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Review

Disease proteomics of high-molecular-mass proteins by two-dimensional gel electrophoresis with agarose gels in the first dimension (Agarose 2-DE)☆

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Abstract

Agarose gel is the preferred electrophoretic medium currently used for separating high molecular mass (HMM) proteins (MW > 100 kDa). Agarose gels are widely used for both SDS-agarose gel electrophoresis and agarose isoelectric focusing (IEF). A two-dimensional gel electrophoresis method employing agarose gels in the first dimension (agarose 2-DE) that is sufficiently good at separating up to 1.5 mg of HMM proteins with molecular masses as large as 500 kDa has been used to separate proteins from various diseased tissues and cells. Although resolution of the agarose 2-DE pattern always depends on the tissue being analyzed, sample preparation procedures including (i) protein extraction with an SDS sample buffer; (ii) ultracentrifugation of a tissue homogenate; and (iii) 1% SDS in both stacking and separation gels of the second-dimension SDS-PAGE gel, are generally effective for HMM protein detection. In a comprehensive prostate cancer proteome study using agarose 2-DE, the HMM region of the gel was rich in proteins of particular gene/protein expression groups (39.1% of the HMM proteins but only 28.4% of the LMM ones were classified as transcription/translation-related proteins). Examples include transcription factors, DNA or RNA binding proteins, and ribosomal proteins. To understand oxidative stress-induced cellular damage at the protein level, a novel proteomic method, in which protein carbonyls were derivatized with biotin hydrazide followed by agarose 2-DE, was useful for detecting HMM protein carbonyls in tissues of both a diabetes model Ostuka Long-Evans Tokushima Fatty (OLETF) rat and a control Long-Evans Tokushima Otsuka (LETO) rat. In this paper, we review the use of agarose gels for separation of HMM proteins and disease proteomics of HMM proteins in general, with particular attention paid to our proteome analyzes based on the use of agarose 2-DE for protein separation followed by the use of mass spectrometry for protein identification.
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Keywords: Reviews; Two-dimensional gel electrophoresis; Disease proteomics; High-molecular-mass proteins (HMM)

Abbreviations: ACT, actin; Agarose 2-DE, two-dimensional gel electrophoresis with agarose gels in the first dimension; alpha2M, alpha 2-macroglobulin; ATPase, adenosine triphosphatase; BiP, immunoglobulin heavy chain-binding protein; CAP1, adenylyl cyclase-associated protein; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid; 2-DE, two-dimensional gel electrophoresis; 2-D DIGE, fluorescent two-dimensional differential gel electrophoresis; DNA, deoxyribonucleic acid; DTT, dithiothreitol; EGFR, epidermal growth factor receptor; eIF-4H, eukaryotic translation initiation factor 4H; GRP94, endoplasmin; HDL, high density lipoprotein; HMM, high-molecular-mass; Hp2-1, haptoglobulin phenotype 2-1; HSA, human serum albumin; HSP70, heat shock protein 70; IEF, isoelectric focusing; IgM, immunoglobulin M; IPG, immobilized pH gradient; IPG-Dalt, immobilized pH gradient two-dimensional gel electrophoresis; LC, liquid chromatography; LETO, Long-Evans Tokushima Otsuka; LMM, low-molecular-mass; LNCaP, an androgen-sensitive human prostate cancer cell line; 2-ME, 2-mercaptoethanol; MHC, myosin heavy chain; MS, mass-spectrometry; NCC27, nuclear chloride ion channel protein; Mr, molecular mass; mRNA, messenger RNA; OLETF, Otsuka Long-Evans Tokushima Fatty; P4HB, prolyl-4-hydroxylase beta subunit; p*I*, isoelectric point; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; PTMs, post-translational modifications; PVDF, polyvinylidine difluoride; RBCs, red blood cells; RNA, ribonucleic acid; RSA, rat serum albumin; SDS, sodium dodecyl sulfate, sodium lauryl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tg, thyroglobulin; TPI1, triosephosphate isomerase 1; Tris-HCl, Tris(hydroxymethyl)aminomethane hydrochloride; VDAC1, voltage-dependent anion channel 1; VHMM, very high molecular mass

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1. Introduction

Proteomics [1], a new post-genomic field that encompasses protein expression and function in a cell or tissue, is a promising approach for better understanding the biology of organisms including human. Since an organism's proteome depends dynamically on the particular state of that organism, proteomic studies may reveal pathologic states of diseases, including the amounts of proteins present, subcellular localization of the proteins, extent of post-translational modifications and patterns of protein associations. The proteomic approach is, therefore, conceptually attractive because it directly elucidates protein expression, accumulation, and degradation profiles in tissues.

High-molecular-mass (HMM) proteins (MW > 100 kDa) are known to be involved in a number of human diseases, including Duchene and Becker muscular dystrophies (dystrophin: Mr ∼400 kDa) [2], hypothyroidism (thyroglobulin: Mr ∼330 kDa) [3], and cardiomyopathies (cardiac myosin heavy chain: Mr ∼200 kDa) [4]. Though a variety of proteomic techniques have been described, at the moment purification of HMM proteins remains a difficult task. O'Farrell [5] was the first to devise a 2-DE technique capable of detecting more than 1000 spots in a gel. Though it is evident that this method is quite powerful, it is not particularly suitable for analyzing HMM proteins larger than 200 kDa. Hirabayashi [6] was the first to develop a 2-DE method that could analyze HMM proteins as large as 500 kDa, including myosin heavy chain [6] and dystrophin [7]. His insight was to use an agarose gel instead of a polyacrylamide gel for the isoelectric focusing (IEF) in the first dimension. Agarose gels, when used for IEF, permit analysis of much larger proteins than polyacrylamide gels can. Oh-Ishi and Hirabayashi [8] further improved this method by adding 1 M thiourea and 5 M urea into the agarose IEF medium. Thiourea, in combination with urea, was reported to be a potent protein solubilizing reagent [9], especially effective for HMM proteins that could enter the first-dimensional agarose IEF gel [10].

Recently, defects in alternative splicing of mRNA [11] and aberrant post-translational modifications of a protein [12] were shown to be important causes of disease. Detection and identification of a protein spot having an apparent molecular mass and p*I* different from those predicted from a protein database are important applications of agarose 2-DE. In a comprehensive cancer proteome study using agarose 2-DE, the HMM region of the gel is rich in proteins of particular gene/protein expression groups. Examples include transcription factors, DNA or RNA binding proteins, and ribosomal proteins [13].

In this paper, we review the use of agarose gels for HMM protein separation and disease proteomics of HMM proteins in general, with particular attention paid to our proteome analyzes based on the use of agarose 2-DE for protein separation, followed by the use of mass spectrometry for protein identification.

