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Contributed Paper

Proteomic Analysis of Deglycosylated Proteins in Normal Human Serum Using Anhydrous Hydrogen Fluoride Treatment

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ABSTRACT

Deglycosylation with anhydrous hydrogen fluoride (HF) is an alternative method for removing oligosaccharides from glycoproteins which can be extremely useful for identification of proteins and the biological roles of post-translational modifications. In this study, a glycosylated proteome of human serum was treated by anhydrous HF to deglycosylate the number of oligosaccharides from glycoproteins which facilitated protein identification using proteomic analysis. In the preliminary result, the high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS) showed that there was no cleavage of disulfide bonds in HF-treated insulin as a negative control. The effect of HF deglycosylation on electrophoresis pattern was studied by resolving on one-dimensional (1-D) and two-dimensional (2-D) gels. Deglycosylation of glycoproteins in human serum resulted in different protein patterns on 1-DE and 2-DE gels with the clearer protein patterns and low amount of complexity. The deglycosylated serum proteins could be enriched and identified by MS analysis. Using this approach, it indicated that the proteins in human serum have some glycosylation that affected to the protein analysis and might possess the diverse biological functions. Therefore, this deglycosylation technique is an effective method to solve the problem of oligosaccharide interference in proteome analysis and be able to use for further glycan analysis.

Keywords: glycoprotein, deglycosylation, anhydrous hydrogen fluoride, human serum, two-dimensional electrophoresis.

1. INTRODUCTION

Many proteins in eukaryotes are glycosylated proteins that are occurred inside the cells, both in cytoplasm and subcellular organelles. Protein expression is important in many disease states, but the post-translational modification (PTMs) of proteins also play a major role in the biological system and mostly occurred during disease development and

progression [1,2]. Glycosylation is one of a majority of PTMs in eukaryotic cells and consists of mainly two types of protein glycosylation: N-glycosylation, the structure of the linkage region between carbohydrate and protein is β-glycosidically attached via N-acetylglucosamine (GlcNAc) to the amide group of asparagine residues; and O-glycosylation, the glycans attached at a serine, theronine, hydroxylysine, or hydroxylproline [3,4]. However, several methods have been developed for the analysis of glycoproteins by proteomic approaches. Gel electrophoresis is one of the most widely employed biochemical techniques for detection of glycoprotein from various biological materials [5-7]. Although glycoproteins can be analyzed and separated by protein separation techniques including two-dimensional gel electrophoresis (2-DE), which separated the proteins according to their molecular weight and p*I* values, the number of oligosaccharides composed in glycoproteins often resulted in many glycoforms of glycoprotein spots and/or bands, board protein bands and even smear bands or streaks on 2-D gel due to the heterogeneous glycosylation pattern [8,9].

Deglycosylation technique has been used in attempts to remove the glycan chains attached to proteins in order to reduce the carbohydrate interactions and proteome complexity. Therefore, deglycosylation technique may become an efficient way to remove the oligosaccharides resulted in multiple forms of glycoproteins and to obtain a single protein. It may also offer the key to obtain high quality of protein pattern for the next step of protein identification. In general, the carbohydrate chains that covalently linked to protein or peptide backbone can be released by either enzymatic or chemical method that has been used to identify the glycosylation site in cellular organelles or surface membrane. Many reports have been presented the removal of glycan groups from glycoprotein by chemical methods to effect the intact polysaccharide according to the analysis by molecular mass determination and amino acid sequencing [10-12]. Among these methods, the deglycosylation with chemical reagent is one of the most effective methods that can be completely removed all carbohydrate group both N-linked and O-linked glycosylation in only one step.

In the present study, we focused on the removal of all oligosaccharides that attached to proteins by chemical deglycosylation method using anhydrous HF reagent treated to samples prior to analysis. The effects of HF treatment on the cleavage of oligosaccharides and the change of molecular weight and/or p*I* value of proteins in samples were determined using proteomic approach. After treatment, the protein pattern in human serum sample could be visible on 1-DE and 2-DE gel patterns with the unique protein spots and/or bands. Therefore, this strategy can be used in sample preparation that would enable to release of carbohydrate chains from different biological materials.

2. MATERIALS AND METHODS

2.1 Chemicals and Materials

Standard glycoprotein alpha-1-acid glycoprotein (Orosomucoid, 99% purity) and PNGase F from *Elizabethkingia (Chryseobacterium/Flavobacterium) meningosepticum* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anhydrous hydrogen fluoride was purchased from Matheson Gas Products Inc. (Ottawa, ON, USA). HF cleavage apparatus (model FC2002S) was obtained from TOHO Co. (Chigasaki, Japan). Human serum samples (healthy donors) were provided from Lampang Regional Cancer Center, Lampang, Thailand. A commercial protein assay kit was purchased from Bio-Rad (Hercules, CA, USA). Gel electrophoresis running system and

reagents were obtained from GE Healthcare (Uppsala, Sweden). Sypro® Ruby was purchased from Molecular Probes (Eugene, OR, USA).

