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Review

Separation techniques for high-molecular-mass proteins

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Abstract

Many high-molecular-mass (HMM) proteins (MW>100 kDa) are known to be involved in cytoskeleton, defence and immunity, transcription and translation in higher eukaryotic organisms. Even in the post-genomic era, purification of HMM protein is the first important step to analyze protein composition in a tissue or a cell (proteomics), to determine protein tertiary structure (structural biology), and to investigate protein function (functional genomics). To separate a HMM protein from a protein mixture, ions, chaotropes (urea and thiourea), detergents and protease inhibitors in extraction media and buffer solutions either for liquid chromatography or for gel electrophoresis should be carefully chosen, since HMM proteins tend to be aggregates under denatured condition and their long polypeptide chains are easily attacked by intrinsic proteases during separation procedure. Among many liquid chromatography techniques, affinity chromatography either with sequence-specific DNA for transcription factor, or with monoclonal antibody specific for myosin heavy chain has been used for preparative isolation of the respective HMM proteins. Though SDS–PAGE could analyze the size and the quantity of megadalton proteins, the resolution of HMM proteins is relatively poor. A newly developed pulse SDS–PAGE would be able to raise the resolution of HMM proteins compared with the conventional SDS–PAGE. The 2-DE method is not particularly suitable in analyzing HMM proteins larger than 200 kDa. However, a 2-DE method that uses an agarose IEF gel in the first dimension (agarose 2-DE) has been shown to produce significant improvements in 2-DE separation of HMM proteins larger than 150 kDa and up to 500 kDa. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Whole genome sequencing projects have resulted in the accumulation of DNA and protein sequence data at an incredibly rapid pace. The genome-wide studies allow the prediction the entire protein components of organisms. Proteins cannot be amplified by a chemical reaction like the PCR method for DNA or mRNA, but it is inevitable to avoid analyzing protein itself; because (i) the level of mRNA does not allow to predict the expression level of the protein in a cell, (ii) the amount of active protein is controlled both by production and degradation rates, and (iii) protein function is controlled by many post-translational modifications. Even in the post-genome era, purification of protein is the first important step to analyze the protein composition in a tissue or a cell, to determine the protein tertiary structure, and to investigate the protein functions.

Knowledge of the genome sequences of human, fly, worm, yeast and dozens of prokaryotes enables us to compare certain proteins between these organisms. When compared a prokaryote proteome with a eukaryote one, the former (*E. coli*, *H. influenzae*, *M. genitarium*, *M. pneumoniae*, *M. jannaschii* and *Synechocystis* sp.) consists of proteins mostly smaller than 100 kDa [1]. On the other hand, eukaryotes develop multi-functional proteins having several functional domains and the number of HMM proteins increases during the evolutionary process. In fact, a human has many more multi-functional proteins, involved in cytoskeleton, defence and immunity, and transcription and translation than an invertebrates [2].

Among HMM proteins, thyroglobulin (330 kDa) [3], myosin heavy chain (200 kDa) [4] and dystrophin (400 kDa) [5] have been known to be responsible for inherent diseases. Since these HMM proteins would be targets for genomic therapeutics, separation and purification techniques of HMM proteins are required for examining these molecular features.

Though a variety of protein separation techniques have been described, at the moment purification of a HMM protein remains a difficult task: (i) solubilization of the HMM protein greatly depends on each protein's physical and chemical properties, (ii) denatured HMM protein, in case of having hydrophobic regions, tends to aggregate in aqueous solution, and (iii) HMM protein, having a long polypeptide chain, would be a target of endogenous proteases during purification procedure.

The methodology to purify HMM proteins by liquid chromatography and by one- or two-dimensional gel electrophoresis is the subject of this section. An agarose 2-DE system (2-DE with agarose gels in the first dimension) would be preferable when (i) HMM proteins (MW 100–500 kDa) are to be

quantitatively analyzed and (ii) larger amounts of protein up to 1.5 mg in total are to be micropreparatively separated [6].

2. Solubilization of high-molecular-mass proteins

2.1. Ions

Myosin has been extracted at high ionic strength (0.5–0.6 *M* KCl) solution from skeletal muscle homogenate, but other HMM proteins, including M-protein, C-protein, and α-actinin, were also extracted in this condition. For separation of myosin from other HMM proteins, a low ionic strength solution (0.1–0.2 *M* KCl) is recommended.

