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Protein Precipitation Method for Salivary Proteins and Rehydration Buffer for Two-Dimensional Electrophoresis

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Abstract: Precipitants for salivary proteins and rehydration buffers for two-dimensional electrophoresis (2-DE) analysis were, respectively compared and evaluated. Five different protein precipitants: TCA, TCA-acetone-DTT, TCA-acetone-mercaptoethanol, acetone and alcohol were used to precipitate proteins of the saliva samples. The efficiency of the precipitants was evaluated from protein content of the precipitate reflecting protein recovery. The precipitate with the highest protein content was subsequently solubilized using different rehydration buffers (RB1, RB2, RB3 and RB4) before being subjected to the 2-DE. The efficiency of the different rehydration buffers was compared with respect to the resolution and focusing time taken to attain the maximum voltage. Each of the saliva samples was subjected to the above experiments, carried out in triplicates. The precipitant containing TCA-acetone-DTT exhibited the highest protein recovery (82.2%) demonstrating significant difference when compared with the other precipitants ($p<0.05$). The RB4 containing DTT (reducing agent) and 0.5% IPG buffer 3-10 non-linear (carrier ampholyte) exhibited more protein spots indicating better separation resolution. The results obtained suggested that protein recovery depends on the precipitant used in the precipitation and resolution of proteins separation is influenced by the reducing agent and the ampholyte used in the rehydration buffer.

Key words: Salivary proteins, precipitants, reducing agent, carrier ampholytes

INTRODUCTION

Human saliva has great potential for clinical diseases diagnostics and prognostic monitoring (Hofman, 2001; Lawrence, 2002). Saliva testing is noninvasive, simple, safe to handle and cost effective (Tabak, 2001; Mandel, 1990) and the relationship of saliva with plasma levels make saliva an attractive diagnostic tool.

Two-Dimensional Electrophoresis (2-DE) is capable of resolving thousands of proteins in a single separation procedure and together with Mass Spectrometry (MS) have allowed the analysis and identification of human saliva proteins. There has been an increasing interest in the study of human proteome and exploration of the use of salivary protein biomarkers for the detection of human disease such as cancers and autoimmune diseases. Therefore, analysis and cataloguing of the human salivary proteome is a necessary first step to identify potential protein biomarkers in saliva (Xie *et al*., 2005; Hu *et al*., 2005).

Analysis of salivary proteins requires a number of aspects to consider. These include the standardization of saliva collection, sample preparation which includes precipitation procedure and protein precipitant and rehydration buffer for solubilizing the precipitate prior to the 2-DE. The standardization of salivary collection plays a significant role in saliva analysis because several factors may affect salivary flow and composition (Dawes, 2004). Human saliva is easy to collect, but careful attention must be considered to limit variation in sample integrity. Unstimulated whole saliva is more representative of the oral milieu and can be collected by passive drooling (no oral movements), allowing the saliva to drain off the lower lip into a container or by spitting directly into a container (Nurkka *et al*., 2003; Dawes *et al*., 2001; Hodinka *et al*., 1998). Spitting is much easier but specimens collected by spitting may contain up to 14 times more bacteria than those collected by drooling (Nurkka *et al*., 2003). These bacteria may secrete protease which can lead to the degradation of salivary proteins and hence will affect the analysis of salivary proteome.

Sample preparation is one of the most crucial steps for high quality resolution of protein separation and a great number of spots of proteomic maps in the 2-DE. Protein samples should be free of salt and other disturbing agents such as ionic detergents, nucleic acids,

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lipids, phenolic compounds and have an appropriate concentration to be suitable for 2-DE. Protein concentration and desalting method by precipitation is generally employed to separate proteins from non-protein contaminants. Precipitation has an advantage over dialysis or desalting methods in that it enables concentration of the protein sample and also purification from undesirable or interfering substances.

