Improved resolution of the human adipose tissue proteome at alkaline and wide range pH by the addition of hydroxyethyl disulfide

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Running title: 2-DE of human adipose tissue proteins at alkaline and wide pH.

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Abbreviations: **BPB**, bromophenol blue; **HED,** Hydroxyethyl disulfide; **PCOS**, Polycystic ovary syndrome.

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The illustration in Figure 1C could be used as the "graphical abstract".

Abstract

The polycystic ovary syndrome is a very common disease affecting 6.5% of women which is frequently associated with obesity, the link between both disorders being insulin resistance. From morbidly obese women submitted for surgical treatment of obesity, we obtain intra-abdominal adipose tissue samples in order to compare protein patterns between women with and without polycystic ovary syndrome by 2-DE analysis. Adipose tissue is a complex material extremely rich in lipids, and to improve protein solubilisation a lysis buffer including 8.4 M urea, 2.4 M thiourea, 5% CHAPS and 50mM DTT was used followed by ultracentrifugation to remove fat. Hydroxyethyl disulfide was added to increase resolution in the alkaline region [1], and this product demonstrates also capable to improve resolution at wide range pH 3-10. Our work shows for the first time 2-DE maps and identification of some proteins by MS, from human adipose tissue and lays the ground for future studies in metabolic diseases such as polycystic ovary syndrome, obesity, hypertension and type 2 diabetes.

Proteomics could be described as the global analysis of gene expression at the protein level from a whole organism, a cell culture or a tissue. The combined use of 2-DE and MS stands as one of the methods of choice for the characterization of complex samples. Recent technical improvements related to 2-DE and MS have resulted in increased sensitivity, reproducibility and throughput of proteome analysis thereby providing an established technological platform [2]. However, additional improvements are needed for the analysis of specific proteins such as low-abundant, high molecular weight or highly hydrophobic species. On the other hand, while wide range pH gradients and ultra-zoom gels appear to work properly below pH 7, obtaining a comparable resolution in the alkaline pH region is not straightforward [3, 4, 5]. Moreover, samples such as serum, cerebrospinal fluid or adipose tissue are especially difficult to study [6]. The biochemistry of adipose tissue is of great interest, especially because the prevalence of obesity is increasing exponentially in developed countries, and obesity plays an important role in the development of type 2 diabetes, dyslipidemia, polycystic ovary syndrome (PCOS) and cardiovascular diseases [7]. Therefore obesity research focuses on the identification of regulatory systems and potential targets for drug development. Such targets could be found among the genes and proteins expressed in the adipose tissue. Until recently, the adipose tissue was considered to play a passive role in the body by merely acting as a storage depot for fat; however, adipocytes are involved actively in maintaining the energy balance in the body, participating in processes such as satiety, bone function and reproduction. Most of these functions

are carried out via proteins secreted by the cells from the adipose tissue capable of acting locally or at such other nonadipose tissues as muscle, liver and stomach tissues, as well as on nervous, immune and vascular systems, even on bone turnover [8]. To fully understand the physiological functions of intra-abdominal adipose tissue in pathologies related to insulin resistance as obesity and PCOS, and to address whether in the adipose tissue of obese women with and without PCOS there are differentially expressed proteins, in this preliminary work we have optimised a protocol for IEF with IPGs 3-10 and 6-11 in samples from human intra-abdominal adipose tissue and we have identified some proteins by MALDI-TOF MS.

Samples were obtained from morbidly obese women submitted to surgical treatment for obesity. Samples (from ten unaffected women) were cut into small pieces and frozen at -75°C until used. The homogenisation was performed using a Polytron PT-1200C directly in the lysis buffer: 8.4 M urea, 2.4 M thiourea, 5% CHAPS, 50mM DTT and 1% IPG buffer 3-10 (total volume 2 mL/g fat pad). Rabilloud *et al* [9] reported that thiourea in combination with urea increases the solubility of proteins in IPGs and increases the number of proteins visualised in the second dimension. To improve lysis and protein solubilisation, this suspension was gently shaken for 60 min at room temperature, then centrifuged at 70,000 rpm for 1.5 hours at 19°C using a TL-100 ultracentrifuge. When attempting to solubilise and separate proteins from abdominal omental adipose some difficulties arise due to the presence of large amounts (often > 50% of the tissue volume) of triglycerides. Thus, the soluble infranatant below the fat supernatant was carefully recovered, avoiding the unhomogenized