For IEF or 2-DE separation of proteins, excessive salt concentration in a protein mixture (>50 m*M*) often causes disruption of the gel patterns. Consequently, high salt protein mixture should be avoided for gel electrophoresis. However, sodium chloride treatment of the membrane preparation prior to solubilization with urea caused further enrichment of membrane proteins [7].

Guanidine hydrochloride (GdnHCl), a potent denaturant with strong ionic substance, would be able to solubilize HMM proteins, such as myosin heavy chain (200 kDa) [8], thyroglobulin (330 kDa) [9], spectrin (230 kDa) [10] and collagen (300 kDa) [11]. However, at GdnHCl concentrations above 1 *M*, many HMM proteins lose their secondary and tertiary structures. These phenomena are partly reversible, but their functions are only partially recovered under highly limited conditions [12].

Although 8 *M* GdnHCl is sometimes recommended as a solubilizing reagents of proteins for electrophoresis [13], we do not recommend it. If GdnHCl is used in an extraction medium, removal of the reagent from the sample solutions with extensive dialysis is indispensable before IEF or SDS–PAGE. The dialysis, however, inevitably results in considerable loss of solubilized proteins, in particular HMM proteins. We, therefore, do not recommend use of GdnHCl for extracting proteins in 2-DE [6].

2.2. Organic sorbents

Organic sorbents are widely used in biochemistry for protein precipitation. This means only a limited number of proteins would be extracted under the hydrophobic conditions when sorbents are used. Molloy et al. [14] applied a 1:1 (v/v) solution of chloroform and methanol to extract hydrophobic proteins from whole lyophilized *E. coli* prior to solubilization with urea, thiourea, sulfobetaine detergents and tributyl phosphine. Even though some membrane proteins were analyzed by 2-DE for the first time, HMM proteins (>50 kDa) were not found on the 2-DE patterns.

2.3. Chaotropes

2.3.1. Urea and thiourea

Non-ionic chaotropes, such as urea, are compatible with isoelectric focusing (IEF). High concentration of urea (5–9.5 *M*) is efficient in breaking hydrogen bonds and has been used for extraction media and for IEF gels.

Thiourea, in combination with urea, has been reported to be a more potent denaturant than GdnHCl [15]. Solubilization of proteins in a thiourea–urea mixture is quite efficient especially for HMM proteins [16]. As for skeletal muscle HMM proteins (myosin heavy chain, C-protein and α-actinin), protein amounts of these HMM proteins extracted from rabbit skeletal muscle increased when 1 *M* thiourea and 5 *M* urea was incorporated both in the extraction medium and the first dimension agarose IEF gel of the agarose 2-DE [16].

2.3.2. Optimization of urea and thiourea concentrations

We optimized the usage of urea and thiourea in an extraction medium and agarose IEF gel. The concentration range surveyed was 5.0–8.0 *M* for urea and 0–2 *M* thiourea. The test sample used for optimization of these reagent concentrations was rat liver tissue; each of the frozen tissue pieces of about 10 mg in weight was homogenized in a 20-fold volume of extraction medium containing urea and thiourea of given concentrations. After centrifugation, 100 μl of each sample solution was applied onto the alkaline top of an agarose IEF gel containing the same concentrations of urea and thiourea with those of the extraction medium. Protein concentration was determined with the Bio-Rad protein assay according to the Bradford method [17].

The distinctive features are briefly described as follows:

(1) A 1-*M* thiourea–6-*M* urea extraction medium is only second best in protein extraction efficiency. Protein concentration extracted from rat liver tissue with an extraction medium of 20-fold volume of the tissue was 7.4 mg/ml. However, a 1-*M* thiourea–6-*M* urea agarose 2-DE pattern was best in the present survey (Fig. 1a).

(2) A 8-*M* urea extraction medium is the worst in protein extraction efficiency (5.5 mg/ml). A 8-*M* urea agarose 2-DE does not give a good 2-DE pattern, where focusing of the p150 and other proteins just below p150 was not so good (Fig. 1b).

(3) A 1-*M* thiourea–8-*M* urea extraction medium is good in protein extraction efficiency (7.1 mg/ml), but the 2-DE pattern obtained shows many artifactual spots appearing below p150, suggesting that p150 might form aggregates with other proteins.