2. Physical and chemical properties of HMM proteins

An HMM protein (Mr > 100 kDa) is a very long molecule consisting of several structural and repeating domains, capable of folding and functioning autonomously. HMM protein domains have evolved by insertions or deletions of distinct protein domains [14]. Skeletal myosin heavy chain (MHC) (Mr ∼200 kDa), for example, consists of a head domain, containing the ATPase and actin-binding activities, and a rod domain, composed of 40 repeats of 28 residues each that are predicted to form an alpha-helical coiled coil [15,16]. The molecular mass of an HMM protein, e.g., MHC and dystrophin, is often sev-

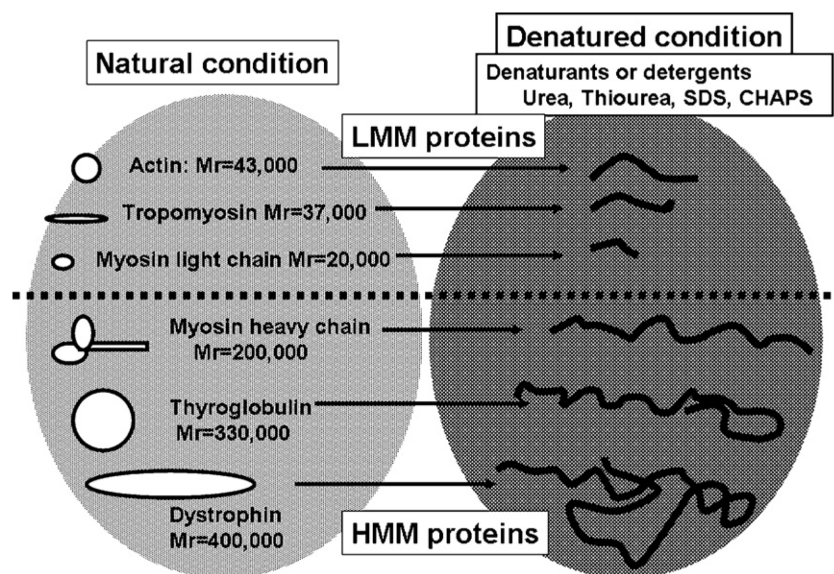

Fig. 1. A schematic drawing of HMM and LMM proteins: comparison of the protein structure between native and denatured conditions. When proteins are to be analyzed by 2-DE, denaturation of the proteins before IEF is essential. HMM proteins (>100 kDa), having long chain and hydrophobic regions, tend to aggregate or tangle easily in the presence of denaturants (urea, thiourea) and/or detergents (SDS, CHAPS).

eral times larger than those of a typical low-molecular-mass (LMM) protein (Mr < 100 kDa), e.g., myosin light chain, actin, and tropomyosin.

Fig. 1 shows a schematic of representative HMM and LMM proteins in native or denatured conditions. In the native state, a protein molecule has a precise three-dimensional structure and a corresponding proper biological function. When total proteins in a cell or tissue homogenate are to be analyzed by 2-DE, denaturation of the proteins, in general, is an essential procedure before IEF. As a result, the solubility of the denatured proteins in aqueous solution may be sharply lowered, causing them to precipitate as insoluble aggregates. In many cases, denaturation is irreversible. HMM proteins that have both a long chain and hydrophobic regions tend to aggregate or tangle easily, even in the presence of powerful denaturants (urea, thiourea) and/or detergents (SDS, CHAPS).

Though a variety of protein separation techniques have been described, purification of HMM proteins remains a difficult task, because (i) solubilization of an HMM protein greatly depends on each protein's physical and chemical properties; (ii) denatured HMM proteins with hydrophobic regions tend to aggregate in aqueous solution; and (iii) an HMM protein, having a long polypeptide chain, is a target of endogenous proteases during purification procedures.

Thyroglobulin (Tg), for example, is a large, 660 kDa globular glycoprotein composed of two identical disulfide-linked subunits (each subunit: 330 kD). Tg, produced by follicular cells of the thyroid gland, is stored within the follicular lumen in a soluble form. Homogenization of thyroid tissue with an extraction medium that included high concentrations of urea and thiourea denatured the Tg molecules, and they became entangled. Not surprisingly, no Tg spot was visible in the immobilized pH gradient 2-DE (IPG-Dalt) gel (but a Tg spot actually was visible using agarose 2-DE) [17].

3. Separation techniques for HMM proteins

3.1. Mass-spectrometry-based proteomics for HMM proteins

Mass-spectrometry (MS)-based proteomics has become an essential tool for the qualitative and quantitative analysis of proteins. "Shotgun proteomics" refers to the automated identification and cataloging of proteins directly from complex protein mixtures in order to rapidly generate a global profile of the proteins in a tissue or cell [18]. In this approach, proteins are enzymatically digested into peptides, separated via reversed-phase liquid chromatography (LC) and analyzed automatically by a mass spectrometer (MS). The method is capable of identifying a large number of proteins, often on the basis of a few peptides only. Consequently, shotgun proteomics is suitable for detecting proteins of lower abundance, and is not biased against certain classes of proteins such as basic proteins, membrane proteins, and HMM proteins, which are difficult to detect by 2-DE [19]. However, a major limitation of MS-based proteomics is that the connection between digested peptides and their assembly into proteins is difficult to achieve if some alternatively-spliced variants encoding a protein are present.

3.2. Gel-based proteomics for HMM proteins

The IPG-Dalt method is not particularly suitable for analyzing HMM proteins larger than 200 kDa [17]. However, a 2-DE method with agarose gels in the first dimension (agarose 2-DE) was shown to be suitable for HMM protein analysis [6,8,10]. The agarose 2-DE method would be preferable when (i) the intact molecular mass and p*I* of HMM proteins are to be determined and (ii) small amounts of HMM protein are to be micro-preparatively separated [17]. HMM proteins detected by agarose