2.2 Deglycosylation of Glycoprotein

2.2.1 Anhydrous Hydrogen Fluoride (HF) Treatment

Deglycosylation of sample using anhydrous HF treatment was performed in a HF cleavage apparatus [13]. Briefly, the glycoprotein sample was added with anhydrous HF and incubated at 0°C for 1 h, with consistent gentle agitation. The reaction was quenched by freezing in liquid N_2 and the remained HF was removed by vacuum pump. The deglycosylated protein was dissolved in distilled water and lyophilized. Since anhydrous HF is extremely hazardous and toxic, the whole apparatus was kept in a fume hood. The protein concentration was determined using a commercial protein assay kit with bovine albumin (BSA) as a standard.

2.2.2 Removal of N-Linked Oligosaccharides by PNGase F Treatment

PNGase F was used to release the N-linked oligosaccharides from glycoprotein. Briefly, 100 μg of lyophilized glycoprotein sample was dissolved in 50 μl of 50 mM phosphate buffer, pH 7.5. The sample was added with the denaturing buffer containing 0.1% SDS and 2% β-mercaptoethanol and heated at 95°C for 5 min. For deglycosylation, the addition of 5 μl of 7.5% NP-40 and 5 Units of PNGase F was performed. The reaction mixture was incubated overnight at 37°C. The free glycan was removed from sample by precipitation with ice-cold ethanol and centrifugation at 10,000 rpm for 15 min [14]. The deglycoslyated protein was desalted by using PD10 desalting column and then lyophilized. The protein concentration was determined using a commercial protein assay kit with bovine albumin (BSA) as a standard.

2.3 Protein Analysis Methods

2.3.1 Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

Analysis of deglycosylated sample was performed using a reverse-phase HPLC column (4.6x250 mm, Nucleosil 7C18) attached to a L-7400 UV-VIS detector and a L-7100 pump (Hitachi model, Tokyo, Japan) with a 20 μL sample injection loop. Two mobile phases, A (0.1% v/v trifluoroacetic acid (TFA) and 5% v/v acetronitrile (ACN)) and B (0.1% v/v TFA and 95% v/v ACN), were used for all samples. All mobile phases were filtered through a 45-μm Millipore filter and degas before use. Each sample was resuspended in mobile phase A , filtered and injected into the HPLC column with a final volume of 10 μL. A linear gradient of 5% ACN to 95% ACN (0-100% mobile phase B) was used for 30 min running time at a flow rate of 1.0 ml/min. Protein was monitored by measuring absorbance at 280 nm.

2.3.2 Liquid Chromatography Mass Spectrometry (LC-MS)

The active compound/protein was analyzed by using a high-resolution ESI-TOF mass spectrometer (BioTOF III; Bruker Bruker Daltonics, Inc.; Billerica, MA, USA).

2.3.3 SDS-PAGE

The lyophilized protein sample was resuspended in 10 mM Tris-HCl, pH 7.5 and desalted with dialysis bag overnight at room temperature, followed to PD-10 desalting column. Samples were separated under denaturing conditions in 15% polyacrylamide gel using a Laemmli buffer system [15]. The samples were dissolved in sample buffer containing 50 mM Tris-HCl, pH 6.8, 0.1 M DTT, 10% glycerol, 2% SDS and 0.1% bromophenol blue at a concentration of 1 mg/ml and heated at 95°C for 5 min. Each sample solution (10 μg/well) was separately

loaded into gel wells. The SDS-PAGE gel was run in a BioRad Mini-Protean II apparatus at 20 mA per gel. After completion of electrophoresis, the protein bands in the gel were visualized by Sypro® Ruby staining and scanned by using a Typhoon 9200 image scanner (GE Healthcare). The Low-range molecular weight calibration kit (GE Healthcare) was used as standard molecular weight protein markers.

