. The density and number of bands of diabetic saliva were greater then that found in non-diabetic saliva.

Keywords*:, human, PAGE, profile, Saliva, SDS*

Abbreviations: EDTA, ethylene diamine tetra-acetic acid. PAGE, polyacrylamide gel electrophoresis. rpm, revolution per minutes. SDS, sodium dodecyl sulfate. TEMED, N,N,N,N′-tetramethylethylenediaminutese.

INTRODUCTION

Human salivary proteins have been studied by electrophoresis in denaturing and non-denaturing polyacrylamide gel electrophoresis (PAGE) as well as by isoelectric focusing (IEF) and two-dimensional procedures, and the clinical applications of this have been reviewed (١).

For determination of many endogenous substances and diseases, saliva is equally suitable or sometimes even superior to plasma or serum as a diagnostic medium (2). The major advantage of saliva is that it can be obtained non-invasively. There are a large number of proteins in human saliva with the concentration ranging from ~0.50 to 3.0 mg/ml. a-Amylase and proline-rich proteins which comprising -25% and ~65% respectively, are the major proteins of glandular origin. The remaining ~ 10% consists of other proteins which include lactoferrin, histatins, cystatins and immunoglobulins. Most of the proteins are salivary gland specific (e.g. -amylase, proline-rich proteins, histamins) and are synthesized within the salivary glands but some, such as albumin, are derived from serum (3). The use of saliva for clinical and diagnostic purposes has opened up a new era in salivary analysis. It has been demonstrated that the composition and flow rate of saliva are altered in several systemic diseases such as Sjogren's syndromes and cystic fibrosis (4). Essential hypertension (5), diabetes mellitus (6), sarcoidosis and inflammatory disease of the salivary glands (7) are known to alter the protein composition of saliva.

Human saliva contains a large number of proteins which can be separated using polyacrylamide gel electrophoresis (PAGE). Electrophoretic methods such as sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been used in the analysis of saliva (8). The salivary analysis, as compared to blood analysis, is more attractive because it offers a simple rapid non-invasive method which is

ISSN (Paper) ISSN
Vol 0, No.0, 2016

suitable for short- and 1ong-term monitoring of pathological disorders and drug therapy. Finestone *et al*. has reported that PAGE of parotid saliva using Buffalo Black staining showed that the y-globulin level of the diabetic patients is higher than that of the non-diabetic subjects, as indicated by diffuse bands in the y-globulin region of the saliva samples from diabetic patients. In our study, the protein in whole saliva of non-diabetic subjects and diabetic patients separated on SDS-PAGE using Coomassie-blue R 250 staining were studied and compared (9).

The present study was designed to explore the possibility of using discontinuous SDS PAGE as diagnostic aid in the laboratory to differentiate electrophoreticaly between the normal patients and the diabetic counterparts.

MATERIALS AND METHODS

Reagents: acrylamide was from Thomas Baker, India. Ammonium persulfate, bromophenol blue, glycine, and N,N´- Methylenebisacrylamide were from BDH, England. Boric acid was from LUDECO, Belgium. Coomassie brilliant blue G-250 was from Sigma, USA. Deionized distilled water was from Iraqi company of medical treatment, Iraq. Disodium ethylene diaminutese tetra-acetic acid (sodium EDTA) was from Scharlau, European Union. Ethanol and 2-mercaptoethanol were from Riedel, USA. Glacial acetic acid was from G.C.C., England. Glycerol was from Watania, Jordon. Sodium dihydrogen phosphate was from Laboratory Rasayan – India. Isopropanol was from Thomas Baker, India. Sodium thiosulfate and Sodium dodecyl sulfate (SDS) were from Carlo Erba reagents, India. TEMED (N,N,N′,N′-tetramethylethylenediaminutese) was from SERVA, Germany. Tris-base was from Fluka, Switzerland.

Apparatus: the equipment used for one-dimentional gel electrophoresis and the preparation of polyacrylamide was from Cleaver Scientific Ltd., UK. This device was described elsewhere (10).

Saliva collection

Saliva samples were donated by diabetes mellitus patients in Hilla city. Normal or non-diabetic saliva samples were obtained from donors who had no history of diabetes mellitus. Saliva samples were collected by expectoration. The samples were incubated in ice within I hour of collection, until centrifuged for 20 min. at 5000 x g and 4˚C. The clear supernatant was collected and stored at -20˚C until SDS-PAGE get ready.

Electrophoresis:

The electrophoresis was taken place in the dual vertical electrophoresis unit (10 × 10 cm gel plates with 1mm spacers).

1. Gel preparation: Laemmli discontinuous method (11) was considered with some modifications, Gel dimensions were 10-cm wide by 8-cm length and 0.1-mm thickness .

