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COMPARISON OF SDS-PAGE PROTEIN PROFILES OF COLORECTAL CANCER TISSUES EXTRACTED USING THREE DIFFERENT EXTRACTION BUFFERS

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Abstrak: Kanser usus merupakan satu daripada kebimbangan umum yang utama disebabkan peningkatan insidennya di seluruh dunia. Pendekatan proteomik boleh dijadikan suatu kaedah yang berguna dalam mengenali pembentukan kanser usus. Analisis ekspresi protein dalam tisu kanser usus dan tisu normal usus akan membawa kefahaman terhadap penyakit ini yang seterusnya boleh menambahbaikkan prognosisnya. Sifat setiap protein berbeza bergantung kepada komposisi asid amino dan lipatan protein. Oleh itu, tiada penampan ekstrak tunggal yang boleh mengekstrak kesemua protein. Dalam kajian ini, kami menggunakan tiga penampan ekstrak untuk mengekstrak protein daripada tisu-tisu usus yang dihancurkan. Profil Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) menunjukkan terdapat protein yang sama dan juga berlainan yang diekstrak oleh setiap penampan daripada tisu. Dengan mengecualikan protein heterogen yang bermigrasi kepada beberapa jalur dalam SDS-PAGE, lima protein dikenal pasti secara eksklusif dalam penampan-penampan ekstrak termasuk alfa-1-antitripsin dan pelopor haptoglobin dalam penampan Fosfat (Ph), keratin 20 dan *smooth muscle cell associated protein-1* (SMAP-1b) dalam penampan Lysis (Ly), dan actin-gamma 1 (ACTG1) protein dalam penampan Thiourea (Thio). Data-data yang didapati menunjukkan kombinasi penampan-penampan yang diperlukan dalam analisis proteomik bagi kanser usus.

Abstract: Colorectal cancer is one of the major public concerns due to its increasing incidents worldwide. Proteomics approach may serve as a useful tool in understanding the development of the disease. Analysis of protein expression in colorectal cancer and normal tissues will lead to the understanding of the disease that may improve its prognosis. The property of proteins varied dependent on their amino acid composition and folding. Therefore, there is no single extraction buffer possible to extract total proteins from the tissues. In this study, we used three extraction buffers to extract proteins from the homogenized colorectal tissues. The Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) profiles of the buffer extracts show common and distinct proteins extracted in each buffer. Excluding the heterogeneous proteins that migrated as multiple bands in SDS-PAGE, five proteins were identified exclusively in the buffers, namely alpha-1-antitrypsin and haptoglobin precursor in Phosphate (Ph) buffer, smooth muscle cell associated protein-1 (SMAP-1b) and keratin 20 in Lysis (Ly) buffer, and actin-gamma 1 (ACTG1) protein in Thiourea (Thio) buffer. The data indicates that the combination of buffers is needed in the proteomic analysis of colorectal tissues.

Keywords: Protein Extraction, Colorectal Cancer, Mass Spectrometric Analysis

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INTRODUCTION

Colorectal cancer is a worldwide public health concern and it is among the best characterized cancers in term of the genetic progression of the disease (Fearon & Vogelstein 1990). The frequency of colon cancer is ten times higher in developed countries than in the developing countries and it is 3.6 times higher in immunosuppressed patients (Birkeland *et al.* 1995). In Malaysia, cancer of colorectal ranks second most common cancers in both male and female. Amongst the three major ethnic groups, Chinese had the highest incident of colorectal cancer followed by Indians and Malays (Gerard & Halimah 2003).

In recent years, proteome analysis for diseases has gained great interest and is presently used as a tool in drug discovery to determine the biochemical processes involved in diseases (Fung *et al.* 2001). It is also used to monitor cellular processes by characterizing protein expression levels in cells (Blackstock & Weir 1999). The comparative characterization of protein patterns in tissues or body fluids of healthy individuals and patients has the potential to serve as the basis for new diagnostic tools development, and the design of disease specific therapy.