2.3.4 2-DE

Three hundred and fifty microgram of proteins was firstly treated with SDS solution buffer containing 0.2% SDS and 2.5 mM dithioerythreitol (DTE) and heated in a heating block at 95°C for 5 min [16]. After treatment, the protein sample was solubilized in 350 µl of lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 4 mM Tris base, 65 mM DTE, 5 mM tributylphosphine (TBP) and 0.5% IPG buffer (pH 4-7 or pH 3-10NL) and followed to incubate for 1 h at room temperature. The sample was centrifuged at 12000 rpm for 20 min and then loaded into an 18 cm IPG strip (pH 4-7 or pH 3-10NL, GE Healthcare). Gel rehydration was carried out for 14 h at 50 V according to the programmed setting: (1) 100 V, 100 Vh; (2) 250 V, 250 Vh; (4) 500 V, 500 Vh; (5) 1000, 1000 Vh; (6) 3000, 3000 Vh; and (7) 8000 V, 60000 Vh. Following IEF, the IPG strips were reduced and alkylated in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6M urea, 30% v/v glycerol, 2% w/v DTE and a trace of bromophenol blue), and then subsequently alkylated in the same buffer that replaced DTE with 2.5% w/v iodoacetamide (IAA) for 15 min. Each equilibrated IPG strip was transferred onto vertical 10-18% linear gradient polyacrylamide gel (18x18x1.5 cm) and covered with 0.5% agarose. The second-dimensional separation was carried out at 45 mA per gel for approximately 5 h at 15°C until the bromophenol blue dye front reached the bottom of the gel. After electrophoresis, the 2-D gels were stained with Sypro® Ruby and scanned using a Typhoon 9200 image scanner at 200 nm resolution. Image analysis was carried out using the ImageMaster 2D Platinum software version 5.0 (GE Healthcare).

2.3.5 Tryptic Digestion

The interested proteins were excised and transferred into a cleaned 0.5 ml siliconized microcentrifuge tube. The gel pieces were extensively washed twice with 200 µl of 50% ACN/25 mM ammonium bicarbonate buffer, pH 8.5, for 15 min each. Then, the gel pieces were washed once with 200 µl of 100% ACN and dried using a Speed-Vacuum concentrator. Reduction and alkylation were accomplished with 50 mM DTE and 100 mM IAA. Dried gel pieces were swollen in 10 µl of 25 mM ammonium bicarbonate buffer, pH 8.5, containing 0.0225 µg trypsin, crushed with siliconized blue stick and incubated at 37°C for at least 16 h. Peptides were subsequently extracted twice with 50 µl of 50% ACN/1% TFA, then the extracted solutions were combined and dried using a Speed-Vacuum concentrator. The peptides or pellets were resuspended in 5 µl of 50% ACN/0.1% TFA.

2.3.6 MALDI-TOF MS and MS/MS

The samples were premixed in a ratio of 1:1 with matrix solution (5 mg/ml CHCA in 50% ACN, 0.1% v/v TFA and 2% w/v ammonium citrate) and spotted onto the 96-wells formatted MALDI sample stage. Data was directed acquisition on the Q-TOF Ultima™ MALDI instrument (M@LDI™; Micromass, Manchester, UK) was fully automated with predefined probe motion pattern and the peak intensity threshold for switching over from MS survey scan to MS/

MS, from one MS/MS to another. Within each well, as many parent ions meeting the predefined criteria (any peak within the m/z 800-3000 range with intensity above 10 count ± include/exclude list) will be selected for CID MS/MS using argon as collision gas and a mass dependent ±5V rolling collision energy until the end of the probe pattern was reached (all details are available at http://proteome.sinica.edu.tw) Proteins were identified from the peptide mass maps using the MASCOT online database (http://www.matrixscience.com) to search the non-redundant protein database.

3. RESULTS AND DISCUSSION

3.1 Effect of HF Deglycosylation on Protein Stability

Chemical deglycosylation method is non-specific for the removal of either N-linked or O-linked glycans. A variety of reagents and treatment conditions are very important for deglycosylation and lead to cause denaturation of proteins [17,18]. In this study, anhydrous HF is a deglycosylated reagent and used to study the effect of chemical deglycosylation on protein stability, especially disulfide bond that can be occurred both inter- and intramolecular polypeptide chain of proteins. Since the structure of insulin is a small proteins containing fifty one amino acid residues and composed of two polypeptide chains that held together by two interchain disulfide bonds [19], it was therefore used as a negative control or standard protein to determine the stability of peptide bonds under the HF-deglycosylation treatment. After treatment, the stability of disulfide bonds in HF-treated insulin was examined by RP-HPLC and the molecular weight of HF-treated insulin was analyzed by LC-MS. The RP-HPLC result showed single peak of HF-treated insulin with the same retention time as standard insulin (Figure 1). It indicated that anhydrous HF reagent could not cleave the disulfide bonds in HF-treated insulin sample. In addition, there was no change of its molecular weight after HF treatment with a constant molecular weight (MW) of approximately 5735 Da (Figure 2). These results indicated that deglycosylation method using HF treatment had no effect on the stability of peptide bonds in standard protein used. Therefore, this deglycosylation technique would be particularly useful for identifying and characterizing the deglycosylated proteome.