(4) A 1.5-*M* thiourea–6-*M* urea extraction medium was the best in protein extraction efficiency (7.5 mg/ml) and the amounts of p150 and other HMM proteins increased. However, the 2-DE pattern obtained showed that each protein did not reach the correct isoelectric point at the end of IEF (Fig. 1c). When we continued IEF longer, proteins appeared as elongated spots in the 2-DE pattern (data not shown). The reason for the elongated spot shape is not clear, but a 1.5-*M* thiourea–6-*M* urea agarose IEF gel, being so fragile, might not be able to hold proteins at their isoelectric points after IEF is finished.

(5) A 2-*M* thiourea–5-*M* urea extraction medium was not bad in protein extraction efficiency (7.0 mg/ml), but the 2-DE pattern obtained was not good (data not shown).

We considered a combination of 1 *M* thiourea–6 *M* urea to be best for extraction medium and agarose IEF gel, though a 1.5-*M* thiourea–6-*M* urea extraction medium was best for protein extraction efficiency.

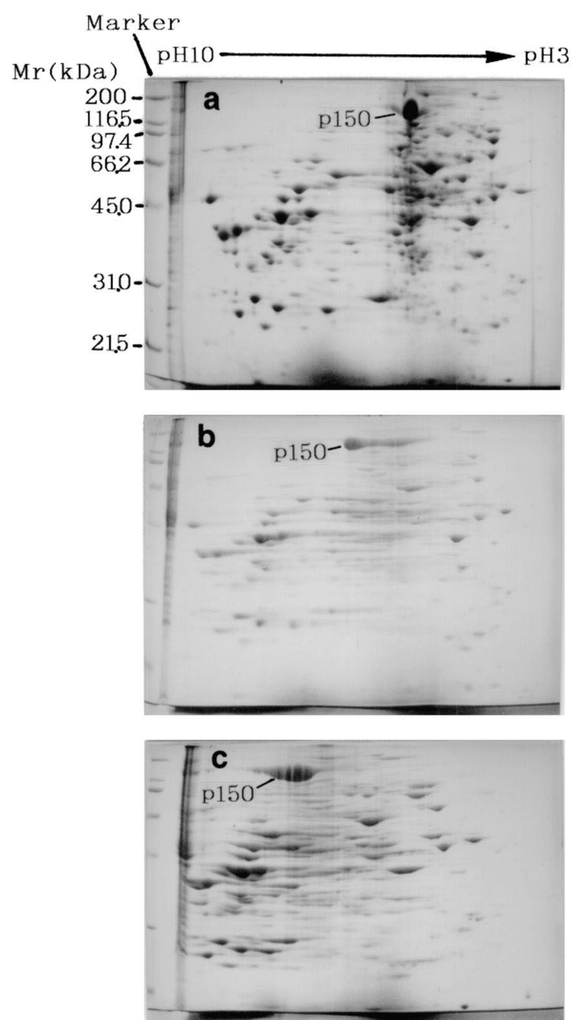

Fig. 1. Protein extraction efficiency of thiourea in combination with urea. Rat liver (10 mg in weight) was homogenized with a 20-fold volume of extraction medium containing different concentration of chaotropes (urea and thiourea). Extraction media and agarose IEF gels contained same concentrations of urea and thiourea. (a) 6 *M* urea and 1 *M* thiourea; (b) 8 *M* urea; (c) 6 *M* urea and 1.5 *M* thiourea. After centrifugation, 100 μl of each sample solution were applied to the agarose IEF gel. Spot p150 is an unidentified 150-kDa liver protein. The gels were stained with PhastGel Blue R.

2.4. Detergents

2.4.1. Solubilization of HMM proteins with detergents

SDS, which was introduced by Shapiro et al. [18], has been widely used in the first stages of solubilization of proteins [19]. Since almost all of the proteins in any tissues and cells are solubilized by SDS, protein mixture can be analyzed by SDS–PAGE.

SDS–protein complexes, having uniformly negatively charged, are not compatible with IEF, and have to be removed from the protein mixture before

IEF. Instead of SDS, non-ionic detergents, such as Triton X-100 and Nonidet P-40, has been used for extraction media and/or IEF gels.

SDS is occasionally used for extraction of HMM proteins such as membrane integral proteins. Harder et al. [20] reported that solubilization of MET6, a methionine synthase (90 kDa), from yeast (*S. cerevisiae*) cells was improved by boiling with 1% SDS, 0.1 *M* Tris–HCl, pH 7.0, followed by dialysis with 9 *M* urea, 1% DTT, 2% CHAPS and 2% carrier ampholytes.