Table 1
HMM proteins (>100 kDa) separated by agarose 2-DE

Tissue (function)	Protein	Experimental MM Da/p*I*	Theoretical MM Da/p*I*	Reference
Rat Thyroid gland				
Hormone production	Thyroglobulin	330000/5.0	304645/5.04	[48]
Chicken skeletal muscle				
Muscle contraction	Myosin heavy chain	200000/6.0	223014/5.63	[6]
Mouse skeletal muscle				
Anchoring the cytoskeleton to the plasma membrane	Dystrophin	350000/5.5	425817/5.66	[7]
Human prostate cancer: LNCaP cells				[35]
Cell division				
	ADP-ribosyltransferase	115000/8.5	113064/9.0	
	Damage-specific DNA binding protein 1	115000/5.4	126893/5.2	
	RAD50 homolog	145000/7.5	153873/6.5	
	Tankyrase 1-binding protein 220	000/4.7	181786/9.0	
Cell signaling/communication				
	Alpha-1 catenin	105000/6.7	100053/6.0	
	Clathrin heavy chain; clathrin, heavy polypeptide-like 2	160000/6.2	191595/5.5	
	Laminin gamma-1 chain precursor	170000/5.1	177588/5.0	
	Nucleoprotein TPR	280000/6.4	265600/5.0	
	Sec23-interacting protein p125	115000/5.5	111058/5.4	
	Spectrin alpha chain	260000/6.2	285046/5.2	
		260000/5.5		
		260000/5.2		
Cell structure/motility				
	Alpha-actinin 1, cytoskeletal isoform	105000/5.3	102956/5.2	
	Alpha-actinin 4	100000/5.4	102250/5.3	
	Chondroitin sulfate proteoglycan 6	135000/7.8	141523/6.8	
	Dynactin 1	145000/6.2	139941/5.5	
	Filamin B, beta	280000/6.3	278174/5.5	
	Myosin heavy chain, nonmuscle type A	200000/7.2	226512/5.5	
		200000/7.7		
		200000/7.6		
		200000/6.7		
		190000/6.8		
	Plectin 1	500000/6.6	518475/5.6	
	Talin	240000/7.2	269697/5.7	
Cell/organism defense				
	Oxygen regulated protein precursor	140000/6.2	111317/5.2	
Gene/protein expression				
	Alanyl tRNA synthetase	110000/5.9	105325/5.3	
	Chromatin-specific transcription elon-gation factor FACT 140 kDa subunit	130000/7.2	119895/5.5	
	Chromosome 20 open reading frame 14	130000/8.0	107095/8.6	
	Coatomer beta subunit 2	105000/6.0	102469/5.2	
	FYVE and coiled-coil domain containing 1	170000/5.0	166963/4.9	
	Glutamyl-prolyl tRNA synthetase	160000/7.5	163007/7.8	
	Hypothetical protein FLJ10839	140000/6.7	132839/5.6	
	Kruppel-type zinc finger protein (PEG3)	180000/6.3	180808/5.3	
	Leucyl tRNA synthetase	120000/7.4	134392/6.8	
	MOP-4	115000/6.0	117979/5.8	
	Puromycin-sensitive aminopeptidase	100000/5.6	103284/5.5	

Table 1 (*Continued*)

Tissue (function)	Protein	Experimental MM Da/p*I*	Theoretical MM Da/p*I*	Reference
	Ran binding protein 2	400000/6.8	358154/5.9	
	RNA helicase (KIAA0801)	130000/7.2	117443/9.3	
	Scaffold attachment factor B (SAFB)	150000/6.5	165120/5.3	
	SMC1 structural maintenance of chromosomes 1-like 1	145000/8.0	143186/7.5	
	SMC2 structural maintenance of chromosomes 2-like 1	130000/8.6	135762/8.7	
	Splicing coactivator subunit SRm300	600000/6.7	299658/12.1	
	Splicing factor 3b, subunit 3, 130 kDa	130000/5.6	135559/5.1	
	Squamous cell carcinoma antigen recognized by T cells 3	125000/6.7	109916/5.5	
	Translation initiation factor 2	165000/6.7	138910/5.4	
	Translation initiation factor 3, subunit 10 theta	160000/7.7	166551/6.4	
	Translation initiation factor 3, subunit 8	110000/6.7	105325/5.5	
	U5 snRNP-specific protein, 116 kD	115000/5.6	109417/5.3	
	Ubiquitin specific protease 7 (herpes virus-associated)	130000/6.0	128253/5.3	
	Ubiquitin-activating enzyme E1	115000/6.0	117771/5.6	
	Valyl tRNA synthetase 2 (Metabolism)	135000/7.6	140475/7.5	
	ATP-citrate (pro-S-)-lyase	115000/7.5	120807/7.0	
	Fatty acid synthase	220000/6.6	273070/6.2	
	Glucosidase II alpha subunit	110000/7.5	109419/5.8	
	Hexokinase 1	110000/6.7	102720/6.3	
	High density lipoprotein binding protein (vigilin)	150000/7.3	141421/6.4	
	Methylenetetrahydrofolate dehydrogenase	105000/7.5	101541/6.8	
	Oxoglutarate dehydrogenase	105000/6.4	113457/6.6	
	Pyruvate carboxylase	115000/6.5	129615/6.4	
	Trifunctional purine biosynthetic protein adenosine-3	110000/6.6	107749/6.3	
Unclassified				
	KIAA0336 gene product	180000/5.5	184639/5.1	
	Leucine-rich PPR-motif containing	135000/6.2	145182/5.5	
	The ha1225 gene product	105000/5.7	106750/5.7	
	Protein for MGC:21133	120000/5.0	128994/5.0	
	[Mouse] alpha-spectrin 1	300000/4.9	279845/4.9	
	[Mouse] murinoglobulin 1	170000/5.8	165139/6.0	
Human esophageal cancer				[50]
	Periplakin	190000/5.2	204506/5.33	
	Vinculin	130000/5.4	116645/5.75	
	Myosin heavy chain nonmuscle form A	130000/4.8	226582/5.41	
	Caldesmon 1 isoform 1	120000/5.5	93176/5.5	
	Smooth muscle myosin heavy chain 11 isoform SM1	110000/4.9	227179/5.29	

Experimental molecular mass (MM) and p*I* were obtained from the protein position on 2-DE gels. Theoretical molecular mass (*MM*) and *pI* were calculated by amino-acid sequence.

2-DE from various cells and tissues are shown collectively in Table 1.

In human plasma, noncovalently associated protein–protein complexes with apparent molecular mass 60–600 kDa can be analyzed by nondenaturing 2-DE [20]. In this system, an agarose gel (1% agarose containing 2.0% Ampholine pH 3.5–10 and 0.5% Ampholine pH 3.5–5) was used for the first-dimensional nondenaturing IEF. In the absence of denaturing agents in the second-dimensional polyacrylamide slab gel, alpha2-macroglobulin (alpha2M), high density lipopro-

tein (HDL) and haptoglobulin phenotype 2-1 (Hp2-1) have apparent molecular masses of about 600, 150 and 212 kDa, respectively. However, when a nondenaturing IEF agarose gel was equilibrated with a 2% SDS solution and then subjected to second-dimensional gel electrophoresis in the presence of SDS, an alpha2M spot appeared at an apparent molecular mass of about 320 kDa, suggesting that it dissociates into its half-molecule in the presence of SDS. This shows that, even if a protein complex in human plasma has a molecular mass larger than 100 kDa, the nondenaturing agarose IEF gel can be useful for separating the complex.