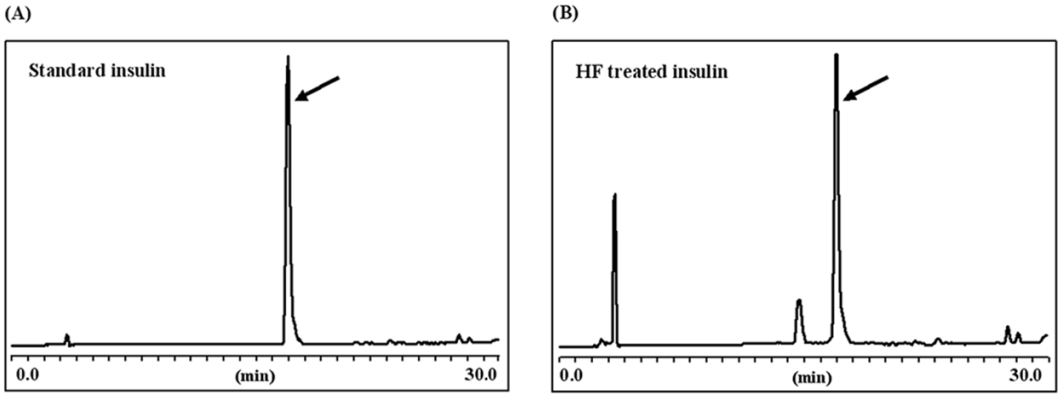


Figure 1. HPLC chromatograms of (A) a standard insulin and (B) HF-treated insulin that analyzed using RP-HPLC for investigation of the disulfide bond digestion, showing a major peak of insulin treated with anhydrous HF and non-treated appearing at the same peak of retention time approximately 18 min, is indicated by the arrow.

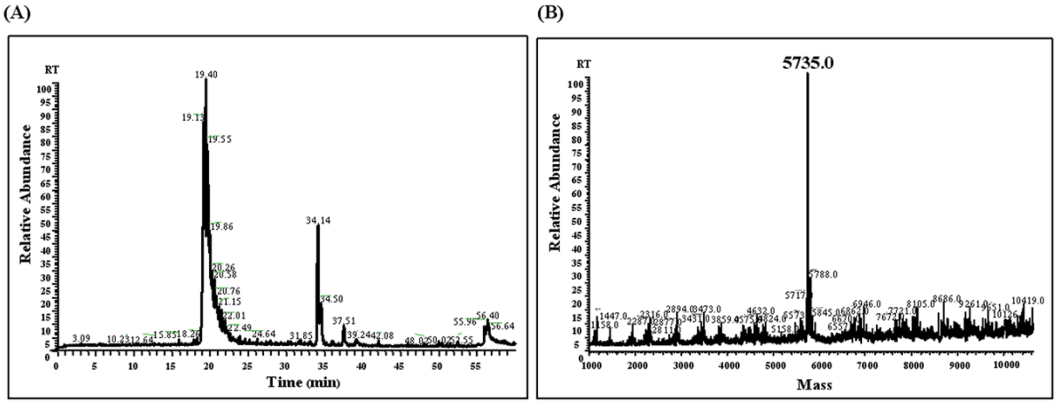


Figure 2. LC-MS profile obtained from the standard insulin treated with anhydrous HF, shown in (A) and (B) shows the signal from the HF-treated insulin which was observed at m/z 5735 Da.