Protein samples need to be efficiently solubilized to produce a maximum number of well-resolved and detectable protein spots in a 2-DE analysis. Thus, proteins in the saliva need to be solubilized, disaggregated, denatured and reduced (Shaw and Riederer, 2003). Mixtures of chaotropic compounds, detergents or surfactants, reducing agents and carrier ampholytes are employed (Molloy, 2000). Chaotropes (urea and thiourea) are used to disrupt the hydrogen bonding, leading to protein unfolding and denaturation. Detergents such as 3-[(3-Cholamidopropyl) dimethylammonio] -1-propane-sulfonate or CHAPS, Sulfobetaine 3-10 (SB3-10) and Triton-X 100 act synergistically with chaotropes to prevent interaction and aggregation of proteins. Reducing agents such as dithiothreitol (DTT) and tributylphosphine (TBP)) are used to break intramolecular and intermolecular disulfide bonds. Carrier ampholytes are used to enhance protein solubility by minimizing protein aggregation due to charge-charge interaction (Shaw and Riederer, 2003) and produce an approximately uniform conductivity across a pH gradient without affecting its shape.

There were two objectives in this study. The first was to compare and select the protein precipitant and precipitation method which will give high protein recovery from saliva samples, as indicated by high protein content. The second was to evaluate the efficiency in the 2-DE analysis, of the different rehydration buffers used to solubilize the protein precipitates prepared using the selected precipitation method as reflected by resolution of the protein separated.

MATERIALS AND METHODS

Chemicals: Chemicals for 2-D gel electrophoresis including acrylamide, bis-acrylamide, Sodium Dodecyl Sulphate (SDS), Tetramethylenediamine (TEMED), ammonium persulfate, thiourea, dithiothreitol (DTT), Immobilized pH Gradient (IPG) strips pH 3-10 non-linear, 13 cm, Immobilized pH Gradient (IPG) buffer 3-10 non-linear were purchased from GE Healthcare (Piscataway, NJ, USA). Urea, CHAPS, TCA, mercaptoethanol, ethanol, acetone, Tris base were purchased from Sigma (St Louis MO, USA) and Complete™ Protease inhibitor Cocktail from Roche, Mannheim, Germany.

Saliva collection: Three volunteers were asked to contribute their saliva samples. Each one of them was asked to abstain from eating and drinking for 2 h before saliva collection and to rinse their mouth with sterile MilliQ water before saliva collection. Whole unstimulated saliva was collected by spitting directly into a pre-chilled, sterile 15 mL falcon tube through sterile, pre-chilled funnels on ice and kept on ice throughout the collection procedure. The samples were kept on ice during the collection procedure. Brief description of the physical appearance of the saliva was recorded and the volume and the flow rate were also recorded. Complete™ Protease Inhibitor Cocktail (Roche) was added to the samples to prevent protein degradation during sample preparation. The saliva processing and storage from the time of collection was limited to 2 h. The collection of saliva samples from volunteers has got the approval of the ethical committee (No. DF OB0703/0004(L)).

Pretreatment of saliva samples for the protein precipitation and solubilization studies: Sterile Milli Q water was added to the saliva sample (1:1 v/v) and vortexed vigorously to reduce the viscosity of the saliva. The sample was then centrifuged at 4,500 rpm for 15 min at 4°C to remove any unwanted particles (e.g., debris or cells). The supernatant with the added Complete™ Protease inhibitor Cocktail was collected, aliquoted in 500 μL aliquots and stored at -80°C for use in the following experiments.

Experimental procedures for the comparison and selection of saliva protein precipitant and precipitation methods: Five different precipitants and precipitation methods were employed: Precipitant and precipitation method A (TCA only), Precipitant and precipitation method B (TCA/acetone/DTT), Precipitant and precipitation method C (TCA/acetone/mercaptoethanol), Precipitant and precipitation method D (acetone only) and Precipitant and precipitation method E (absolute alcohol only). Each of the precipitants and precipitation methods was performed in triplicates for each of the saliva samples.