Extraction of proteins from tissues involved complex chemical processes due to the unique characteristic of each protein. Therefore, a combination of different types of detergents is needed to increase the solubility of the protein. In this study, proteins from cancerous and normal tissues were extracted using three types of extraction buffers that made up of a mixture of salt, chaotropic agents, detergents, polyols and protease inhibitor that were used to increase the solubility of proteins in the buffers. In addition, proteomics approach was used to identify the differentially expressed protein in colorectal cancerous and normal tissues.

MATERIALS AND METHODS

Samples Collection and Isolation

Human normal and cancerous colonic tissues were provided by Hospital Pulau Pinang. The normal tissues collected are the tissues adjacent to the tumor tissue, which is the tissue of the mucosa layer. The normal and cancerous tissues were confirmed by the hospital's pathologist. The tissues were collected after informed consent from the patients was obtained. The tissues were cut into small pieces, weighed and immediately stored at -70^{o}C freezer before analyzed. In this study, four sets of tissues (cancerous and normal) from four patients were analyzed. Patient MMN was at stage IV and with cancer grade well-differentiated adenocarcinoma while patients LKH, CCH and NSC were all of cancer grade moderately-differentiated adenocarcinoma and at stages of II, IV and III, respectively.

Protein Extraction Method
Five hundred milligrams of tissues was subjected to analysis. The deep-frozen colon tissue specimens were disrupted by grinding in a liquid nitrogen-cooled mortar until became powder-form. The powder-form-like tissues were aliquoted in separate Eppendorft tubes. The extraction procedures are described in the following sections. The concentration of protein was determined using RC DC protein assay kit (BioRad).

Phosphate Extraction Buffer
Phosphate extraction buffer (Ph buffer) was prepared according to Hjelmeland (1990) and was further optimized in our laboratory. The optimized Ph buffer contained 0.2 M phosphate buffer (pH 7.2), 150 mM NaCl, 8 M urea, 1% (w/v) CHAPS (detergent), 10% (v/v) glycerol and 2 mM EDTA. A 1000 μl of Ph buffer was added to 500 mg of the freshly homogenized tissue. It was then vortexed for 30 s until the tissue and the extraction buffer were thoroughly mixed. The mixture was then centrifuged (12 000 x g, 15^{o}C, 15 min). The supernatant was collected in aliquots of 50 μl and kept in –70^{o}C.

Lysis Extraction Buffer
Lysis extraction buffer (Ly buffer) was prepared according to O'Farrell (1975) Lysis buffer with slight modification. The composition of Ly buffer is made up of 9 M urea, 1% (w/v) DTT (dithiotreitol), 2% (w/v) CHAPS, 0.2% (v/v) carrier ampholytes and 10 mM EDTA. A 1000 μl of Ly buffer was added to 500 mg of the freshly homogenized tissue. It was then vortexed thoroughly for 30 s. The mixture was then centrifuged (12 000 x g, 15^{o}C, 15 min). The supernatant was stored in aliquots of 50 μl at –70^{o}C until analyzed.

Thiourea Extraction Buffer
A modified thiourea extraction buffer (Thio buffer) of Rabilloud (1998) was used. Thio buffer comprised of 2 M thiourea, 7 M urea, 1% (w/v) DTT, 2% (w/v) CHAPS, 0.2% (v/v) carrier ampholytes and 10 mM EDTA. A 1000 μl of Thio buffer was added to 500 mg of the freshly homogenized tissue. It was then vortexed thoroughly for 30 s. The mixture was then centrifuged (15^{o}C, 12 000 x g, 15 min). The supernatant was stored in aliquots of 50 μl at –70^{o}C until analyzed.

SDS-PAGE
The gel system used is of Laemmli (1970), discontinuous gel system consists of 12% resolving (separating) gel and 4% stacking gel. The gel dimension is 16 cm x 20 cm. Protein samples for SDS-PAGE separation was carried out by adding 20% (v/v) of sample buffer (0.5 M Tris-HCl, 10% (v/v) glycerol, 0.02% (w/v) SDS, 0.1% (w/v) Coomassie blue) to each protein extracts (1 mg) and vortexed for 30 s prior to loading onto the wells of polyacrylamide gel. The separation was carried out at a constant voltage of 200 V until the dye reached the resolving gel and then the voltage is increased to 245 V. The electrophoresis is completed when the tracking dye reached approximately 2 mm from the bottom of gel. The total running time was approximately 3.5 h. After electrophoresis, stacking gel was discarded and the gel was Coomassie blue stained for approximately one

hour with shaking followed by destaining overnight under shaking. Image of the gel was then taken using Versa Doc Imaging System Model 4000 (BioRad). Subsequently, analysis of gel image which includes molecular weight and bands intensity determination were performed using Quantity One Analysis Software.