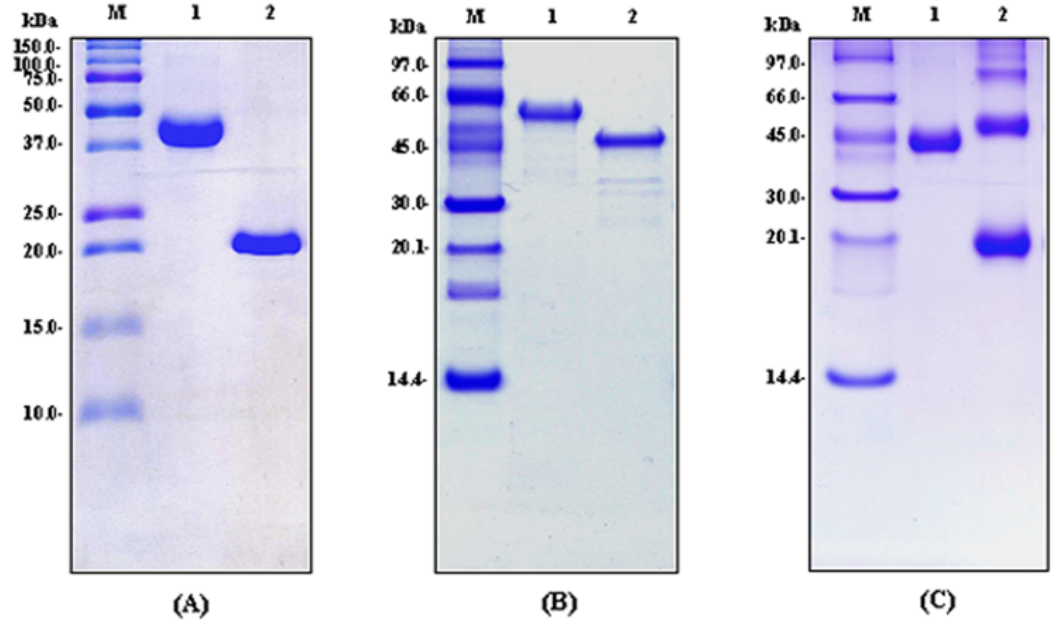
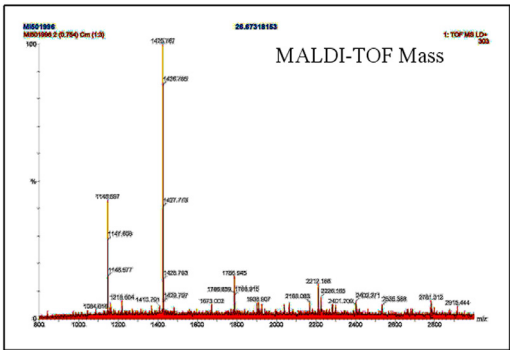
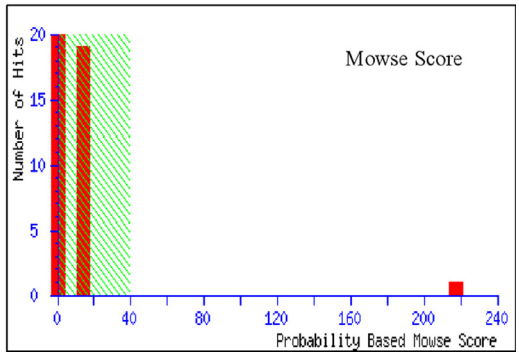


Figure 3. SDS-PAGE results represent the deglycosylated samples by two deglycosylation methods, PNGase F and HF treatment. Two standard glycoproteins of AGP1 and AHSG were used in these treatments and separated on 15% polyacrylamide gel, then stained with Coomassie blue. (A) Deglycosylation of AGP1. Lane 1, standard AGP1; lane 2, AGP1 deglycosylated with PNGase F. (B) Deglycosylation of AHSG with PNGase F. Lane 1, standard AHSG; lane 2, AHSG deglycosylated with PNGase F. (C) Deglycosylation of AGP1 using anhydrous HF treatment. Lane 1, standard AGP1; lane 2, HF-deglycosylated AGP1; M, protein markers.

(A)



(B)



(C)

Protein Identify	NCBI accession no.	Score	Sequence coverage (%)	Queries matched	Matched sequences
α_1- acid glycoprotein	gi-58531035	217	28	4	W FYIGSAFR AIQAAFFYLEPR VESDREHFVDLLLSK NVGVS FYADKPEVTQ EQK

Figure 4. Protein identification result of a HF deglycosylated AGP1. (A) MALDI-TOF-MS peptide mapping analyses of the deglycosylated AGP1; (B) MS mowse score and (C) summary of protein identification based on NCBI database search.

3.2 Determination of Deglycosylated Standard Glycoprotein

Comparison of deglycosylation methods between enzymatic deglycosylation (PNGase F) and chemical deglycosylation (HF treatment) was carried out by using two standard glycoproteins, alpha-1-acid glycoprotein (AGP1) containing N-linked glycans and alpha-2-HS glycoprotein (AHSG) containing both N- and O-linked glycans [20,21].The deglycosylated AGP1 and AHSG samples under both treatments were resolved on SDS-PAGE and the change of protein's molecular weight was examined. After PNGase F treatment, the protein band of AGP1 generally located at 45 kDa shifted to 20 kDa (Figure 3A) whereas the protein band of AHSG generally located at 57 kDa shifted to 45 kDa (Figure 3B). In addition to HF treatment, the protein band of AGP1 showed a few of deglycosylated protein bands with the major protein band or completed deglycosylated protein band at 20 kDa (Figure 3C). It indicated that the deglycosylation with PNGase F provided the completed deglycosylation of oligosaccharides from both AGP1 and AHSG whereas the deglycosylation with HF treatment provided uncompleted deglycosylation reaction of AGP1. In order to confirm the uncompleted deglycosylation by HF treatment, the upper protein bands over 45 kDa were excised from SDS-PAGE gel and subsequently identified the proteins by MALDI-TOF MS (Figure 4). The HF-deglycosylated AGP1 upper proteins were still identified as AGP1. We suggested that the deglycosylation of AGP1 by HF treatment might not sufficiently remove the glycans from glycoprotein and the HF treatment condition should be optimized to get the high efficiency