Precipitant and precipitation method A: The experiment was carried out using a combination of the methods described by Chen *et al.* (2002), Manadas *et al.* (2006) and Gehrke (2006). Five hundred microliter of pretreated saliva aliquot was mixed with 500 μL of TCA (20% w/v) and the mixture was vortexed to mix thoroughly and allowed to precipitate overnight at -20°C. This was followed by centrifugation at 15,000 rpm, 4°C for 30 min (Chen *et al.*, 2002). The supernatant was decanted and the pellet obtained was washed twice with 200 μL of cold acetone. For each wash, the pellet suspended in the cold acetone

was sonicated for 5 min or more until the whole pellet was fully broken to form pellet suspension (Manadas *et al*., 2006). The pellet suspension was then placed at -20°C for 20 min (Gehrke, 2006) and subsequently centrifuged in a 4°C refrigerated-centrifuge for 5 min at 15,000 rpm. The acetone-containing supernatant was then decanted and the pellet obtained was dried in a SpeedVac for 5 min to remove any residual acetone. The pellets prepared were then stored at -80°C until ready for further use in protein estimation and if necessary, in the 2-DE analysis.

Precipitant and precipitation method B: The experiment was carried out as described above except that acetone and DTT were added to the TCA and the washing procedure was modified. Five hundred microliter of pretreated saliva aliquot was mixed with 500 μL of TCA (20% w/v) -acetone (90% v/v) - DTT (20 mM) mixture, vortexed to mix thoroughly and allowed to precipitate overnight at -20°C. This was followed by centrifugation at 15,000 rpm, 4°C for 30 min. The supernatant was decanted and the pellet was washed twice. In this method, the precipitate obtained was first washed with 200 μL cold acetone (90% v/v) containing 20 mM DTT and the second wash with cold acetone (80% v/v) containing 10 mM DTT (Gehrke, 2006). The subsequent procedure was similar to what was described above and stored until for use in the protein estimation and, if necessary in the 2-DE analysis.

Precipitant and precipitation method C: The experiment was carried out as described above except that acetone and mercaptoethanol were added to the TCA and the washing procedure was modified. Five hundred microliter of the pretreated saliva aliquot was added to 500 μL of TCA (20%)/acetone (90%)/2-mercaptoethanol (0.07%) mixture. The mixture was vortexed to mix thoroughly, incubated overnight at -20°C and centrifuged at 15,000 rpm, 4°C for 30 min. The supernatant was decanted and the pellet was washed twice with 200 μL of cold acetone containing 0.07% 2-mercaptoethanol. The subsequent procedure was similar to what was described above and stored until further use for protein assay and if necessary, in the 2-DE analysis.

Precipitant and precipitation method D: The experiment was carried out according to a modification of the method described by Jiang *et al*. (2004). Two hundred and fifty microliter of the pretreated saliva aliquot was mixed with three volumes of ice cold acetone (90% v/v). The mixture was vortexed to mix thoroughly, incubated overnight at -20°C and centrifuged at 15,000 rpm, 4°C for 30 min. The supernatant was discarded and the pellet was dried in the SpeedVac for 5 min and stored for further use such as in protein assay and if necessary, in the 2-DE analysis.

Precipitant and precipitation method E: The procedure described in precipitant and precipitation method D was repeated except that the acetone was replaced with absolute alcohol. Two hundred and fifty microliter of the pretreated saliva aliquot was mixed with three volumes of cold absolute ethanol. The mixture was vortexed to mix thoroughly, incubated overnight at -20°C and centrifuged at 15,000 rpm, 4°C for 30 min. The supernatant was then discarded and the pellet dried in the SpeedVac for 5 min and stored for further use such as in protein assay and if necessary, in the 2-DE analysis.

Assay for protein estimation: The protein pellets obtained in all of the above methods were pretreated with 10 μL of 0.2 M NaOH for 2 min at room temperature prior to the addition of 250 μL of rehydration buffer (7 M Urea, 2 M Thiourea, 4% CHAPS). This was because the pellets are fairly insoluble in acidic condition (Nandakumar *et al*., 2003). The solubilized protein precipitate was then left at room temperature for 1 h and vortexed periodically every 10 min, followed by centrifugation at 10,000 rpm for 10 min at 10°C to remove any insoluble materials. The supernatant collected was then used in the assay for protein content according to the Bradford protein assay method (Bio-Rad) (Bradford, 1976).

Preparation of the rehydration buffers: Rehydration buffer was used to solubilize the protein preciptates and to rehydrate the gel strips used in 2-DE. Rehydration buffer can also be referred as solubilization buffer.

Four rehydration buffers RB1, RB2, RB3 and RB4 were prepared accordingly and their compositions are as given in Table 1.