Almost all proteins in a eukaryotic organism are modified from their mRNA-translated polypeptide by post-translational modifications (PTMs), which result in a protein diversity far exceeding the complexity of the genome [21]. One of the advantages of 2-DE is that it can resolve post-translationally modified (PTM) proteins into multiple spots, providing useful information when comparing the abundance of PTM proteins to that of the corresponding mRNA [22]. Specialized methods have been developed to study phosphorylation (phosphoproteome) [23] and glycosylation (glycoproteome) [24]. When we compare the gel-estimated Mr/p*I* of each HMM protein with the theoretical Mr/p*I* of the HMM protein calculated from a protein database, we notice that discrepancies can occur between the two results. These discrepancies occur partly because the electrophoretic mobility of the HMM protein is highly sensitive to PTM. This makes it possible to assess the modification state of the protein.

Roncada et al. [25] reported that the resolution of HMM proteins analyzed by SDS-PAGE could be improved by using gels that are copolymers of acrylamide and allyl agarose instead of pure polyacrylamide. This improvement is partly due to the higher average pore size of the copolymer gel, which does not entrap HMM proteins at the top. To investigate the performance of such copolymer gels for the second-dimension of 2-DE, they performed 2-DE of proteins extracted from the membranes of red blood cells (RBCs). The 2-DE map provided by the acrylamide/agarose copolymer showed well-defined spots in the 160–220 kDa range (spectrin alpha chain).

4. Analytical aspects and benefits of agarose gels

4.1. Comparison of agarose and polyacrylamide gels for protein separation

Agarose gel is the preferred chromatographic medium used for separating biological molecules of molecular mass >250 kDa, for which minimal nonspecific binding and retention of biological activity is required [26].

One of the reasons that agarose gels are suitable media for analyzing HMM proteins is the pore size of agarose gels, which was reported to be much larger than that of the polyacrylamide gels. The pore size of agarose gels at 0.5%–3.0% concentrations was 800 to 260 nm, respectively [27], while the apparent polyacrylamide gel pore radii ranged from 21 nm in gels containing 10.5% T, 5% C to 200 nm in gels with 4.6% T, 2% C [28].

In conventional SDS gel electrophoresis, the most commonly used separation matrix is a cross-linked polyacrylamide gel, typically at concentrations higher than 5%, or, when used in a gradient format, up to 25% [29]. The acrylamide concentration is selected to maximize resolution of the proteins of interest. A typical gel of 7% acrylamide composition nicely separates proteins with molecular mass between 45 and 200 kDa. In order to nicely separate smaller HMM proteins (100–200 kD), the concentration of acrylamide needs to be less than 5%. Note that, although the lowest percentage of acrylamide that will allow gelation is around 3%, gels made with less than 4% acrylamide are extremely difficult to work with, and thus rarely used [30].

4.2. SDS-agarose gel electrophoresis

Polyacrylamide-based SDS gels with very high molecular mass (VHMM) proteins (>500 kDa) is highly problematic, since protein migration is very limited in them. Attempts to overcome this problem through the use of large porosity gels are compromised by such gels' mechanical instability [31]. In addition, the transfer efficiency of HMM proteins out of polyacrylamide gels is low, which makes Western blotting a difficult challenge. By contrast, major advantages of agarose gels include increased mobility of VHMM proteins, improved resolution of isoforms, higher mechanically stability of the gels, and a much higher transfer efficiency to Western blots [31].

Various types of agarose gels have been used for separation of HMM proteins. SDS-agarose gels have been commercially available from FMC Bioproducts (Rockland, ME, USA) for over 10 years. Warren et al. [31] reported the SeaKem Gold agarose gel (Biowhittaker Cell Biology Products, Walkerville, MD, USA) worked best among various types of agarose or polyacrylamide gels for separating titin (3000–4000 kDa), novex-3 (700 kDa) and neblin isoforms (600–900 kDa) from skeletal and cardiac muscle extracts. The ProSieve resolving gel has been used horizontally to separate glycoproteins in a 150–220 kDa range [32]. Gabriel and Zentner [33] reported that SDS agarose gel electrophoresis and electroelution are suitable methods for studying very large molecular mass salivary mucin glycoproteins (>450 kDa). The superior mechanical strength of agarose, even at low gel concentrations, its biological inertness, and its stability in the pH range of 4–9 make it a popular electrophoresis separation matrix [29].

4.3. Agarose IEF

Agarose gels for isoelectric focusing (IEF) have been used for protein separation since the 1970s. Rosen et al. [34] reported that the resolving power of agarose IEF was comparable to that obtained in thin-layer IEF in polyacrylamide gels when native 19S IgM and alpha 2-macroglobulin were separated. However, agarose-based IEF has not been widely used for protein separation, mainly because the agarose gels are fragile and pose handling difficulties when compared with polyacrylamide gel-based IEF.

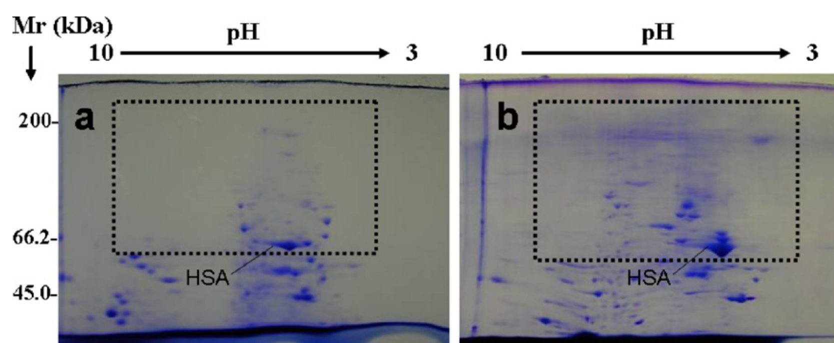

Fig. 2. Protein extraction efficiency of SDS in combination with urea and thiourea. (a) Human prostate cancer cells LNCaP (50 mg in weight) was homogenized with a 20-fold volume of extraction medium (7 M urea, 2 M thiourea, 2% w/v CHAPS, 0.1 M DTT, 2.5% w/v Pharmalyte pH 3–10 and protease inhibitors (Complete Mini EDTA-free; Roche Diagnostics, Mannheim, Germany). After centrifugation, 200 μL of the sample solution was applied to the agarose IEF gel. (b) LNCaP (50 mg in weight) was homogenized with a 10-fold volume of SDS sample buffer (2% SDS, 10% w/v glycerol, 5% w/v 2-ME, 0.0625 M Tris–HCl, pH 6.8). A 10-fold volume of the extraction medium was put into the homogenate, mixed well, then centrifuged with Ultrafree-0.5 centrifugal filter units (Millipore, Billerica, MA, USA) until the solution volume was reduced to one-fifth of the original. After centrifugation, 100 μL of the sample solution was applied to the agarose IEF gel. The gels were stained with PhastGel Blue R. HSA: human serum albumin.