In-Gel Digestion

The SDS-Polyacrylamide gel was washed thoroughly in 100 mM NH_4HCO_3 and the differentially expressed protein bands from either normal or cancerous colon tissues were then excised from the gel. In-gel digestion using trypsin was performed according to Gam and Aishah (2002). The gel pieces were first excised and shrunk by dehydration in acetonitrile. The solvent was then discarded and the gel pieces were dried in a vacuum centrifuge. A volume of 10 mM DTT in 100 mM NH_4HCO_3 sufficient to cover the gel pieces was added, and the protein was reduced for one hour at 56^oC. After cooling to room temperature, the DTT solution was replaced with a same volume of 55 mM iodoacetic acid in 100 mM NH_4HCO_3. After 45 min incubation at ambient temperature in dark with occasional vortexing, the gel pieces were washed with 50–100 μl of 100 mM NH_4HCO_3 for 10 min, dehydrated with acetonitrile, rehydrated in 100 mM NH_4HCO_3 and dehydrated in the same volume of acetonitrile. For each dehydration step, the gel was dried in a vacuum centrifuge. The gel pieces were swollen in digestion buffer containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$, and 12.5 ng/μl of trypsin in an ice-cold bath. After 45 min, the supernatant was removed and replaced with 10 μl of the same buffer but without trypsin to keep the gel pieces wet during enzymatic cleavage at 37^oC overnight. Peptides were extracted from the gel matrix by adding 15 μl of 20 mM NH_4HCO_3, vortexed and incubated at room temperature for 10 min and the supernatant was recovered after a brief spin. This was followed by adding (1 to 2 times the volume of gel pieces) 5% (v/v) formic acid in acetonitrile:water mixture (70:30), vortexed and incubated for 20 min at room temperature. It was then spun down and the supernatant was recovered. These steps were repeated three times. Pooled extracts were dried in a vacuum centrifuge and stored at –20^oC.

Mass Spectrometric Analysis

The dried sample was reconstituted in 30 μl of deionized water (Millipore, USA) and centrifuged (500 x g, 15^oC, 5 min). The supernatant was transferred to a clean vial and subjected to LC/MS/MS analysis. 5 μl of the sample was injected into a RPC-column (C_{18} 300 A, 5 μm, 1 x 150 mm) connected to a HPLC (1100 Series, Agilent, Germany). A capillary pump was used to pump the mobile phase at 10 μl/min flow rate, the linear gradient used was 5% B to 95% B in 65 min. Mobile phase A was 0.05% formic acid in deionized water and B was 0.05% formic acid in ACN. The HPLC was interfaced to an ion-trap mass spectrometer. The dry gas temperature for MS was set at 300°C with gas flow rate of 8.0 l/min, nebulizer pressure of 30.0 psi. The peptides were ionized using electrospray soft ionization technique (ESI). Data dependent experimental method consisting of two scan events was used. The first scan event was a full scan MS and the second was the data dependent MS/MS scan which is dependent on the results

of the first scan event. The parameters set for data dependent scan (MS/MS scan) were default collision energy (voltage) = 1.15 V, charge state = 2, minimum threshold = 3000 counts, and the isolation width = 2 m/z.

Mascot Protein Identification

Protein was identified through Mascot Protein Database Search engine *(www.matrix-science.com)*. The Peptide Mass Tolerance was set as ± 2 Da and ± 0.8 Da was set for the Fragment Mass Tolerance and only one missed cleavage was allowed. Matches were computed using a probability-based Mowse score defined as $-10 \times \log(P)$, where P is the probability that the observed match was a random event (Perkins *et al.* 1999). The proteins' functions and their characteristics were obtained from protein database searches including SwissProt *(http://www.expasy.org)*, PubMed *(http://www.ncbi.nlm.nih.gov)*, Human Protein Reference Database *(http://www.hprd.org)* and The Protein Information Resource *(http://www.pir.georgetown.edu)*.