Table 1: Composition of the four rehydration buffers

	Rehydration Buffers (RBs)			
Chemical	RB1	RB2	RB3	RB4
Chatropes	5 M Urea 2 M Thiourea	5 M Urea 2 M Thiourea	7 M Urea 2 M Thiourea	7 M Urea 2 M Thiourea
Detergents	4% CHAPS 2% SB3-10	4% CHAPS 2%SB3-10	4% CHAPS	4% CHAPS
Reducing agent	5 mM TBP	65 mM DTT	65 mM DTT	65 mM DTT
Carrier ampholytes	1.6% pH 5-8 Bio-lytes	1.6% pH 5-8 Bio-lytes	1.6% pH 5-8 Bio-lytes	0.5% IPG buffer
	0.4% pH 3-10 Bio-lytes	0.4% pH 3-10 Bio-lytes	0.4% pH 3-10 Bio-lytes	pH 3-10 NL
References	Modified from Huang (2004)	Modified from Huang (2004)	Modified from Ryu *et al*. (2006)	Modified from Ryu *et al*. (2006)

RB1 was prepared according to a modification of the method described by Huang (2004). One of the detergents used in the preparation of the buffers, the 2% SB3-10 cannot dissolve in 7 M urea and hence in this study, the urea concentration was reduced to 5 M, a concentration which allows the detergent to dissolve. RB2 was prepared by the modification of RB1, in which the reducing agent TBP (5 mM) was replaced with DTT (65 mM). RB3 was prepared according to a modification of the method described by Ryu *et al.* (2006). In the preparation of this buffer, the concentration of the reducing agent DTT (0.5% DTT) was increased to 65 mM DTT and the carrier ampholyte 2% pH 3-10 pharmalyte was replaced with 1.6% pH 5-8 Bio-lytes, 0.4% pH 3-10 Bio-lytes. RB4 was a modification of the RB3 in which the carrier ampholyte 0.5% IPG buffer with pH 3-10 non-linear (NL) gradient was used instead of the 1.6% pH 5-8 Bio-lytes, 0.4% pH 3-10 Bio-lytes. RB1 and RB2 differ in the reducing agent while RB3 and RB4 differ in the ampholyte used in the preparation of the buffers.

Determination of the protein resolution in Two-Dimensional Electrophoresis (2-DE) with different rehydration buffers (RBs): From the analysis of protein content, the protein precipitated using precipitant and precipitation method B was selected and used further in the 2-DE analysis. The dried protein pellets were solubilized according to the procedure described for protein analysis, that is, by pretreating the precipitate with 10 μL 0.2 M NaOH for 2 min at room temperature prior to the addition of 250 μL rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS). The solubilized protein precipitate was left at room temperature for 1 h and vortexed periodically every 10 min, followed by centrifugation at 10,000 rpm for 10 min at 10°C to discard any insoluble materials. The supernatant was subsequently used in the 2-D gel electrophoresis analysis.

For isoelectrofocusing (IEF), 13 cm IPG strips pH 3-10 NL gradient were rehydrated overnight at room temperature with 250 μL of RB1, RB2, RB3 and RB4, respectively; each containing an estimated 50 μg protein under mineral oil. IEF was performed at 20°C on IPGphor™ IEF System (Amersham Bioscience) with the following parameters: 500 V for 1 h, 1000 V for 1 h and 8000 V for 2 h and 30 min (steady-state-level; total of 20 kV). The current was limited to 50 μA/strip. Upon completion of the first dimension, the strips were stored at -20°C until analysis by SDS-PAGE.

The rehydrated strips were incubated with gentle shaking in an equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M Urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue dye) containing 65 mM DTT for 15 min and thereafter in the same buffer containing 13.5 mM iodoacetamide for 15 min. When the proteins were solubilized using RB1 containing TBP, the strips were incubated for 20 min in the same SDS equilibrium buffer as above except that DTT was replaced by 5 mM TBP. For second dimension, the strips were washed in SDS electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS), placed on top of 12.5% polyacrylamide gels containing SDS (SDS-PAGE) and sealed in place with sealing solution (0.5% low-melting agarose in SDS electrophoresis buffer). Gels were run using SE 600 Ruby set (Amersham Bioscience), fixed and proteins were detected by silver staining and then scanned using Image scanner. Results were analyzed using ImageMAsterTM 2-D Elite software (Amersham Bioscience).