Resolving gel preparation and pouring: (The resolving gel concentration used in these experiments was 12%). 4 ml of 29/1 acrylamide – bisacrylamide (30%) stock solution was mixed with 6 ml 1 x of resolving gel buffer (0.1% SDS, 0.375 M tris-HCl, pH 8.8) and filtered by Whatman filter paper (No. 1). Freshly prepared 100 µl (10%) ammonium persulfate and 10 µl TEMED were added to the gel solution. The mixture was mixed briefly (that depends on the quality of ammonium persulfate and TEMED used). 10 ml syringe was used, which was inverted, to expel any air entered syringe barrel. Using the same syringe, the acrylamide gel solution was expelled into the space between the two glass plates carefully on the margin of the glass plates to avoid generating air bobbles formed. Gel pouring was continued until the gel level reached 1 cm below the region where the comb teeth finish (which was provided enough space for pouring of stacking gel). 1 ml of isopropanol was added at the top of the gel to exclude oxygen from the surface. The acrylamide gel solution was allowed to polymerize for

ISSN (Paper) ISSN
Vol 0, No.0, 2016

30 minute at room temperature. Before pouring stacking gel, distilled water and any remaining un-polymerized gel was drew.

Stacking gel preparation and pouring: (The stacking gel {0.125 M, pH 6.8, 0.1% SDS}concentration used in these experiments was 5 %). 0.5 ml acrylamide – bisacrylamide (30%) stock solution was mixed with 2.5 ml (1x) stacking gel buffer (0.1% SDS, 0.125 M, pH 6.8,) and filtered by Whatman filter paper (No. 1). Freshly prepared 30 µl (10%) ammonium persulfate and 5 µl TEMED were added to the gel solution. The acrylamide gel solution was mixed briefly. 5 ml syringe was used, which was inverted. Using the same syringe, the acrylamide gel solution was expelled into the space between the two glass plates carefully. Once the acrylamide gel solution reached its estimated level at the top of the notched glass plates, the comb teeth was inserted carefully from one end of the glass plates (45˚) to avoid generating air bobbles. Since the stacking gel polymerization takes longer time compared to resolving gel counterpart, the acrylamide gel solution was allowed to polymerize for 50 minutes at room temperature.

2. Loading samples: After removing the comb, 10 µl (4X) loading buffer (40% glycerol, 20% 2-mercaptoethanol, 12% SDS, 50% 4x stacking tris, 0. 3% bromophenol blue) was added to 30 µl of saliva sample. The mixture was mixed briefly, and boiled immediately for 2 minutes at 80˚C. To remove the precipitants, all saliva samples except one were centrifuged for 1 minutes at 12000 rpm in a microcentrifuge at 17˚C . 25µl of each sample was loaded into the wells of the gel.

3. Gel electrophoresis conditions: Electrophoresis was taken place in mini-slab gel (purchased from CleaverScientific – UK) and performed at room temperature using pre-cold 1X electrophoresis buffer (0.1% SDS, 0.144% glycine, and 0.003% tris base {wt/vol each} pH 8.3) at 75 V / 10 mA for stacking gel at the beginning of run (0 hour), 75 V / 5 mA for stacking gel – resolving gel attachment point (30 minutes), and 100 V / 15 mA for resolving gel until the tracking dye reached three quarts of the resolving gel. When electrophoresis was completed, the gel slab and the reservoir buffer were relatively warm, but Rodger and Holmes (1979) mentioned that such warming did not appear to be harmful to the resolution (12).

4. Staining by Coommasie brilliant blue G-250: The Standard staining method of Sambrook and Rushell (13) was considered with some modifications correlated with the dilution of Coommasie brilliant blue G-250 dye concentration to 10 folds (14). Polyacrylamide gel was placed in 100 ml Coommasie staining solution (0.025 % w/v Coommasie brilliant blue, 45 % methanol, 10 % glacial acetic acid) and gently rotated (revolution per one second) at room temperature on a rotary shaker (Karl Kalb – Germany) for 30 minutes. During shaking, stacking gel was discarded. When intensely stained bands appeared against a relatively clear background, the resolving gel was submerged in 100 ml of Coommasie destaining solution (45 % methanol, 10 % glacial acetic acid) and rotated in the same conditions for 30 minutes. Gel was positioned in a thin white plate and picture was taken immediately by 7.2 M.P. digital camera (Sony – China).