RESULTS

Three types of extraction buffers, namely Ph, Ly and Thio buffer were used to extract proteins from the cancerous and normal colonic tissues. Figure 1 shows the profiles of proteins extracted using the three buffers from the tissue of the same patient. A distinct pattern of protein profiles can be seen in each lane which was loaded with three buffer extracts at similar concentration. Besides the common proteins, certain proteins were found uniquely in one particular buffer. When compare between the cancerous and normal tissues, differential proteins expression can be detected between the two tissues types of extracted using the similar extraction buffer. In all the three types of buffer extracts, the proteins at lower molecular weights were expressed in greater intensity in cancerous tissue compared to the normal tissue.

Figures 2, 3 and 4 show the protein profiles of proteins extracted from tissues of different patients using Ph buffer, Ly buffer and Thio buffer, respectively. A consistent profile shown by each buffer on the four patients confirmed the stability of the extraction procedure. Amongst the four patients tested, only the cancerous tissue from patient MMN was well-differentiated cancer grade whereas the rest of the tissues were well-differentiated cancerous tissues. Nevertheless, the difference in tissues pathological status cannot be differentiated by their protein expression pattern as all the four patients exhibited similar protein profiles in respective types of buffer extracts. However, due to small number of the tissues analyzed, the results shown in this study represent only a preliminary observation which needs to be confirmed with greater number of tissues. The mobility of the protein bands was measured by the Quantity One Software (BioRed) with reference to the protein molecular weights markers and the protein bands that were uniquely and differentially expressed in either cancerous or normal tissues were subjected to further analysis.

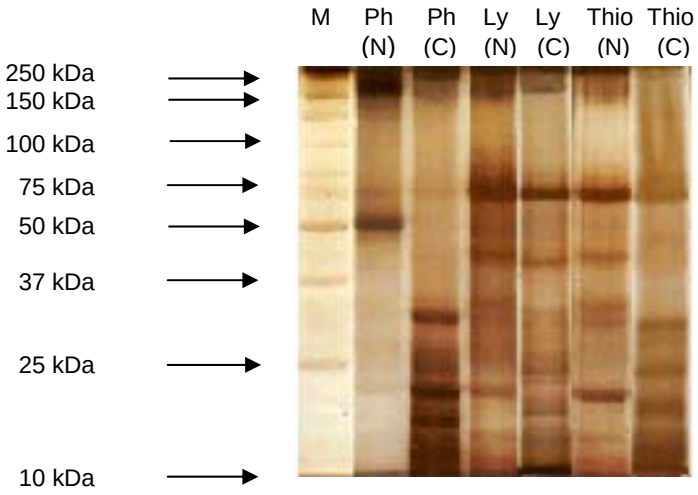

Figure 1: Comparison of the protein profiles of Ph, Ly and Thio buffer extracts from both normal and cancerous tissues. M: Marker; N: Normal Tissue; C: Cancer Tissue.