material at the bottom of the centrifuge tube. The protein concentration in the collected fraction was measured according to the Bradford dye-binding procedure [10]. The original protein extract was diluted in the rehydration solution containing 8 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer, 0.002% BPB and 10mM DTT (or 50mM DTT or 100 mM of hydroxyethyl dishulphide, HED, depending of the gel) before the IEF. For IPG 6-11, 100 µg of proteins diluted in 100 µl of rehydration solution were applied by cup-loading close to the anode [2]. For IPG 3-10, 80 µg of proteins were applied by in-gel sample rehydration [11,12] in 350 µl of the above solution. The experiments were performed in duplicate for each condition and pH range. The strips (18 cm) were focused at 0.05 mA/IPG strip in the IPGphor IEF System (Amersham Biosciences, AB). The running conditions for IPG 6-11 were: 0 - 300 V in 1 min, 300 V for 3h, 300 - 600 V for 2 h, 600 - 1000 V for 2 h, 1000 - 8000 V for 3 h and 8000 V to reach about 55 - 58 kVh. The running conditions for IPG strips 3-10 were those reported by Görg *et al*., [2] with minor modifications, including an additional step at 120 V for 1 h, and a 90 min ramp from 1000 V to 8000 V, instead of 30 min. The step at 8000 V is maintained until reaching about 35 kVh. After IEF, the strips (pH 6-11 and 3-10) were equilibrated and SDS-PAGE was performed using a Protean II XL system (Bio-Rad). Gels (12.5%) in a format of 185 x 200 x 1 mm were fixed overnight and silver stained.

Horizontal streaking at the alkaline region of 2-DE maps is very often observed. This streaking is due to: (i) the loss of reducing agent in the basic part of the IPG strip, together with oxidation of the protein thiol

groups [2]; and (ii) water transport to the anode due to a reverse electroendosmotic flow [3]. To circumvent the first drawback, some authors introduce a paper wick immersed in DTT at the cathode during focusing to alleviate the loss of DTT in the basic part [4, 13]. With regard to water transport, addition of 10% isopropanol [3] or an isopropanol/glycerol mixture to the rehydration solution has been proposed as a solution [4]. Other authors have simplified the procedure for analysing very alkaline proteins by decreasing the total focusing time to a minimum (increasing the voltage to 8000 V to shorten the time required to reach the steady state) [5, 14].

For samples from human abdominal omental adipose tissue, replacing DTT by HED (DeStreak Reagent from AB) in the rehydration and sample solution when using Immobiline DryStrips 6-11, resulted in a relevant improvement in separation efficiency (Fig. 1), confirming the findings of Olsson and co-workers in which HED was shown to eliminate the horizontal streaking and to simplify the spot patterns in basic gradient [1]. Fig. 1 shows that horizontal streaking along with numerous spot trains disappear when HED is employed instead of DTT. More proteins can be visualised, as the intensity and number of spots increase. The majority of these spots are better defined and smaller. Moreover, the 2-DE map in Fig. 1C is better focused as compared to the maps in Fig. 1A and 1B. HED quantitatively oxidizes protein thiol groups to disulfide yielding a single protein species without any unwanted side reactions, therefore improving gel reproducibility [1].

As shown in Fig. 2, 2-DE maps are notably improved when HED is added to the rehydration solution of wide IPG 3-10 strips. In this case in-gel sample rehydration [11, 12] was employed instead of cup-loading, as in our hands the former performs better in the aforementioned pH range; however, the resulting spot patterns are comparable (data not shown). Serum albumin is the most abundant protein in the adipose tissue (spot labelled as number 1 in Fig. 2). Despite that 2-DE gels easily become overloaded with this protein, the protocol used to optimise IEF for adipose tissue has greatly enhanced the focusing of serum albumin.

In the rehydration solution for both IPG 3-10 and 6-11, HED concentrations other than 100mM, namely 50 mM and 200 mM, were tested, and the three conditions led to very similar spot patterns (results not shown).

The employ of PDQuest software (Bio-Rad) to detect spots in the gel images was assayed according to the manufacturer's instructions. For IPG 3-10, the program detected 110 and 123 more spots when HED was added to the rehydration solution instead of 10 mM DTT and 50mM DTT, respectively, using the same spot detection parameters. For IPG 6-11 only the map in Fig. 1C was analysed with PDQuest, which detected 488 spots.