RESULTS AND DISCUSION

All papers that dealt with saliva by SDS-PAGE used sophisticated, tedious and multistage, concentration of saliva samples with cost effective devices like column fractionation (15), or lypholization (16), or gel filtration chromatography (17). In this study, no previous concentration of applied saliva samples in electrophoresis was used. This, in turn, was simplified the procedure and minimized the time required for pre-electrophoresis preparations. The possibility of electrophoresis of non-concentrated saliva specimens was familiarized SDS-PAGE utilization in routine clinical laboratories. The only step in this study happened before electrophoresis for saliva specimens represented by centrifugation, which is very important to remove any insoluble materials to avoid its interference in results.

ISSN (Paper) ISSN
Vol 0, No.0, 2016

The un-concentrated electrophoresed saliva specimens showed significant variation between the normal and diabetic saliva. This results appeared in-coincidence with the result of Hoe *et al.*, 1997 (18).

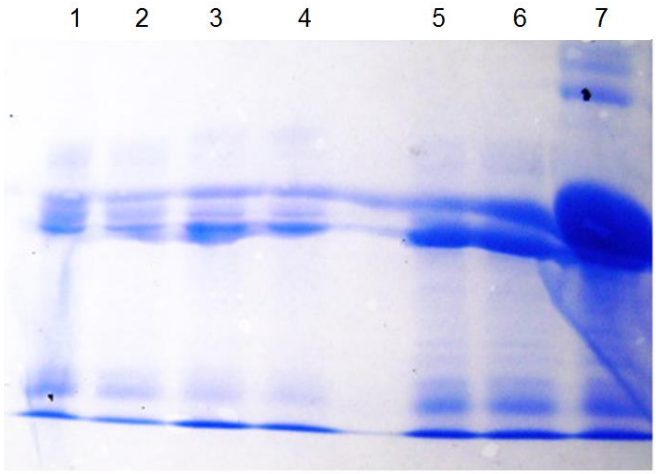

Figure (1): Electrophoresis of saliva specimens on SDS-discontinues polyacrylamide gel, 5% stacking gel (discarded) and 12% resolving gel. All lanes were run at 100 V/15mA for resolving gel. Saliva specimens were made visible after staining with 0.025% Coomassie brilliant blue G-250. Lanes 1 - 4: saliva of non-diabetic individuals , lanes 5 – 6 centrifuged saliva of diabetic patients, lane 7: un-centrifuged saliva of diabetic patient. The direction of electrophoresis was from top to bottom.

There are some dense bands detected on the gel for diabetic patients. This study showed that the distribution of these diabetic bands despite of being consistent with non-diabetic bands but they were showed to have more dense bands than the others. In addition, the saliva from diabetic patients showed to have more bands in low molecular weight region (Fig I. Lanes 5-7), while saliva from non-diabetic subjects (Fig. 1. Lane 1-4) showed less variable bands at this region. Hoe *et al*, (1997) mentioned that these bands were reported to be the proline-rich proteins and they has metachromatic effect. The metachromatic effect was found to be specific for Coomassie Blue R-250. If

All the non-diabetic samples were showed approximately six blue stained protein bands. Some considerable individual-to-individual variations were observed. Certain bands which were strongly stained in some individuals were either less prominent or not detectable in others.
the Coomassie Blue G-250 is used, the metachromasia only occurs when the destaining solution is free from organic solvent (19).

SDS-PAGE has some disadvantages correlated with the collection of saliva where many patients were reluctant to spit for sample collection. The high viscosity, potential discoloration, and particles from food intake could cause discomfort for the potential sources of interference during processing. So particular procedures should be taken to overcome these difficulties (20). Represented by collection of samples after at least two hours of any meal, and centrifuging them to get rid off the insoluble particles. As it was seen in sample 7, the absence of centrifugation of saliva sample will interfere fiercely with the results of the gel (figure 1), pre-electrophoretic centrifugation step was a necessary step to avoid such irregular results.

Since the SDS-PAGE reflect the real image of every protein profile, diabetic saliva were showed more dense bands then the normal saliva counterparts. Despite of the individual variations that identified in figure 1, there was a unique salivary profile was determined for diabetic patients in such away it could be possible to conclude that these ultra-unique profile for these samples to estimate the level of diabetic progression in the body. Thus, more intensive researches should be undertaken to give us more details about how much the diabetic sample was aggressive in this case.

I recommend using two dimensional gel electrophoresis in addition to SDS-PAGE to more possibly prognoses the aggressiveness of diabetes in the sample submitted to examination. But this study proved that SDS-PAGE alone could give prominent clue about the existence of diabetes in a noninvasive, inexpensive, and comfortable method. Since the extreme sensitivity SDS-PAGE it is recommended to

apply this technique to predict the early stages of diabetes through reading salivary protein profile variations.

Acknowledgements: I am grateful to the dean of college of science for women Dr. Abdul-Kareem Al-Bermani for his continuous financial support for biotechnology laboratory in which this study has taken place.

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