RESULTS

Comparison of the protein content of the saliva samples prepared by different protein precipitation methods: The precipitant and precipitation method B gave the highest protein recovery (82.8%) followed by precipitant and precipitation method C (70.7%) and precipitant and precipitation method D (52.2%). The precipitant and precipitation method A and E gave less than 50 % protein recovery where the precipitant and precipitation method A gave 46.5% and the precipitant and precipitation E, 33.9% (Fig. 1).

The protein recovery between precipitant and precipitation method A and those of the precipitants and precipitation methods B, C, D and E was evaluated and compared. It was shown that the different precipitants and precipitation methods with the exception of between A and D and between B and C are statistically significant (Table 2).

Comparison of the protein resolutions obtained with different rehydration buffers used in the solubilization of the protein precipitate prior to 2-DE: The results obtained showed that the reducing agent 65 mM DTT in RB2 (Fig. 2b) compared to 5 mM TBP with respect to the

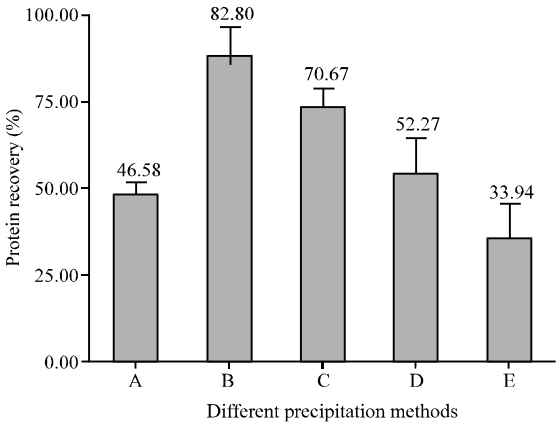

Fig. 1: The protein recovery with the different precipitation methods

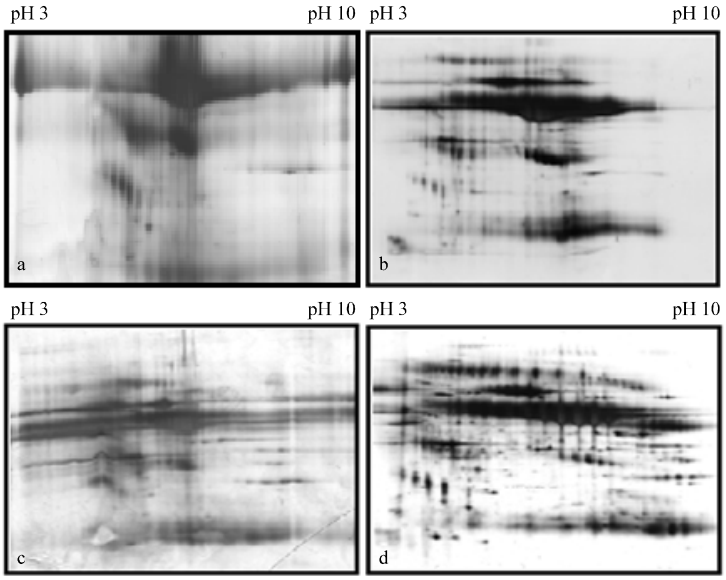

Fig. 2: The effect of different reducing agents: 5 mM TBP in RB1 (electrophogram a) and 65 mM DTT in RB2 (electrophogram b) and ampholyte carrier: 1.6% pH 5-8 Bio-lytes, 0.4% pH 3-10 Bio-lytes in RB3 (electrophogram c) and 0.5% IPG buffer NL in RB4 (electrophogram d) on protein resolutions in 2-D PAGE. 50 μg protein was solubilised in RB1 and RB2 which contain 5 M urea, 2 M thiourea 4% CHAPS, 2% SB 3-10 but differ in reducing agents and RB3 and RB4 which contain 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT but differ in ampholyte carriers on IPG strip pH 3-10, 13 cm

Table 2: Statistical analysis (ANOVA) of the protein recovery between different precipitants and precipitation methods (A, B, C, D and E) The precipitant and precipitation method B gave higher protein recovery than those using precipitant and precipitation method A, D and E