Hirabayashi [6] was the first to develop a 2-DE method using agarose IEF in the first dimension. Oh-Ishi and Hirabayashi [8] further improved the method by adding 1 M thiourea and 5 M urea into an agarose IEF medium. A thiourea-urea agarose IEF solution does not gel at room temperature but at 4 °C, and the gel formed at 4 °C does not melt when the gel is returned to room temperature [35]. From the practical point of view, the 1 M thiourea-5 M urea-agarose IEF gel was a tremendous improvement over the 7 M urea-agarose IEF gel originally developed [6], because the agarose solution temperature no longer needed to be kept above 40 °C when preparing agarose IEF gels.

Mukai et al. [36] selected agarose gel IEF among various techniques employing charge differences for protein separation, since it has the following advantages: (i) isoelectric focusing is superior in resolution and in the separable p*I* range of proteins than ion-exchanging chromatography or other charge-based chromatographic or electrophoretic techniques; and (ii) an agarose gel has a larger average pore size than a polyacrylamide gel, thus it is better suited for separating HMM protein complexes.

5. 2-DE with agarose gels in the first dimension (Agarose 2-DE)

5.1. Importance of solubilization of HMM proteins for agarose 2-DE

The use of different solubilization conditions for HMM proteins can increase the total number of protein spots detected on a 2-DE gel. Since the overall resolution of a 2-DE pattern always depends on the tissue being analyzed, the researcher will need to investigate various solubilization alternatives in order to find the methods and reagents that meet their requirements and optimize conditions for their study.

Thiourea, when used in combination with urea, was reported to be an efficient solubilizing reagent for HMM proteins [10]. For example, in skeletal muscle HMM proteins (myosin heavy chain, C-protein and alpha-actinin), the available amounts of these HMM proteins increased significantly when 1 M thiourea and 5 M urea were used for protein extraction.

SDS, a negatively charged ionic detergent, has been widely used in the first stages of solubilization of proteins [37]. Since almost all the proteins in any tissue or cell are solubilized by SDS, protein mixtures are often analyzed by SDS-PAGE. However, SDS-protein complexes, being uniformly negatively charged, are not compatible with IEF, and must be removed from the protein mixture before IEF [17]. Instead of SDS, non-ionic detergents, such as Triton X-100 and Nonidet P-40, or zwitterionic detergents, such as CHAPS, have been used for extraction media and/or IEF gels.

SDS is occasionally used for extraction of HMM proteins such as membrane integral proteins [38]. We tested the usage of SDS in HMM protein extraction medium in our laboratory. Fig. 2 shows agarose 2-DE patterns for human prostate cancer cells obtained in the absence (Fig. 2a) or presence (Fig. 2b) of SDS in the extraction media. In the former case, human prostate cancer cells LNCaP (50 mg in weight) were homogenized with a 20-fold volume of a 7-M urea-2-M thiourea extraction medium (7 M urea, 2 M thiourea, 2% w/v CHAPS, 0.1 M DTT, 2.5% w/v Pharmalyte pH 3–10 and protease inhibitors: Complete Mini EDTA-free; Roche Diagnostics, Mannheim, Germany). After centrifugation, 200 μL of the sample solution was applied to the agarose IEF gel. In the latter case, LNCaP (50 mg in weight) was homogenized with a 10-fold volume of SDS sample buffer (2% SDS, 10% w/v glycerol, 5% w/v 2-ME, 0.0625 M Tris–HCl, pH 6.8). Since SDS occasionally disrupts a 2-DE pattern, extensive removal of SDS before IEF is an essential procedure. To accomplish this, a 10-fold volume of the 7-M urea-2-M thiourea extraction medium was put into the SDS-treated homogenate, mixed well, then centrifuged using Ultrafree-0.5 centrifugal filter units (Millipore, Billerica, MA, USA) until the solution volume reduced to one-fifth of the original. After centrifugation, 100 μL of the sample solution was applied to the agarose IEF gel. The 7-M urea-2 M thiourea extraction was not as effective (Fig. 2a) as SDS extraction (Fig. 2b) in solubilizing these HMM proteins.

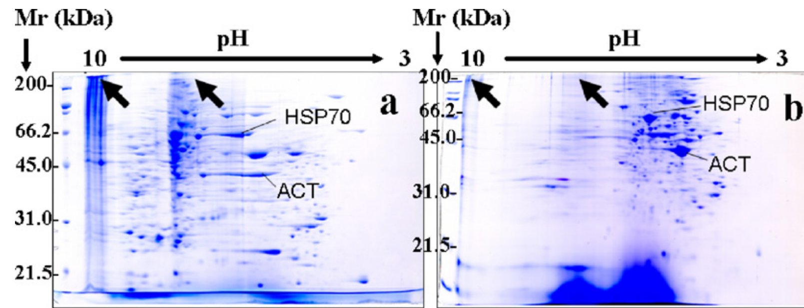

Fig. 3. Effect of ultracentrifugation. (a) Rat testis was homogenized with the extraction medium. The homogenate was centrifuged with a TOMY TMA-6 rotor at 15 000 rpm (30 000 × g) for 20 min. (b) The homogenate was centrifuged with a Beckman Coulter TLA-55 rotor at 50 000 rpm (112 000 × g) for 20 min. After centrifugation, 100 μL of each sample solution were applied to the agarose IEF gel. Arrows point to vertical streaks at the alkaline side of the second-dimensional SDS-gel, which came from proteins trapped on the alkaline top of the first-dimensional agarose gels. ACT: actin; HSP70: heat shock protein 70.

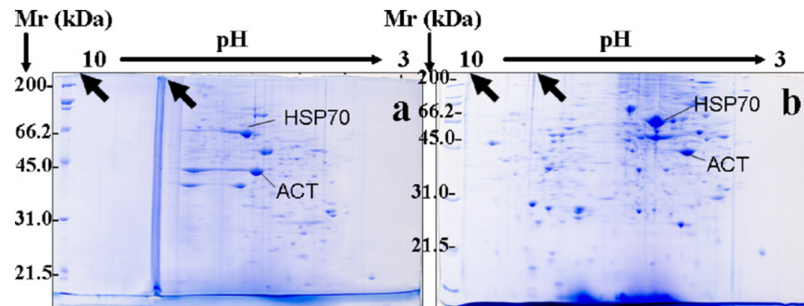

Fig. 4. Effect of ultracentrifugation. (a) Rat thymus was homogenized with the extraction medium. The homogenate was centrifuged with a TOMY TMA-6 rotor at 15 000 rpm (30 000 × g) for 20 min. (b) The homogenate was centrifuged with a Beckman Coulter TLA-55 rotor at 50 000 rpm (112 000 × g) for 20 min. The clear supernatant was centrifuged again with the same rotor at 50 000 rpm for 20 min. After centrifugation, 100 μL of each sample solution were applied to the agarose IEF gel. Arrows point to vertical streaks at the alkaline side of the second-dimensional SDS-gel. ACT: actin; HSP70: heat shock protein 70.