The spots of interest were excised from the gel and submitted to tryptic digestion according to a protocol based on that from Shevchenko *et al*. [15] with minor variations. The resulting digestion solutions were collected and spotted on a 400 m AnchorChip MALDI probe (Bruker Daltonics, Bremen, Germany) together with the appropriate matrix solution and allowed to dry at room temperature. Samples were measured on a

Bruker Reflex IV MALDI-TOF mass spectrometer (Bruker-Daltonics) in positive ion reflector mode. Mass measurements were performed automatically under the control of a fuzzy logic engine. Each spectrum was internally calibrated with the masses of two trypsin autolysis products at m/z = 842.510 and m/z = 2211.105 to reach a typical mass measurement accuracy of ±30 ppm. The measured tryptic peptide masses were transferred through MS BioTools program (Bruker-Daltonics) as inputs to search the NCBInr database using Mascot software (MatrixScience, London, UK). No restrictions were placed on the species of origin of the protein and no variable modifications were allowed. Up to one missed tryptic cleavage was considered and a mass accuracy of 50 ppm was used for all tryptic-mass searches. Some of the identified proteins are listed in Table 1.

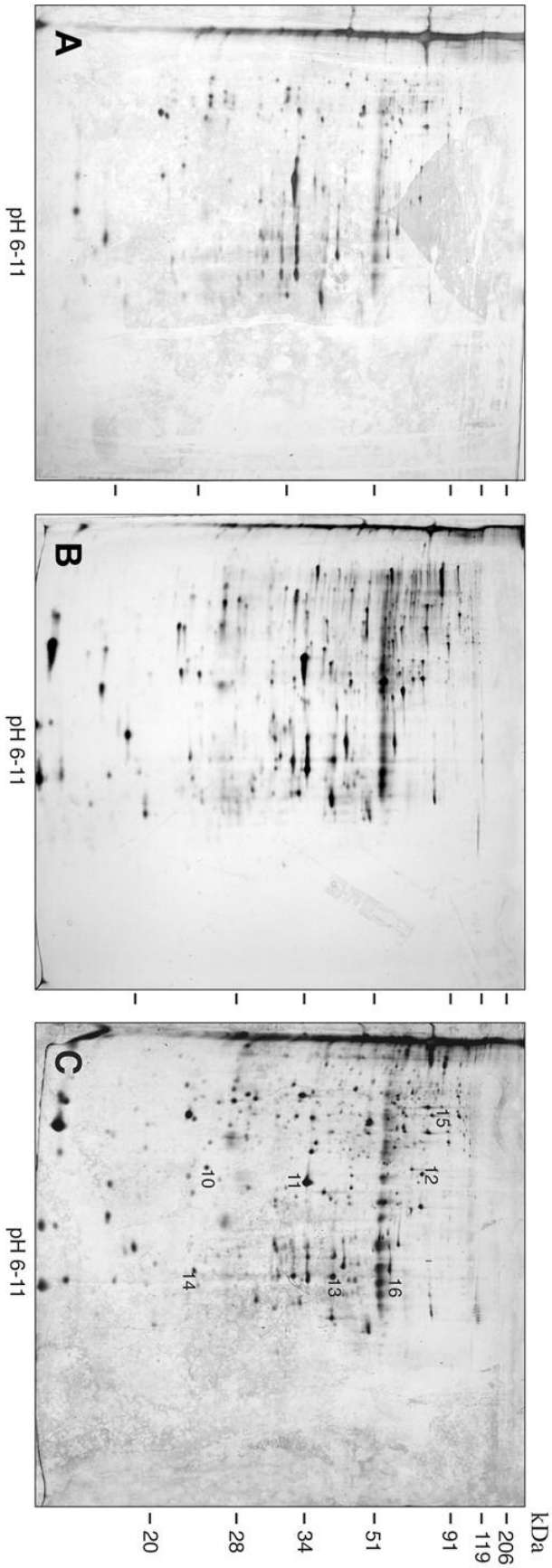
Several 2-DE maps of mouse white adipose tissue proteins have been reported [6, 16], while related proteomic approaches have been applied to human cultured preadipocytes and adipocytes exclusively [17]. In this study we have optimized the sample preparation procedure for the proteomic analysis of human intra-abdominal adipose tissue using two different IPG strips and extended the analysis region to include the alkaline range.

Legends to the Figures

Figure 1. 2-DE gel (pH 6-11) from 100 µg of human intra-abdominal adipose tissue focused in the presence of 10 mM DTT (A), 50 mM DTT (B) and 100 mM HED (C) in the rehydration and sample solutions. The indicated spots were picked and digested for protein identification (Table 1).

Figure 2. Protein pattern (pH 3-10) from 80 µg of human intra-abdominal adipose tissue focused in the presence of 10mM DTT (A), 50 mM DTT (B) and 100 mM HED (C) in the rehydration solution. The indicated spots were picked and digested for protein identification (Table 1).