Methods		p-value	Significant
A	B	0.000	p<0.05
	C	0.000	p<0.05
	D	0.677	p>0.05
	E	0.035	p<0.05
B	A	0.000	p<0.05
	C	0.061	p>0.05
	D	0.000	p<0.05
	E	0.000	p<0.05
C	A	0.000	p<0.05
	B	0.061	p>0.05
	D	0.003	p<0.05
	E	0.000	p<0.05
D	A	0.677	p>0.05
	B	0.000	p<0.05
	C	0.003	p<0.05
	E	0.011	p<0.05
E	A	0.035	p<0.05
	B	0.000	p<0.05
	C	0.000	p<0.05
	D	0.011	p<0.05

RB1 (Fig. 2a) exhibited better separation of proteins. With ampholyte carrier, it was shown that 0.5% IPG buffer pH 3-10 NL (RB4) demonstrated the best protein separation with the highest number of spots (Fig. 2d) compared to 1.6% pH 5-8 Bio-lytes, 0.4% pH 3-10 Bio-lytes (RB3) (Fig. 2c). With the RB2 and RB4, less streaking of the protein spots was observed. It was also shown that the RB4 attained maximum voltage (8000 V) within the least focusing time (4 h and 23 min) (Table 3).

Table 3: The maximum voltage attained and the total focusing time in relation to the different reducing agents and ampholytes used in the rehydration buffers

RB	Maximum voltage reached (V)	Total focusing time (h:min)
1	5000	8:53
2	7000	5:23
3	1733	11:15
4	8000	4:23

DISCUSSION

The procedures frequently used to treat samples containing protein include using TCA as protein precipitant and washing the precipitate using ice-cold acetone, prior to solubilization of the precipitate for use in 2-DE analysis (Jiang *et al*., 2004; Guy *et al*., 1994). In this study, the efficiency of five different precipitants and washing procedures was evaluated to select the most efficient precipitation method for the preparation of saliva proteins prior to 2-DE. It was observed that the protein recovery using precipitant and washing procedure in

methods B and C is better compared to that in methods A, D and E, with method B (82.2%), giving the most protein recovery (Fig. 1). In method B, the saliva protein was precipitated using TCA containing acetone and DTT and the precipitate collected was washed twice with ice-cold acetone, each wash using different concentration of DTT and acetone and this is in accordance with the method described by Gehrke (2006). In method C, which gave 70.6% protein recovery, DTT was replaced with 2-mercaptoethanol. The presence of reducing agent (DTT and 2-mercaptoethanol respectively) in precipitant B and C demonstrates better protein recovery. The small difference observed in the protein recovery between the two methods could be due to the different reducing agent used. The use of TCA-acetone mixture and the addition of reducing agent is a combination of procedures advocated by Chen *et al*. (2002). From this result, the washed TCA precipitated protein from method B was subsequently selected for use in the subsequent study, that is, comparison of the protein resolution obtained in the 2-DE with different rehydration buffers. The rehydration buffers were compared with respect to the reducing agents (RB1 and RB2) and ampholytes (RB3 and RB4) used.

The protein precipitate was neutralized with sodium hydroxide prior to its solubilization in rehydration buffer. This has been recommended by Nandakumar *et al*. (2003), who claimed that the precipitate dissolved better after being neutralized by sodium hydroxide. From the results shown in Fig. 2, the RB4 showed to give a better protein resolution compared to the RB1, RB2 and RB3. The RB2 gave a better resolution compared to RB1 and RB3 but lesser number of spots compared to RB4. A good solubilization of protein may play a role in producing a good 2-DE gels protein resolution which otherwise may result in horizontal streaking (Fig. 2a, c).