5.2. Importance of ultracentrifugation of samples

Our experience using 2-DE with various cells and tissues tells us that optimum sample preparation procedure depends on the sample. Proteome analysis of tissues with high DNA content, such as testis and thymus, is a very challenging task, because (i) many DNA binding proteins, including histones and transcriptional factors, are tightly complexed with DNA molecules; (ii) DNA molecules, which are acidic substances, can rush into the agarose IEF gel and destroy it before any proteins enter the gel, if the tissue extract is applied at the alkaline end of the gel; and (iii) the long-chain DNA can entangle with other proteins at the alkaline end of the IEF gel. Thus, removal of DNA molecules from the tissue extracts before sample loading is an essential process.

Figs. 3 and 4 show the effectiveness of ultracentrifugation for removing DNA from DNA-abundant homogenates before IEF. Ultracentrifugation with Beckman Coulter TLA-55 rotor at 50 000 rpm (112 000 × g) for 20 min was effective in subsequently obtained a well-resolved agarose 2-DE pattern (Fig. 3b). In the case of the thymus tissue extracts (Fig. 4), in which DNA is highly abundant, the clear supernatant should be centrifuged again at 50 000 rpm (112 000 × g) for 20 min before IEF (Fig. 4b). The right arrow in Fig. 4a shows the alka-

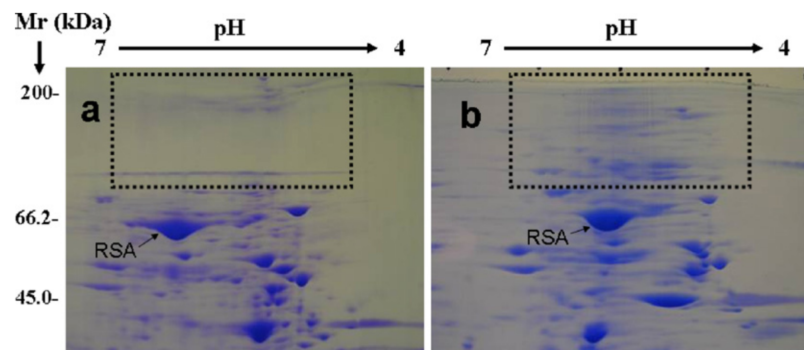

Fig. 5. Effect of 1% SDS in the second-dimensional SDS-polyacrylamide gel for analyzing HMM proteins of rat submaxillary gland. (a) The second-dimensional polyacrylamide gel includes 0.1% SDS according to the method of Laemmli. (b) 1% SDS was both in the stacking and separation gels. Note that protein spot densities are different in the square regions (HMM protein regions) surrounded by dotted lines in (a) and (b). RSA: rat serum albumin.

line end of the agarose IEF gel. Note how the IEF gel was shortened because of the highly abundant DNA in the thymus tissue.

5.3. Importance of SDS concentration of the second-dimensional SDS-polyacrylamide gel

O'Farrell applied the stacking gel system of Laemmli to the second-dimensional SDS-PAGE of his 2-DE system [5]. However, we noticed that 0.1% SDS concentration in the gel was not sufficient to solubilize HMM proteins in the first dimensional agarose IEF gel and to maintain solubility during the second-dimensional SDS-PAGE. Fig. 5 shows the effectiveness of 1% SDS in the second-dimensional SDS-polyacrylamide gel for analyzing HMM proteins of rat submaxillary gland. By comparing Coomassie-stained 2-DE patterns, we easily observed that the HMM protein spot densities are in general much thinner in Fig. 5a than in Fig. 5b (the square regions surrounded by dotted lines in Fig. 5a and b). Among tissues inspected, we noticed that tissues such as the submaxillary gland, sublingual gland and intestinal mucus, which bear abundant mucins (major glycoprotein components of mucus), are likely to distort the HMM protein region of an agarose 2-DE pattern.

6. Use of agarose 2-DE in cancer proteomics of HMM proteins

Cancer proteomics is expected to be useful for discovering new biomarkers and drug targets, present in serum and other tissues, that would give us means for early diagnosis of cancer, inventive cancer therapeutics and drug discovery [39]. One of the most prevalent and successful models in cancer research is that cancer involves changes in gene expression. However, given recent indications that alternative splicing is a widespread mechanism of functional regulation in the human genome, it is interesting to ask whether cancer might involve changes in mRNA splicing [40]. Whereas in normal cells such failures of the splice site selection machinery rarely occur [41], their frequency can be greatly elevated in tumors [42]. Cancer-associated mRNA splice variants have been reported for HMM proteins such as EGFR (Mr ∼170 kDa), lactate dehydrogenase (Mr ∼142 kDa), cadherin-11 (Mr ∼120 kDa), and fibronectin (Mr ∼440 kDa) [43]. Since gene expression profiles based on mRNA analysis do not always correlate with protein expression data, detection and identification of a cancer-specific isoform of HMM proteins, which then could be useful targets of cancer proteomics for early detection of cancer and for evaluation of tumor progression, is needed.

6.1. Prostate cancer

Although PSA has been a useful biomarker for prostate cancer, PSA in the "gray-zone" from 4.1 to 10 ng/mL is not particularly effective for differentiating prostate cancer from benign prostate diseases [44,45].

We made a comprehensive study of protein expression of androgen-independent (AI) prostate cancer (LNCaP human prostate tumors) by means of agarose 2-DE followed by liquid chromatography–tandem mass spectrometric analysis [46]. About 500 protein spots could be seen on a 2-DE gel stained with PhastGel Blue R. We successfully identified 295 proteins (91.0%) out of 324 spots excised. 233 out of 295 were non-redundant. Within this set, we divided the 226 cancer-related proteins into 64 high-molecular-mass (HMM) and 162 low-molecular-mass (LMM) groups based on their molecular masses being above or below 100 kDa. HMM and LMM proteins were observed to have different compositions with regard to functional classification: 39.1% of the HMM proteins but only 28.4% of the LMM ones were classified as transcription/translation-related proteins. Successfully obtaining this result was due to our usage of an agarose 2-DE method for protein separation. This method was sufficiently good at separating HMM proteins as large as 500 kDa and as much as 1.5 mg of total protein.

In light of this demanding situation, we expected that our agarose 2-DE maps would be a reasonable starting point for discovering new prostate cancer biomarkers. We identified 12 proteins that had not been reported previously to bear any relation to cancer and 35 more that had been considered to have something to do with cancer in general, but not with prostate cancer in particular [46]. Some of these proteins might be candidates as new biomarkers for prostate cancer, and further investigation is ongoing in our laboratory.