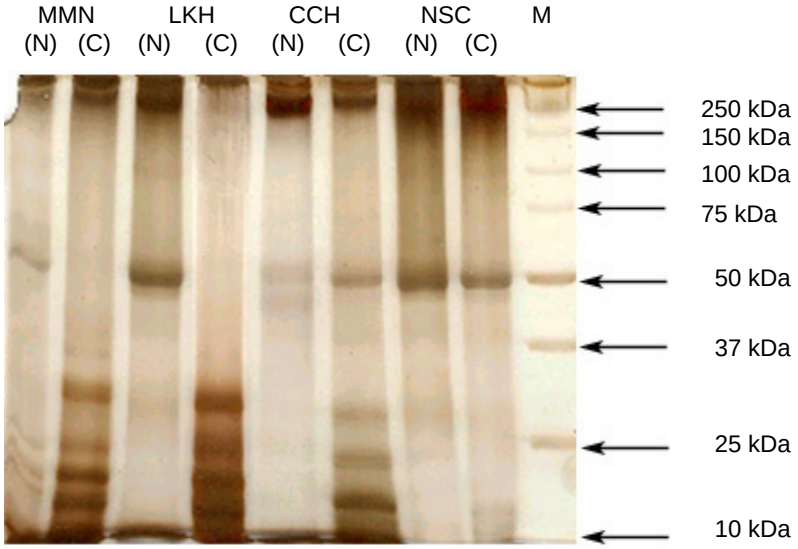

Figure 2: Protein profiles of normal and cancerous colon tissues from four different patients. The proteins were extracted using Ph buffer. N: Normal colon tissues; C: Cancer colon tissues; M: Marker; Lanes 1 and 2: MMN samples; Lanes 3 and 4: LKH samples; Lanes 5 and 6: CCH samples and Lanes 7 and 8: NSC samples.

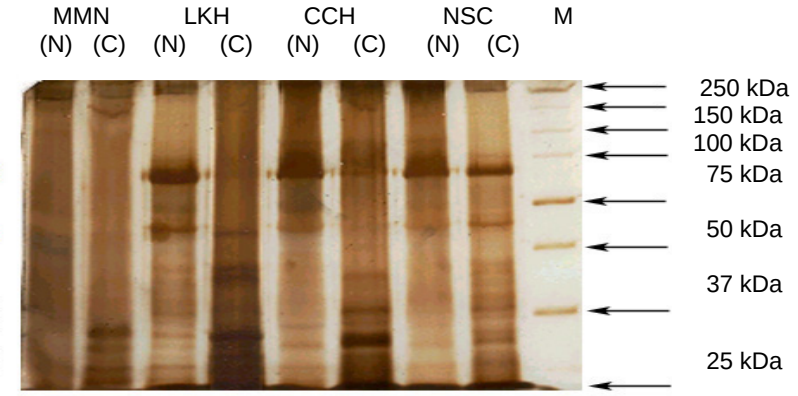

Figure 3: Protein profiles of normal and cancerous colon tissues from four different patients. The proteins were extracted using Ly buffer. N: Normal colon tissues; C: Cancer colon tissues; M: Marker; Lanes 1 and 2: MMN samples; Lanes 3 and 4: LKH samples; Lanes 5 and 6: CCH samples and Lanes 7 and 8: NSC samples.

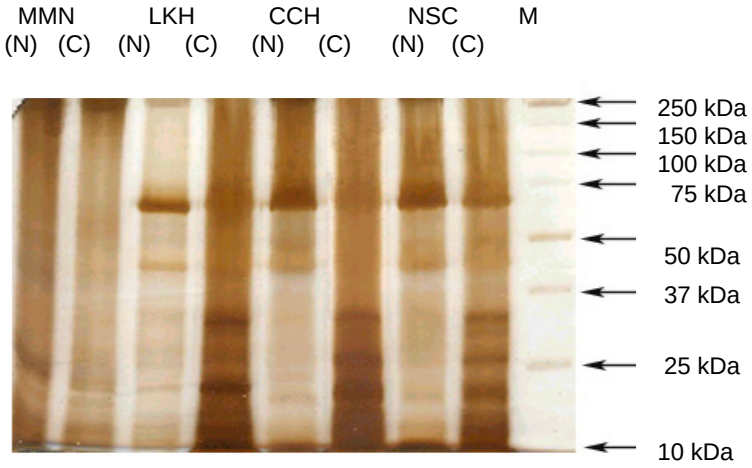

Figure 4: Protein profiles of normal and cancerous colon tissues from four different patients. The proteins were extracted using Thio buffer. N: Normal colon tissues; C: Cancer colon tissues; M: Marker; Lanes 1 and 2: MMN samples; Lanes 3 and 4: LKH samples; Lanes 5 and 6: CCH samples and Lanes 7 and 8: NSC samples.

Figure 5 shows the example of LC/MS/MS data of one of the proteins identified in the study. Prior to mass spectrometric analysis, the proteins were digested with trypsin into peptides of specific N-terminal. After HPLC separation, the eluent was directed to an ion-trap mass spectrometer which is interfaced with the HPLC. The peptides were ionized by electrospray ionization to form single and multiple charged ions. All the positively charged ions were scanned by the MS and the most abundance ion in the scan will be isolated and excited to the collision induced dissociation forming a series of fragment ions in MS/MS scan.