The RB1 and RB2 have similar composition except for the reducing agent in which TBP was replaced with DTT in RB2. The DTT has been widely used as a reducing agent but it tends to be charged especially at alkaline pH causing the migration of protein off the IPG strip during IEF, which will result in loss of some proteins. In contrast, the TBP lacks a free thiol group, is neutral and hence does not migrate during IEF. This property of TBP helps to maintain the reducing condition over the entire IEF. However, TBP has a low solubility, is unstable, volatile and toxic (Rabilloud, 1996; Herbert *et al*., 1998; Molly *et al*., 1998; Berkelman and Stenstedt, 2002). A comparison of these reducing agents used in rehydration buffer to separate proteins from the same sample showed that DTT in RB2 gave better protein resolutions and separation compared to the TBP in RB1. Hence the results obtained in this study did not support the claim by Herbert *et al*. (1998) that TBP serves as a better reducing agent compared to DTT but is in accordance with the findings reported for myelin proteins and membrane proteins by Taylor and Pfeiffer (2003), Marqui *et al*. (2006) and Ruan and Wan (2007).

In this study, it was found that the 2% SB3-10 did not dissolve in the rehydration buffer containing 7 M urea and this was not observed by Huang (2004). The rehydration buffer was then modified by using a lower concentration of urea (5 M), the concentration that is considered the limit for the solubility of SB 3-10 (Herbert, 1999; Gorg and Weiss, 1999).

Comparison of the ampholytes used in the rehydration buffer with respect to protein separation and resolution and focusing time, showed that 0.5% IPG buffer pH 3-10 NL (RB4) is better than 1.6% pH 5-8 Bio-lytes and 0.4% pH 3-10 Bio-lytes (RB3). These two buffers used 7 M urea as they did not contain SB 3-10 and hence the solubility of SB 3-10 did not arise. Of all the RBs, the RB4 exhibited better efficiency and is cost effective compared to the other 3 RBs. This is because cost-wise, IPG buffer is much cheaper to purchase compared to the Bio-lytes that was used in the other 3 RBs.

In term of focusing time, the RB4 demonstrated better efficiency in which it only requires 4 h 23 min to attain the maximum voltage of 8000 V compared to the RB2 which required 5 h 23 min to only attain 5000 V. The RB2 which used Bio-lytes as the carrier ampholyte may not be conducive for the 2-DE. This is because the concentration of ampholyte used is high (1.6% biolyte 5-8 and 0.4% biolytes 3-10) which will affect the conductivity of the IPG strip. It has been reported that the concentration of IPG buffer or pharmalyte which is higher than 0.5% increases the conductivity of the IPG strip, resulting in a lower final voltage when the system is limited by the maximum current setting (Garfin, 2003). This could explain the longer focusing time observed for the RB1, RB2 and RB3.

CONCLUSION

The recovery of salivary proteins from the precipitation procedure is influenced by the type of precipitant and washing procedure employed. The resolution of protein separation in the 2-DE analysis is determined by the type of reducing agent and carrier ampholyte used in the rehydration buffer for the solubilization of the precipitates.

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REFERENCES

Berkelman, T. and T. Stenstedt, 2002. 2-D Electrophoresis Using Immobilized Ph Gradients. Principles and Methods. 1st Edn., Amersham Biosci., Uppsala, Sweden.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem., 72: 248-254.

Chen, S.T., T.L. Pan, Y.C. Tsai and C.M. Huang, 2002. Proteomics reveals protein profile changes in doxorubicin-treated MCF-7 human breast cells. Cancer, Lett., 181: 95-107.

Dawes, C., R.W. Tsang and T. Sueltlze, 2001. The effect of gum chewing, four oral hygiene procedures and two saliva collection techniques on the output of bacteria into human whole saliva. Arch. Oral. Biol., 46: 625-632.

Dawes, C., 2004. Factors Influencing Salivary Flow Rate and Composition. In: Saliva and Oral Health, Edgars M., C. Dawes and D.O'Mullane, (Eds.). British Dental Association, British, ISBN: 0904588742, pp: 32-49.

Garfin, D.E., 2003. Two-dimensional gel electrophoresis: An overview. Trenes. Analyt. Chem., 22: 263-272.

Gehrke, C.W., 2006. Total protein extraction for IEF and 2-D SDS-PAGE. proteomics.missouri.edu/protocols /IEF protein extraction (Nov 06).pdf.

Gorg, A. and W. Weiss, 1999. Analytical IPG-Dalt. Methods. Mol. Biol., 112: 189-195.