It should be noted that we did not identify certain well-known prostate cancer biomarkers such as PSA, PSMA, p53, vp27, Bcl-2, Ki-67, and E-cadherin [47] that had been found using genomic approaches. We believe that these discrepancies were due to the following: it was reported that the correlation between mRNA and protein levels was insufficient for reliably predicting protein expression levels from quantitative mRNA data [48,49]. Consequently, this proteomic approach may also have the capability of finding biomarkers that are different than those found by genomic approach [13]. The discrepancies might be attributed to the low sensitivity of our methods for protein detection with Coomassie Blue staining. Silver stain is one to two orders of magnitude more sensitive for detection of protein than Coomassie Blue staining. In fact, we could find at least several hundred protein spots when the silver staining method was used for protein detection. We used a 2D-DIGE system with agarose 2-DE for detection of human esophageal cancer proteins [50]. Our technique is thus also compatible with the sensitive CyeDye detection system.

6.2. Colorectal cancer

Although extensive proteome studies of colorectal cancer have been performed by several investigators, only a limited number of proteins have been identified, probably due to small loading capacity [51–55]. In order to significantly increase the amount of starting protein, we used the agarose 2-DE method to compare the protein expression profiles in normal and colorectal cancer tissues [56]. For HMM proteins, no significant difference between the tumor tissue and adjacent normal mucosa was observed. On the other hand, we did identify 36 LMM

proteins whose expression is altered in tumor tissue compared with adjacent normal mucosa. They include novel proteins such as eukaryotic translation initiation factor 4H (eIF-4H), inorganic pyrophosphatase, voltage-dependent anion channel 1 (VDAC1), adenylyl cyclase-associated protein (CAP1) and nuclear chloride ion channel protein (NCC27). The elevated expression of these proteins in cancer tissues was confirmed by Western blot. Moreover, post-translational modifications of prolyl-4-hydroxylase beta subunit (P4HB), annexin A2 and triosephosphate isomerase 1 (TPI1) were also identified. Interestingly, cleavage of P4HB was tumor-specific, whereas annexin A2 was resistant to proteolytic cleavage in tumor tissues. These novel findings demonstrated that the higher loading capacity of agarose 2-DE helps make it a powerful technique for looking for not only quantitative but also key qualitative changes in the biological process of interest. These capabilities will undoubtedly contribute to our understanding of the underlying mechanisms of diseases such as cancer.

6.3. Esophageal cancer

Esophageal cancer is one of the most lethal malignant tumors. The aggressive behavior of this tumor is often associated with systemic spread of the disease at the time of diagnosis [57]. Esophageal cancer has a high incidence of locoregional recurrence after radical surgery, and long-term survival is low [58]. Thus, to improve the prognosis of esophageal cancer, the development of early detection methods is critical. We identified novel proteins with altered expression in primary esophageal cancer using agarose 2-DE and agarose 2-D DIGE [50]. Thirty-three proteins out of 74 spots with altered expression in tumors were identified. They include five HMM proteins periplakin (∼195 kDa), vinculin (∼130 kDa), myosin heavy chain nonmuscle form A (∼130 kDa), caldesmon 1 isoform 1 (∼120 kDa), and smooth muscle myosin heavy chain 11 isoform SM1 (∼110 kD). Among them, a membrane-associated, 195 kDa protein, periplakin, was significantly downregulated in esophageal cancer, which was confirmed by immunoblotting. Immunohistochemistry showed that periplakin was mainly localized at cell–cell boundaries in normal epithelium and dysplastic lesions, while it disappeared from cell boundaries and shifted to the cytoplasm in early cancers and was scarcely expressed in advanced cancers [50]. These results suggest that periplakin could be a useful marker for detection of early esophageal cancer and evaluation of tumor progression.

7. Endocrine disease proteomics of HMM proteins

The human endocrine system is a complicated network of hormone-producing organs that control metabolism, growth, and development. Too much or too little of a certain hormone has deleterious effects throughout the body and causes various endocrine disorders. Endocrine diseases, including hypothyroidism and diabetes, show abnormalities in many different organs and tissues. Endocrine proteomics is expected to be useful for discovering new biomarkers and drug targets present in serum and various tissues or organs. Such discoveries would give us new means for early diagnosis of endocrine disorders, as well as inventive therapeutics and drug discovery. Although many organs can be affected by hormonal disorders, there are currently few validated biomarkers useful for evaluation of individual risk and staging the degree of target organ abnormality. Detection and identification of proteins related to endocrine diseases requires an extensive proteomic analysis of various tissues and organs of an appropriate animal model.

7.1. Hypothyroidism and dwarfism

Because hormone abnormalities affect many organs and tissues, agarose 2-DE in an endocrine system proteome project is expected to reveal virtually all proteins present in associated cells and tissues at any given time.

We searched for proteins known to be related to hereditary dwarfism in the *rdw* rat (gene symbol: *rdw*) in various tissues of the rat with agarose 2-DE followed by immunoblotting and microsequencing [59]. Among 10 tissues inspected, only the pituitary and thyroid glands showed abnormalities in protein contents. The abnormalities in the *rdw* thyroid were far more serious than in the pituitary. Fig. 6a shows that at least 18 protein levels in the *rdw* thyroid were above, and 17 were below, normal (Fig. 6b). Those identified among the increased proteins were endoplasmin (GRP94), immunoglobulin heavy chain binding protein (BiP/GRP78) and heat shock protein 70 (hsp70), the contents of which, respectively, were 40×, 10× and more than 50× higher in the *rdw* thyroid as in the normal tissue. These increased proteins are known to be molecular chaperone proteins. The observation of a decrease in thyroglobulin (Tg: Mr ∼330 kDa/subunit, 660 kD total) content in the *rdw* thyroid, as shown in Fig. 6a and b, suggests that thyroid hormone production is reduced in the *rdw* thyroid. Increased levels of the chaperone proteins and the pH shift of Tg in the *rdw* thyroid suggest an accumulation of misfolded, aberrantly glycosylated, or unassembled Tg. In fact, the primary cause is a single point mutation in the

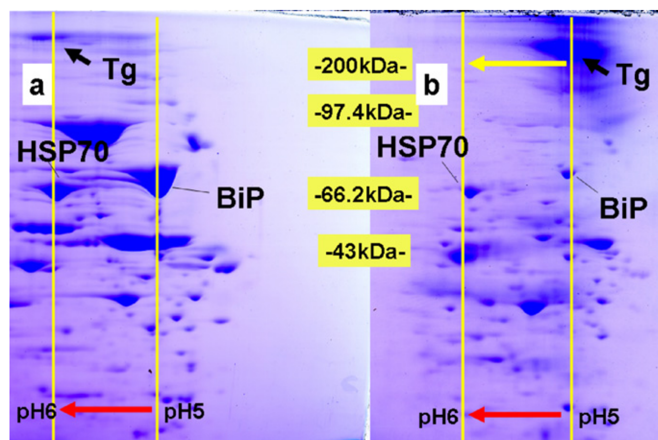

Fig. 6. Comparison of agarose 2-DE patterns of *rdw* (a) and the normal thyroid extracts (b). The 2-DE gels were stained with PhastGel Blue R. Tg: thyroglobulin (Mr ∼330 kDa/subunit; 660 kD total); HSP70: heat shock protein 70; BiP immunoglobulin heavy chain-binding protein. Note that the p*I* of *rdw* Tg was shifted from pH 5–6.