The fragment ions contain information of the amino acid sequence of the peptide. The MS/MS data generated was then subjected to Mascot protein database search engine, where matching of the MS/MS data with the pre-existing protein database was carried out for protein identification. Using this technique, the identity of all the proteins was identified.

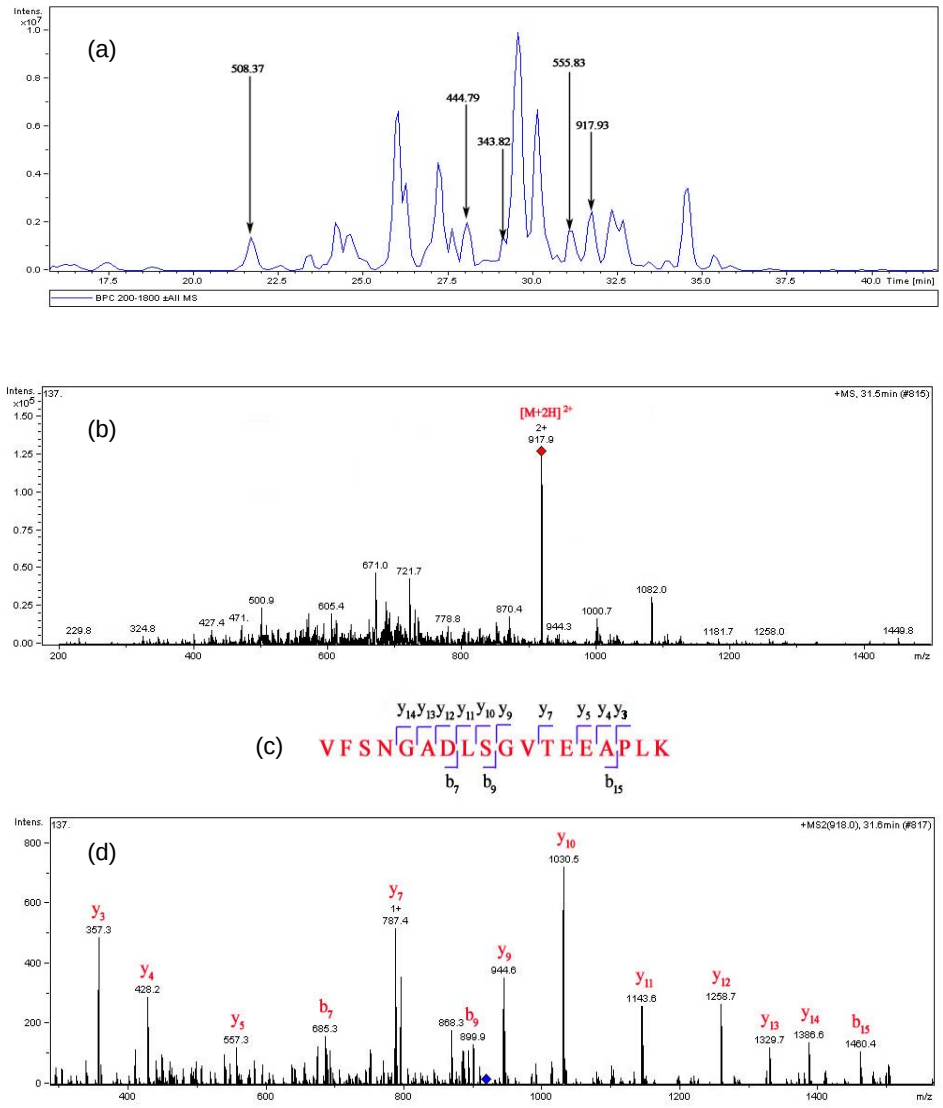

Figure 5: Identification of alpha-1-antitrypsin precursor (a) the base peak chromatogram; (b) the full scan MS; (c) the amino acid sequence derived from the MS/MS spectra; (d) the MS/MS spectrum of 917.93 precursor ion.