Guy, G.R., R. Philip and Y.H. Tan, 1994. Analysis of cellular phosphoproteins by two-dimensional gel electrophoresis: Applications for cell signaling in normal and cancer cells. Electrophoresis, 15: 417-440.

Herbert, B.R., M.P. Molloy, A.A. Gooley, B.J. Walsh, W.G. Bryson and K.L. Williams, 1998. Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent. Electrophoresis, 19: 845-851.

Herbert, B., 1999. Advances in protein solubilization for two-dimensional electrophoresis. Electrophoresis, 20: 660-663.

Hodinka, R.L, T. Nagashunmugam and D. Malamud, 1998. Detection of human immunodeficiency virus antibodies in oral fluids. Clin. Diagn. Lab. Immunol., 5: 419-426.

Hofman, L.F., 2001. Human saliva as a diagnostic specimen. Rev. J. Nutr., 131: 1621S-1625S.

Hu, S., Y. Xie, P. Ramachandran, R.R. Ogorzalek Loo and Y. Li *et al*., 2005. Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. Proteomics, 5: 1714-1728.

Huang, C.M., 2004. Comparative proteomic análysis of human whole saliva. Arch. Oral Biol., 49: 951-962.

Jiang, L., L. He and M. Fountoulakis, 2004. Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. J. Chromatogr. A, 1023: 317-320.

Lawrence, H.P., 2002. Salivary markers of systemic disease: Noninvasive diagnosis of disease and monitoring of general health. Can. Dent. Assoc., 63: 170-174.

Manadas, B.J., K. Vougas, M. Fountoulakis and C.B. Duarte, 2006. Sample sonication after trichloroacetic acid precipitation increases protein recovery from cultured hippocampal neurons and improve resolution and reproductivity in two- dimensional gel electrophoresis. Electrophoresis, 27: 1825-1831.

Mandel, I.D., 1990. The diagnostic uses of saliva. Rev. J. Oral. Pathol. Med., 19: 119-125.

Marqui, A.B., A. Vidotto, G.M. Polachini, M. Bellato Cde and H. Cabral *et al*., 2006. Solubilization of proteins from human node tissue and two-dimensional gel storage. J. Biochem. Mol. Biol., 39: 216-222.

Molloy, M.P., 2000. Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. Anal. Biochem., 280: 1-10.

Nandakumar, M.P, J. Shen, B. Raman and M.R. Marten, 2003. Solubilization of trichloroacetic acid (TCA) precipitated microbial proteins via NaOH for two-dimensional electrophoresis. J. Proteome. Res., 2: 89-93.

Nurkka, A., J. Obiero, H. Kaythy and A.G. Scott, 2003. Effects of sample collection and storage methods on antipneumococcal immunoglobulin A in saliva. lin. Diagn. Lab. Immunol., 10: 357-361.

Rabilloud, T., 1996. Solubilization of protein for electrophoretic analysis. Electrophoresi, 17: 813-829.

Ruan, Y. and M. Wan, 2007. An optimized procedure for solubilization, reduction, and transfer of human breast cancer membrane-enriched fraction by 2-DE. Electrophoresis, 28: 3333-3340.

Ryu, O.H., J.C. Atkinson, G.T. Hoehn, G.G. Illei and T.C. Hart, 2006. Identification of paratoid salivary biomarkers in Sjogren's syndrome by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional diference gel electrophoresis. Rheumatology, 45: 1077-1086.

Shaw, M.M. and B.M. Riederer, 2003. Sample preparation for two-dimensional gel electrophoresi. Proteomics, 3: 1408-1417.

Tabak, L.A., 2001. A revolution in biomedical assessment: The development of salivary diagnostics. J. Dent. Edu., 65: 1335-1339.

Taylor, C.M. and S.E. Pfeiffer, 2003. Enhanced resolution of glycosylphosphatidylinositol-anchored and transmembrane proteins from the lipid-rich myelin membrane by two-dimensional gel electrophoresis. Proteomics, 3: 1303-1312.

Xie, H., N.L. Rhodus, R.J. Griffin, J.V. Carlis and T.J. Griffin, 2005. A catalogue of human saliva proteins identified by free flow electrophoresis-based peptide separation and tandem mass spectrometry. Mol. Cell Proteomics, 4: 1826-1830.