of deglycosylation. Although the enzymatic treatment has a high specificity of an appropriate enzyme that allowed to cleavage of a definite saccharide depending on glycosylation sites [22] and gave the completely deglycosylation result rather than HF treatment, the enzymatic treatment still had a limitation of individual enzyme and necessary to use multiple enzymes to remove several types of glycan chains attached on glycoproteins [23,24]. In contrast, deglycosylation by HF treatment has the advantage in removal of all glycans in glycoprotein regardless of structure or glycosylation site. However, the chemical deglycosylation technique using HF reagent can be used as an alternative method for further study of deglycosylated proteome in human serum samples.

3.3 Effect of HF Deglycosylation on Glycoproteins in Human Serum

Human serum is a complex biological sample containing a large number of proteins and glycoproteins. Many proteins in serum present complex combinations of post-translational modifications (PTMs) , such as glycosylation that can be discriminated by 2-DE [25-27]. Due to the complexity of human serum, a number of variables need to be considered including sample preparation prior to analyisis. Deglycosylation technique is thus an alternative method for enriching the amount of proteins and determining the structure of glycoproteins in human proteome. In the present study, we used anhydrous HF reagent to deglycosylate the glycoproteins in normal human serum and examined the deglycosylated proteome patterns. The pattern of proteins between untreated serum sample (control) and HF-deglycosylated serum sample were investigated by proteomic approaches. The deglycosylated proteins in human serum samples resulted in different protein patterns

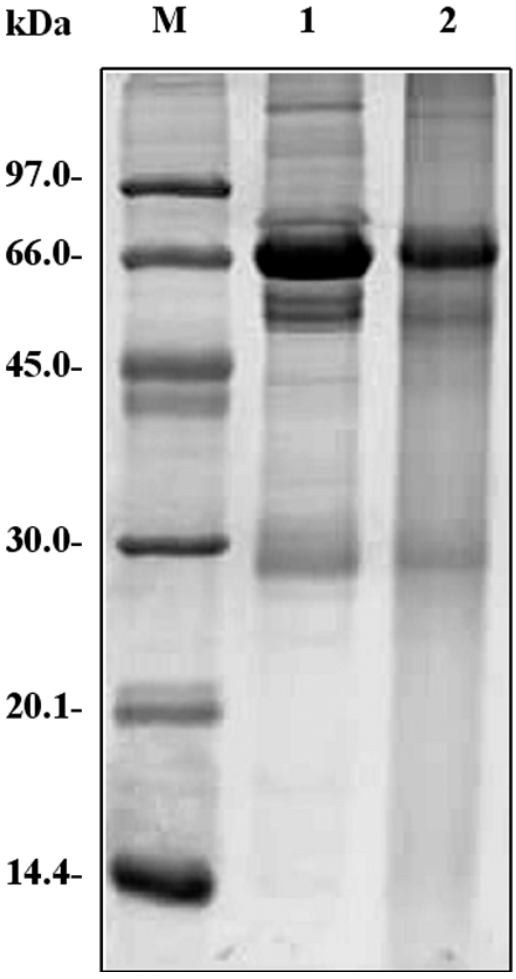


Figure 5. Analysis of normal human serum sample before and after deglycosylation with anhydrous HF by a 12.5% SDS–PAGE. Following electrophoresis, the gel was stained directly by Sypro® Ruby staining. Labels; M, protein markers; Lane 1, normal human serum; lane 2, HF-treated human serum sample.

on SDS-PAGE (Figure 5). Besides the use of 2-DE, it is another way to separate and analyze the isoforms of deglycosylated proteins with the high-resolution of separation. Using 2-DE, both 2-DE gel patterns of HF-treated sample in a wide pH range of 3-10 NL and a narrow pH range of 4-7 were distinctly different from untreated sample, in which the