Tg gene [60]. This was originally inferred from the proteomic study [59] and later confirmed by sequencing the Tg cDNA [60]. Although a missense mutation (G2320R) in the Tg gene is the primary cause of the hereditary disorder, the deficiency of thyroid hormone (T4) also influences the developmental process of the *rdw* rats.

7.2. Diabetes

Proteins related to diabetes in the model Otsuka Long-Evans Tokushima Fatty (OLETF) rat, which resembles human diabetes mellitus Type II, were searched for in various tissues of the rat by a combined method of agarose 2-DE and agarose 2D DIGE. Long-Evans Tokushima Otsuka (LETO) rat was used as a control. This method allowed us to investigate differences in the 2-DE patterns from tissue extracts of OLETF and LETO rats, including changes in the amount of several proteins larger than 100 kDa (HMM proteins). In Fig. 7, red spots indicate proteins that are upregulated and green ones those that are downregulated in the liver from a 50-week-old OLETF rat. Identification of these proteins is now underway, and we believe that the results will be useful for finding new marker proteins for diabetes.

Oxidative stress is implicated in a broad variety of chronic and acute diseases, such as diabetes. To understand, at the protein level, cellular damage caused by this stress, we developed a proteomic method in which protein carbonyls were derivatized with biotin hydrazide followed by 2-DE [61]. Agarose 2-DE was chosen for its capability of analyzing HMM proteins. This method was applied to detecting protein carbonyls in skeletal and cardiac muscles of a diabetes model OLETF rat and a control LETO rat. A number of proteins, including mitochondrial ATP synthase beta-chain, desmin, actin, creatine kinase, and myosin heavy chain (MHC: Mr ~200 kDa), were found to be carbonylated. In chronological studies of protein carbonylation in gastrocnemius muscle of the OLETF rat, the carbonyl content of MHC changes with aging: MHC carbonyls stay low at 4 to 12 weeks of age, but rose and stayed high after 20 weeks of age. MHC carbonyl content in gastrocnemius muscle was the highest in the 50 week old rat [61].

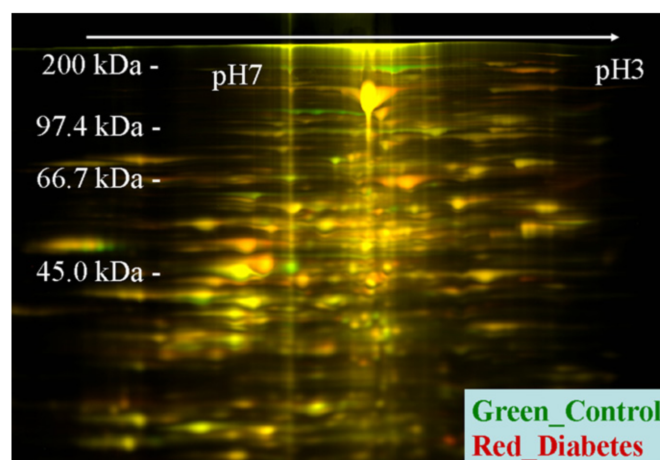

Fig. 7. An agarose-2D-DIGE pattern of rat liver proteins. Proteins extracted from the liver of the 50-week-old control LETO rat were labeled with green (Cy3) and those from the diabetic OLETF rat with red (Cy5). Note that HMM proteins can be successfully analyzed by this method of detection.

Fig. 8 shows 2-DE patterns for the testis of the 50 week old LETO (Fig. 8a and b) and OLETF (Fig. 8c and d) rats. The left panels of Fig. 8 stand for protein amounts (PhastGel Blue R staining) and the right ones for protein carbonyl contents. The square regions surrounded by dotted lines in the left panels correspond to the right ones. The left panels (Fig. 8a and

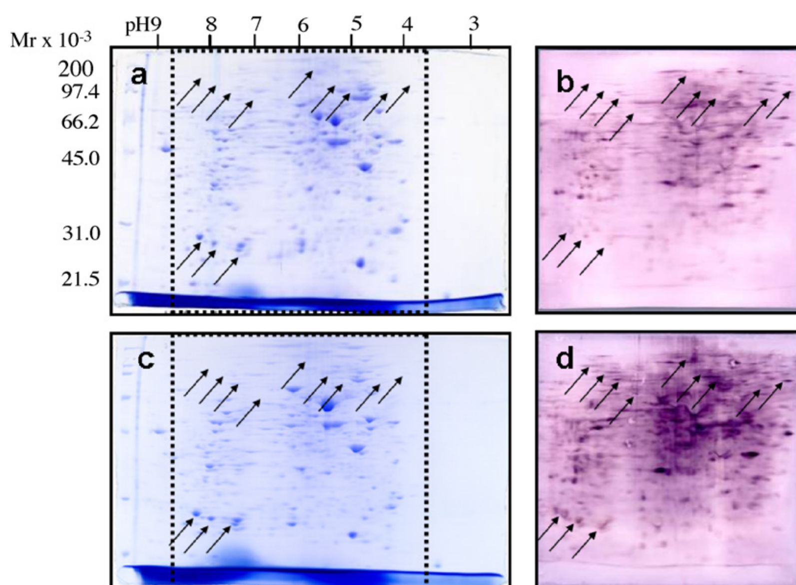

Fig. 8. Protein carbonyls detected in agarose 2-DE of testis extracts. (a) and (b) 50-Week-old normal LETO rat; (c) and (d) 50-week-old diabetic OLETF rat. (a) and (c) Protein amounts by PhastGel Blue R staining of 2-DE gels; (b) and (d) Protein carbonyl contents by carbonyl-specific color development on PVDF membranes. The square regions surrounded by dotted lines in the left panels correspond to the right ones. Major differences in protein carbonyl contents between the LETO and OLETF extracts are indicated by arrows.

c) are quite similar to each other, but the right panels (Fig. 8b and d) are not. The right panels of Fig. 8 clearly show that carbonyl contents of proteins in testis are higher in the OLETF rat than in the LETO. Protein carbonyls of the HMM proteins indicated by arrows were detected only in the OLETF rat. Unfortunately, since the amounts of the HMM proteins were very low, we could not identify these HMM proteins. However, this method would be a candidate for one of the proteomic tools that could individually analyze oxidative damage on these HMM proteins.

In conclusion, this proteomic method, utilizing protein carbonyl derivitization, could provide a means towards attaining a comprehensive view of oxidative modifications of proteins during the long progression of age-related diseases such as diabetes, as well as understanding the mechanisms involved in the onset, progression and complication of these diseases.

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