Despite efforts to solubilize extensively integral membrane proteins, protein-solubilizing techniques with non-ionic detergents are of poor efficiency for the analysis of membrane proteins in 2-DE [21]. The use of zwittterionic detergents, such as CHAPS, has been recently described for the analysis of membrane proteins in 2-DE [22]. Friso and Wikstrom [7] improved solubilization of membrane proteins with the combination of CHAPS, Tris, thiourea and urea. In fact, a marker membrane protein, the NR1 subunit of the *N*-methyl-D-aspartate receptor and a 120-kDa hydrophobic protein, was identified using a monoclonal antibody in combination with Western blotting on a 2-DE pattern.

Chevallet et al. [23] tested denaturing medium containing various detergents and chaotropes. Best results were obtained with a denaturing solution containing urea, thiourea, and zwitterionic detergents. Among the dozen detergents synthesized and tested, amidosulfobetaines with an alkyl tail containing 14–16 carbons proved most efficient, solubilizing previously undetected membrane proteins.

Fibronectin (MW 240 kDa), a glycoprotein found in the extracellular matrix, is present in fairly high concentration (milligram range), while it is undetectable in 2-DE gels. With a combination of zwitterionic and chaotropic substances, Musante et al. [24] obtained a good resolution of the protein in 2-DE gels containing 0.5 *M* thiourea in combination with 8 *M* urea.

2.4.2. Optimization of detergent usage

We tested the usage of detergents such as Nonidet P-40, Triton X-100 and CHAPS in the 1-*M* thiourea–6-*M* urea extraction medium and agarose IEF gel. The problem we wanted to solve is the dark vertical streaks at the alkaline end of Fig. 1, which testifies that some of the proteins applied on the alkaline top of an agarose IEF gel was trapped on the spot. We assumed that 10% of the applied proteins is left on top of an 1-*M* thiourea–6-*M* urea agarose IEF gel. In order to reduce the trapped proteins on top of an agarose IEF gel, we added one of the three detergents, Nonidet P-40, Triton X-100 and CHAPS, to either or both of an 1-*M* thiourea–6-*M* urea extraction medium and agarose IEF gel. Final detergent concentrations we tested were 1, 3 and 5% for each detergent.

Fig. 2 shows agarose 2-DE patterns obtained in the presence of either 3% Triton X-100 (Fig. 2a), 3% CHAPS (Fig. 2b), or 1% Triton X-100 and 3% CHAPS in combination (Fig. 2c) in the extraction media.

Fig. 2a shows that 3% Triton X-100 removed the vertical streak lines at the alkaline end of the agarose 2-DE gel (arrows in Fig. 2a). 3% Nonidet P-40 showed similar results.

Fig. 2b shows that 3% CHAPS in an extraction medium is not as effective as Triton X-100 in removing the vertical streak lines at the alkaline end of a 2-DE gel, but spot focussing of 150-kDa rat liver proteins is better than with the other two detergents.

Simple increase in detergent concentration above 3% is in general not recommended, because 5% Triton X-100 in the extraction medium and/or in the agarose IEF gel disrupted the 2-DE patterns in the following way: (1) horizontal streaks were seen in the upper region of 2-DE patterns, (2) protein spots in the low-molecular-mass region became dim, and (3) the front lines in 2-DE patterns were disturbed.

We further investigated if we could improve the 3% CHAPS result by reducing the trapped amount of proteins at the alkaline end of the agarose IEF gel while keeping the quality of spot focusing. We tested using two pairs of detergents in the 1 *M* thiourea–6 *M* urea extraction medium, i.e. 3% CHAPS–1% Triton X-100 as shown in Fig. 2c. This combination gave better results than in 3% CHAPS alone (Fig. 2b) when we concentrated on reducing the vertical streak lines at the alkaline end of the agarose gel. However, in Fig. 2c, there was evident of increasing vertical streaking on p150. In addition, the focus of