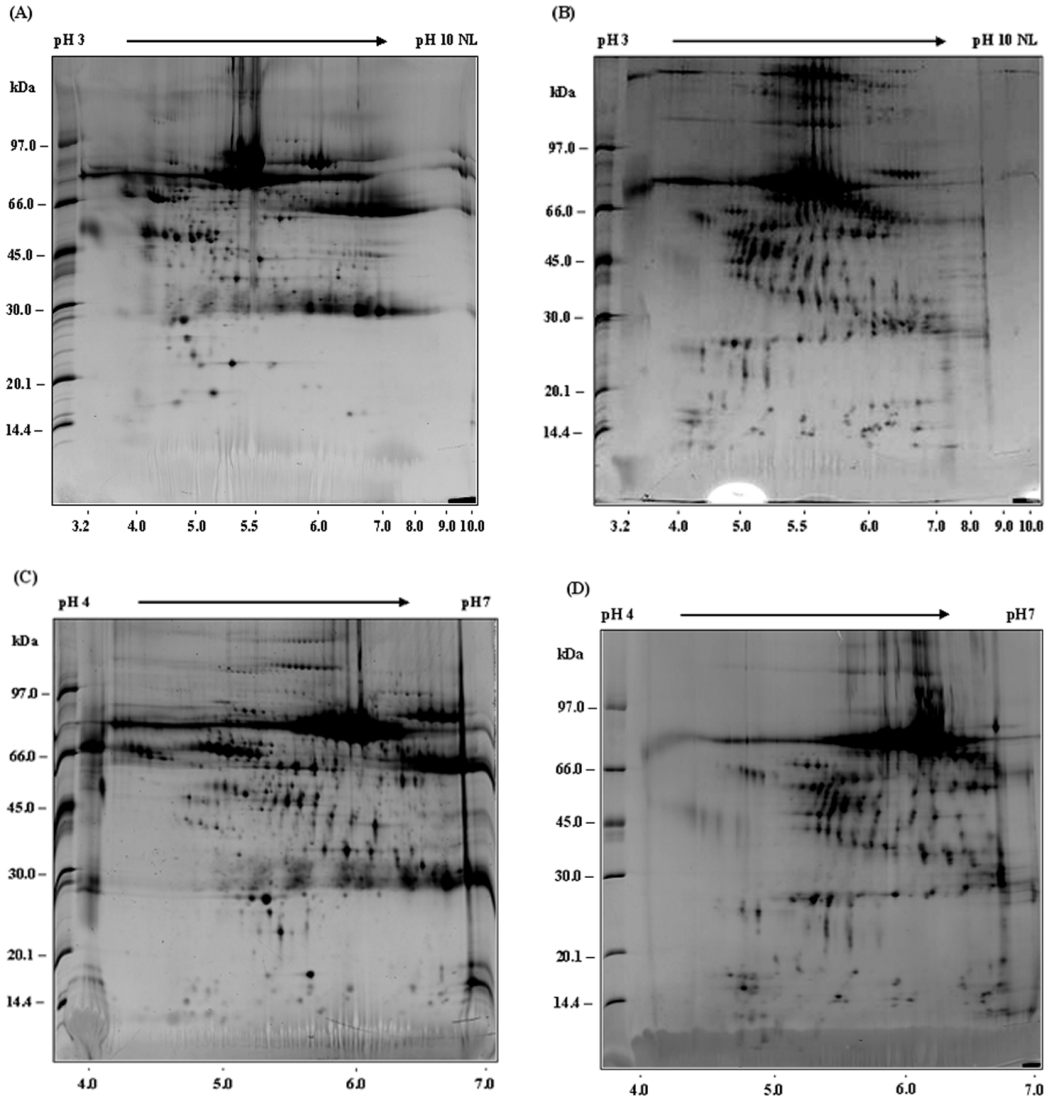


Figure 6. 2-DE image of normal human serum as control and HF-deglycosylated serum sample. The samples were applied to a pH 3-10 NL IPG strip (A, B) and a narrow range pH 4–7 IPG strip (C, D) for the first dimension and the 10-18% SDS–PAGE for the second dimension and stained with SYPRO Ruby gel staining.

complexity of protein patterns was reduced and the neat or unique proteins could be enriched (Figure 6). Figure 7 shows a representative example of the protein spots on a narrow range 2-D gel image that were matched between untreated and treated with anhydrous HF in normal human serum using Image Master software. After matching analysis, there was significant change in the total number of detectable protein spots by 2-DE analysis. Approximately 46 total match of protein spots were detected whereas the control sample exhibits the reduction of total protein spots were observed from 364 to 222 protein spots in HF deglycosylated serum sample.