DISCUSSION

The proteome of a tissue can be analyzed when its proteins were extracted through solubilization in the extraction buffer. As the result of vast characteristic of proteins properties that confer their solubility. A combination of extraction buffers that differ in ionic strength should be used to extract proteins from the tissue. There are mainly two types of proteins reside in cells, namely aqueous soluble proteins and membrane-linked proteins. The aqueous soluble proteins are proteins with hydrophilic properties consisted of cytosolic protein and nucleus protein, whilst the membrane-linked proteins are protein with hydrophobic properties consisted of membranes proteins and membrane-associated proteins (Huber *et al.* 2003). Due to the wide range of proteins in tissue, we believe that no single buffer is able to extract total proteins from the tissues. In this study, we employed three extraction buffers made up of different chemical compositions to extract proteins from the tissues.

Ph buffer contained urea, CHAPS, EDTA, glycerol and NaCl. Chaotropic agent (urea) disrupts hydrogen bonds and is used when hydrogen bonding causes unwanted aggregation or formation of secondary structures that affect protein mobility. Detergent disrupts hydrophobic interactions and increase solubility of certain types of proteins, especially membrane protein (Hjelmeland 1990). The presence of NaCl is to prevent precipitation of proteins at their respective pI. NaCl is also used to solubilize membrane proteins that display ionic and hydrophobic interactions. Glycerol is used to promote solubilization and stability of proteins and it does not denature proteins at any concentration (Gekko & Timasheff 1981). The used of protease inhibitor such as EDTA is to inhibit the inherent proteolytic activity associated with the sample (Lindwall *et al.* 2000). The combination of these reagents allows the extraction of hydrophobic proteins as well as hydrophilic proteins.

Ly buffer is made up of urea, DTT, CHAPS, carrier ampholytes and EDTA. The combination of urea and CHAPS disturbs the rugged protein-protein interaction found in structural and cytoskeletal proteins. However, even in the presence of these reagents, certain proteins need stringent salt (carrier ampholytes) to maintain their solubility. Reducing agent such as dithiothreitol is used to disrupt disulfide bonds, which is important for analyzing proteins as single subunits. Therefore, Ly buffer extracts both aqueous soluble and proteins with intermediate hydrophobicity (Gorg *et al.* 2000).

The uniqueness of Thio buffer is that it is made up of a mixture of urea and thiourea, a combination that was recommended to enhance the solubilization of highly stringent proteins from the tissue (Rabilloud 1998). Thiourea is used to solubilize many otherwise intractable proteins. Therefore, Thio buffer is not only restricted to membrane proteins, but also sparingly soluble proteins such as nuclear proteins, which are prone to isoelectric precipitation (Rabilloud *et al.* 1997). Although most of the reagents used in the extraction buffers were meant for subsequent 2D gel separation, however for the ease of comparison, SDS-PAGE was employed, where a direct comparison of protein profiles can be made and very minimal deteriorating effects on the polyacrylamide gel caused by the reagents was seen throughout the study.

Direct comparison of protein profiles can be done effectively when proteins were separated on SDS-PAGE. This is because several protein extracts can be run concurrently in the same gel and therefore changes the running condition of the gel would not influence the comparison analysis. Furthermore, the limitation on gel resolution of SDS-PAGE was compensated for by using LC/MS/MS analysis that is a reliable and sensitive instrument for proteins and peptides detection (Ducret *et al.* 1998; Govorukhina *et al*. 2003).

The different protein profiles exhibited by the three buffer extracts indicate the different types of proteins were extracted by each buffer. Certain protein bands were found common in all the buffer extracts, these proteins represent the proteins that can be extracted by all the three buffers. In addition, unique protein bands can also be found exclusive in each buffer extracts, these proteins may be the proteins that were uniquely extracted by the buffer. However, we found that some of the unique protein bands belong to proteins with variable electrophoretic mobility in SDS-PAGE that migrated as multiple bands, which is described as heterogeneous. Heterogeneity of proteins can be influenced by protein phosphorylation and glycosylation (Duvet *et al.* 2002). In addition, limited proteolytic cleavage was also being reported to cause heterogeneity in some proteins (Fountoulakis *et al.* 2004). Most of the heterogeneous proteins detected in the tissues analysis of differentially expressed proteins in cancerous and normal colonic tissues were hemoglobins, actin gamma, serum albumin and serum albumin precursor.