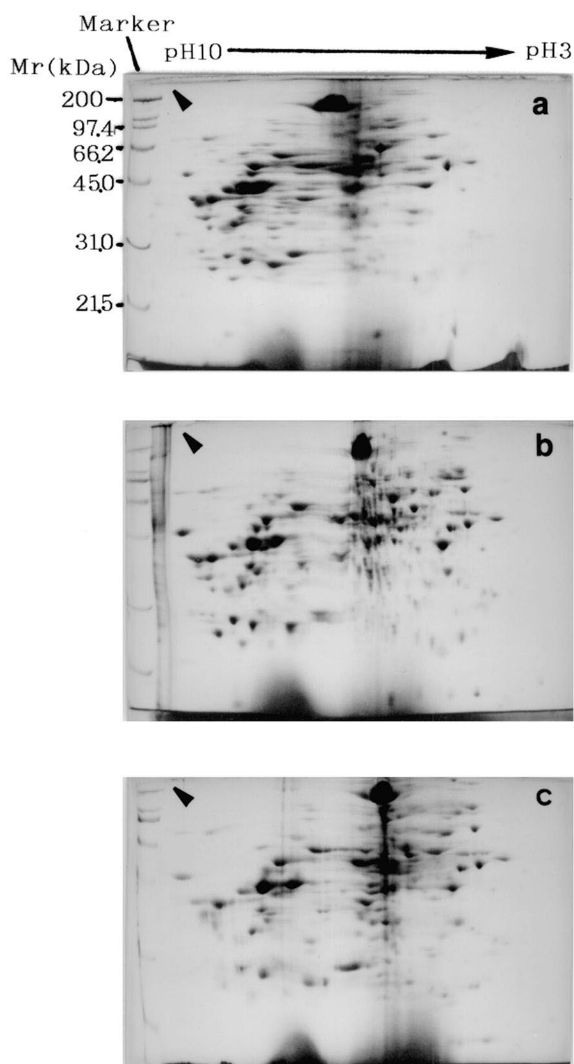

Fig. 2. Optimization usage of Triton X-100 or CHAPS through the quality of 2-DE patterns. The tissue sample used is rat liver. In addition to chaotropes (6 *M* urea and 1 *M* thiourea), (a) 3% Triton X-100, (b) 3% CHAPS, and (c) 1% Triton X-100 and 3% CHAPS were included only in the agarose IEF gels. Arrowheads point to vertical streaks at the alkaline and second-dimensional SDS–PAGE, which came from proteins trapped on the alkaline top of the first-dimensional agarose gels.

the spots in the basic region of the gel was also compromised.

We also report on the problem caused by detergent usage: i.e. where the amount of proteins trapped on top of the second dimensional 12% separation gel is increased by the presence of detergents in the first dimensional agarose IEF gel. Our solution to this problem is to let Triton X-100 penetrate on top of the separation gel. Just after gellation of 12% acrylamide solution, we covered the gel top with 1 ml of 5% Triton X-100, and kept it overnight at room temperature.

This procedure reduced the amount of proteins trapped on top of the second dimensional polyacrylamide gel, while keeping the quality of the agarose 2-DE patterns of rat liver (Fig. 3). However, if Fig. 3b and a are compared in more in detail, some HMM proteins in Fig. 3b were not focused and appeared blurred.

2.4.3. Sequential extraction of proteins

A sequential extraction method, aimed for a complete proteome analysis, is a new trial for collecting as many kinds of proteins as possible from a tissue or cells with the use of different extraction media.

Molloy et al. [14] reported a simple three-step sequential solubilization protocol applicable for whole cell lysates. As the first step, Tris-base was used to solubilize many cytosolic proteins. The resultant pellet was then subjected to conventional solubilizing solutions including urea, CHAPS, DTT, Tris and carrier ampholytes. Finally, the membrane protein rich pellet was partially solubilized using a combination of urea, thiourea, tributyl phosphine and multiple zwitterionic detergents.

2.5. Inhibition of proteolysis

Contamination of proteases in a tissue extract often causes proteolytic degradation of proteins, in particular HMM proteins. Proteolysis can be avoided by the use of protease inhibitors. Various protease inhibitors or premixed inhibitor cocktails are commercially available and can be added to the extracts.

For liquid chromatographic separation, protease inhibitors should be added both in binding and elution buffers and the buffers should be kept at 0–4 °C to minimize the proteolytic degradation.