The appearance of broad albumin band

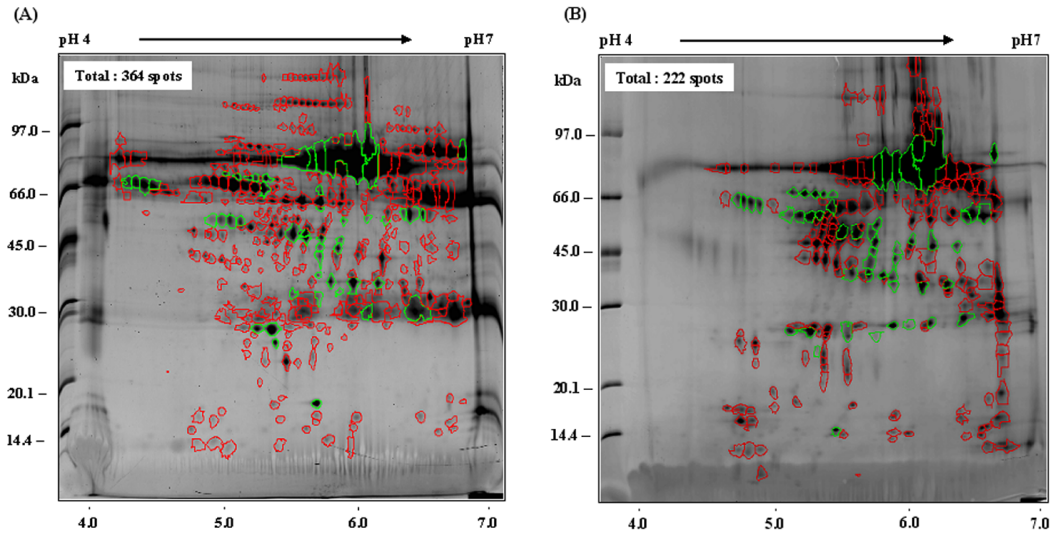


Figure 7. Spot detection using ImageMaster software for comparison of the narrow range 2-DE gel image between (A) normal human serum and (B) HF-treated human serum sample. The marked spots in green color were proteins that showed the match protein spots. Additionally, the differential expressed spots in HF treatment sample were indicated by red color.

in both treated and untreated serum samples was still revealed on 2-DE gel image with the high molecular weight at 65 kDa because the albumin is the highest abundant protein in human serum and the deglycosylated albumin still contained the major protein that located at the same position and seemed to be not different. In contrast to the high abundant haptoglobin at the MW approximately 45 kDa as to be haptoglobin β-chain, the 2-DE gel images showed the clear protein band, indicating the successfully deglycosylation of haptoglobin and enriching the proteins at that area. In addition, some deglycosylated proteins in HF treated samples had some modifications with MW and p*I* shifts. It indicated that HF could remove the oligosaccharides in glycoproteins and provided the deglycosylated proteins with different p*I* and MW. In addition to these modifications, it may be caused by the strongly acidification of proteins during HF treatment. Unfortunately, the chemical deglycosylation method for deglycosylating glycoproteins in human serum resulted in the incompletely removal of carbohydrate and extensive degradation of peptide core, which is a limitation of this technique. The 2-DE gel patterns also showed a vertically smeared protein bands due to the remaining anhydrous HF in samples. Although the 2-DE results of HF treated serum showed a slightly poor resolution of protein separation, the HF treatment could deglycosylate the oligosaccharides of glycoproteins and enriched the low abundant proteins. Moreover, the deglycosylated proteins in human serum showing in neat proteins may be served as a potential indicators or a wealthy biological source for discovery of biomarker [28, 29]. Therefore, deglycosylation using HF may be an alternative method for deglycosylation of glycoproteins, reducing the sample complexity and enriching the neat proteins. However, the condition of this method still needs to be

optimized for improving the deglycosylation efficiency and then able to use in the first step in the human serum proteome analysis.

4. CONCLUSION

Deglycosylation is an alternatively challenging method for analyzing the size of the protein and the biological role of post-translational modification. After HF deglycosylation, there was no effect of HF on the cleavage of disulfide bonds in the insulin as standard protein. The protein patterns of HF-treated samples showed the high reduction of complexity, in which the contribution of N- and O-linked glycosylations was eliminated, and the enrichment of low abundant proteins or neat proteins. Therefore, this method may be alternative used in the sample preparation of various human materials such as serum and urine that have the high complexity of protein samples. In addition, the anhydrous HF reagent may be used as a valuable chemical reagent for deglycosylation of glycoprotein to determine unknown structures of glycoproteins and to reduce all glycosylation variants. However, our results are the preliminary study of HF deglycosylation in proteome analysis and this method will be further optimized for improving the deglycosylation of glycoprotein prior to protein analysis.

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