Figure 1


A
B
C
pH 6-11
pH 6-11
pH 6-11
15
12
11
10
16
13
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kDa
206
119
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51
34
28
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Figure 2.

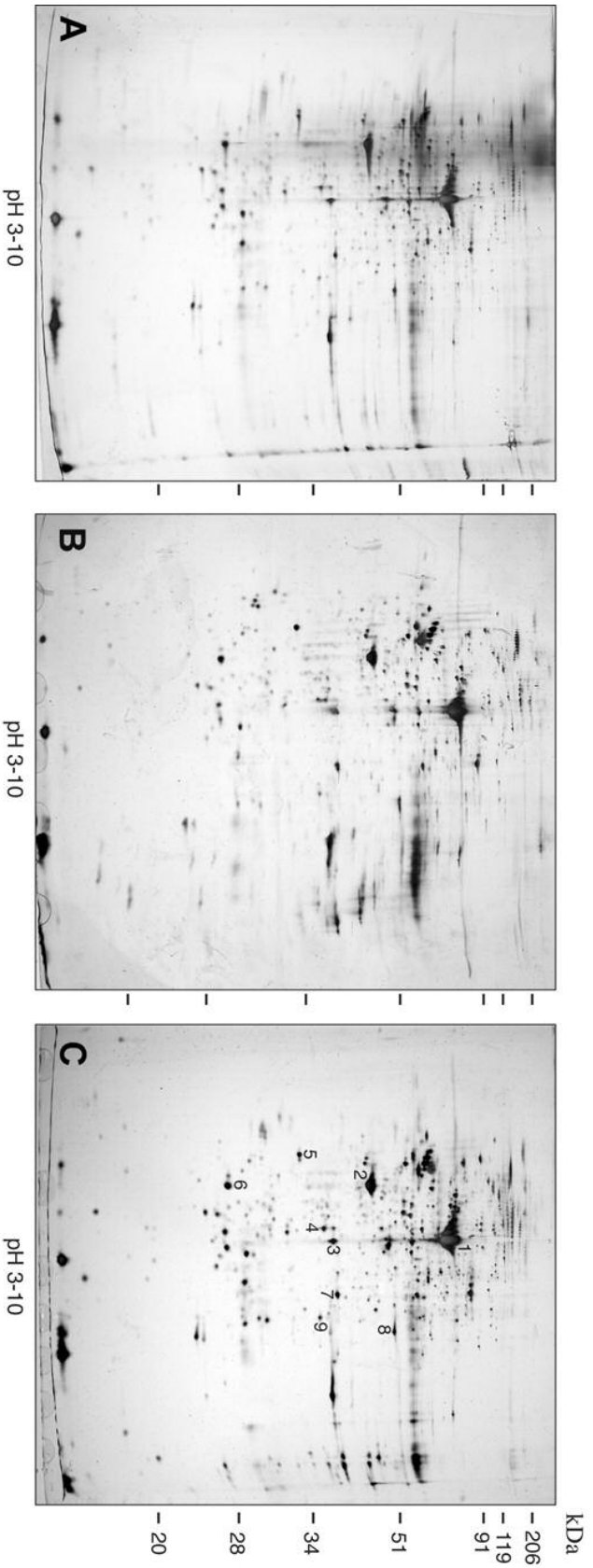

Table 1. Protein identified by MALDI peptide mapping of indicated spots in Fig.1 and 2.

Spot no.	Protein identity	Accesion no.	Theoretical Mr (Da)	Theoretical p*I*
1	Human serum albumin	gi|4389275	67988	5.69
2	Actin, gamma 1 propeptide	gi|4501887	42108	5.31
3	Glycerol-3-phosphate dehydrogenase 1	gi|21594877	38171	5.81
4	Lactate dehydrogenase B	gi|4557032	36900	5.71
5	Annexin V	gi|17391477	35959	4.94
6	Apolipoprotein A1 precursor	gi|4557321	30759	5.56
7	Annexin I	gi|4502101	38918	7.57
8	Enolase 1	gi|4503571	47481	7.01
9	Cytosolic malate dehydrogenase	gi|5174539	36631	6.91
10	Biliverdin reductase B	gi|544759	21960	7.31
11	Annexin A2	gi|16306978	38822	7.57
12	Transketolase	gi|4507521	68519	7.58
13	Aldolase A	gi|229674	39720	8.39
14	Peroxiredoxin 1	gi|4505591	22324	8.27
15	Long-chain fatty-acid-Coenzyme A ligase 2	gi|11276083	78919	6.81
16	Immunoglubulin heavy chain gamma	gi|2765425	53379	8.74

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