The proteins that were exclusively extracted by Ph buffer were alpha-1-antitrypsin precursor (SWISS-PROT accession number: P01009) and haptoglobin precursor (P00738) that were uniquely expressed in normal and cancerous respectively. Keratin 20 (P35900) and SMAP-1b (Q9H3U1) were exclusively extracted in Ly buffer from both normal and cancerous tissues, the expression of keratin 20 and SMAP-1b were higher in cancerous tissues. On the other hand, ACTG1 protein (Q6PJ43) was found only in Thio buffer extracts of both normal and cancerous tissues, however, its expression was greater in cancerous than normal tissues.

Haptoglobin is a defense response protein unique to cancerous tissues in Ph buffer extracts. It is a transport glycoprotein that removes free hemoglobin from the circulation of vertebrates. In human population, haptoglobin exists in polymorphic forms (Brune *et al.* 1984). The roles of haptoglobin in cell migration, arterial restructuring (de Kleijn *et al.* 2002) and its increased expression in oncological tissues, which cell migration and matrix remodeling were important features suggesting its involvement in cancer (Smeets *et al.* 2003). Moreover, higher level of haptoglobin in serum was detected during carcinogenesis (Thompson *et al.* 1991).

Alpha-1-antitrypsin is endopeptidase inhibitor protein extracted in Ph buffer. The absent of alpha-1-antitrypsin leads to destroy of colon and rectum membrane by protease, leaving the colon and rectum vulnerable to cancer development (Yang *et al.* 2000). The main role of alpha-1-antitrypsin is to protect damages of lung tissues that cause by elastase (Rosenberg *et al.* 1984). Alpha-1-antitrypsin was detected in lung cancer (Harris *et al.* 1976) and it was used as a cancer marker for cervical cancer (Govorukhina *et. al.* 2003). Although it was

reported in colon cancer (Friedman *et al.* 2004), we found it unique in normal colonic tissues.

SMAP-1b is a protein-binding protein that highly expressed in cancerous tissues. It was exclusively extracted in Ly buffer. SMAP-1b is associated with nonstriated and involuntary muscle protein in vertebrates (Murray 2000). It was detected in colon tissue (Strausberg *et al.* 2002). Expression of cytoskeletal molecules associated with smooth muscle cell differentiation is reduced in malignant smooth muscle neoplasms (Sprogoe-Jakobsen & Holund 1996; Trzyna *et al.* 1997).

Keratin 20 is a structural protein that made up intermediate filament in cytokeratin family. The cytokeratins are components of the cytoskeleton in virtually all epithelial cells that consist of primordial components of the cytoskeleton and the nuclear envelope (Quinlan *et al*. 1995). The expression pattern of cytokeratins was used to characterize a specific epithelial cell type (Franke *et. al.* 1981). Keratin 20 was found exclusively in Ly buffer and it was greatly expressed in cancerous tissues compared to normal tissues.

ACTG1 is a motor activity proteins that highly expressed in cancerous tissues in Thio buffer extracts. It is involved in the formation of filaments that made up the major components of the cytoskeleton (Kabsch & Vandekerckhove 1992). Its existence in colorectal cancer tissue was reported by Vadlamudi and Shin (1998). Actin-gamma mutations were associated with tumorigenicity particularly in cancer initiation or progression and mutated cytoskeletal genes were suggested by the author to be a class of oncogenes (Chou *et al.* 1987).

CONCLUSION

In this study, not all of the differentially expressed proteins were analyzed as it was not our objective to study the full proteome of the tissues which was reported in our earlier study (Gam *et al.* 2006). However, the protein profiles of Ph, Ly and Thio buffers revealed that the three buffers were capable of extracting both the common and distinct proteins. The distinct proteins were analyzed by LC/MS/MS and the identity of the proteins were identified. The data obtained here showed that the extraction of total proteins from tissues by using single extraction buffer is not possible due to the different property of proteins. Therefore, the proteins should be extracted using different composition of extraction buffers and the combination of data obtained for each buffer extracts will provide a more comprehensive findings that enhance the analysis of tissue's proteins.

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