GdnHCl is quite efficient in inhibiting protease activity and the endogenous proteases in cells could be inhibited completely when the cells are homogenized in GdnHCl. In an attempt to prepare a whole-

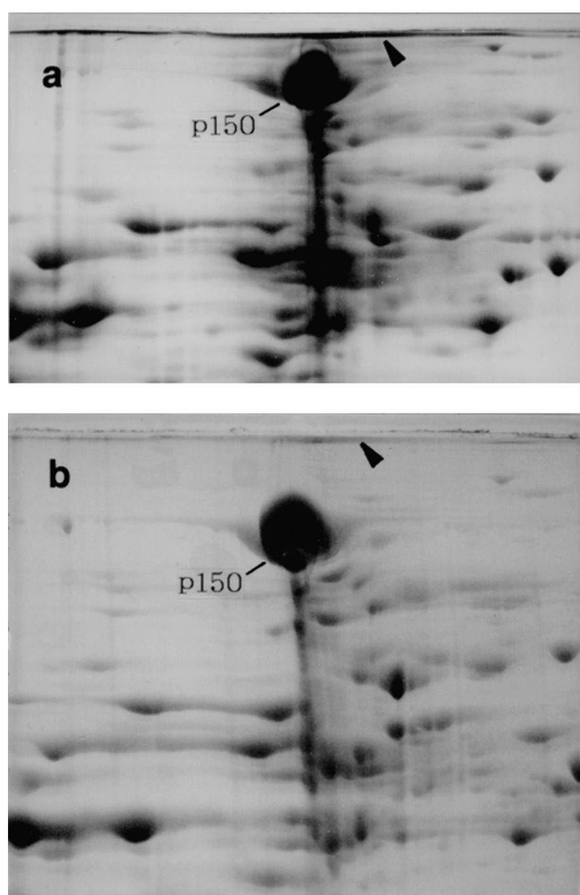

Fig. 3. Reduction of proteins trapped on top of the second-dimensional acrylamide gels. The tissue sample used is rat liver. (a) In addition to chaotropes (6 *M* urea and 1 *M* thiourea), 3% CHAPS and 1% Triton X-100 to an extraction medium. 2-DE gels with agarose gels in the first dimension were used. (b) Same as (a) except that the gel top of the second-dimensional acrylamide gel was incubated overnight at room temperature with 5% CHAPS. By incubating the gel top of the second-dimensional acrylamide gel with 5% CHAPS, the trapped proteins were reduced (arrowheads in upper and lower panels). p150, an unidentified 150 kDa protein. The gels were stained with PhastGel Blue R.

cell extract of *Tetrahymena*, a free-living protist, protease activity was so high that HMM proteins homogenized in an extraction buffer including 2% SDS were quickly digested by the endogenous proteases. Hirabayashi et al. [25] employed a 8-*M* GdnHCl extraction medium containing 10% 2-ME and 0.1 *M* Tris buffer, pH 7.5, for the *Tetrahymena* cells, and the degradation of HMM proteins could be prevented perfectly.

For sample preparation of crude extracts for 2-DE, endogenous protease activities should be inhibited completely when protease-containing tissues, such as brain and intestine, are analyzed by 2-DE. Though GdnHCl is the choice for extraction medium of 2-DE, some HMM proteins would be lost during dialysis against urea and thiourea before IEF.

Shortening of time for sample preparation is an important factor for minimizing proteolytic degradation.

In our experience, HMM proteins (>100 kDa) tend to be degraded even in an ice-cold extraction medium with 6 *M* urea and 1 *M* thiourea including a protease inhibitor cocktails (Complete Mini EDTA-free; Roche, Mannheim, Germany). Fig. 4a seems to be very different from Fig. 4b, but both are 2-DE patterns obtained from the same sample extracted from mouse intestine. After homogenization and centrifugation with a TOMY TMA-6 rotor at 15 000 rpm for 20 min, a 100-μl protein sample solution was applied at the top of an agarose IEF gel. IEF was started immediately (Fig. 4a), or 3 h later (Fig. 4b). Although the sample solution was kept in an ice-bath before the IEF started, HMM proteins were preferentially degraded (indicated by arrows in Fig. 4a and b).

3. Liquid chromatography

3.1. LC for HMM proteins

For isolation of a HMM protein from tissue extracts, several kinds of liquid chromatography, based on different principles, such as ion-exchange, size exclusion, reversed-phase, hydrophobic, hydroxylapatite and bioaffinity are used sequentially. For example, a 120-kDa protein factor P, which is responsible for the binding to specific DNA, was purified by fractionating a yeast crude extract through DEAE–cellulose, heparin ultrogel, Mono Q, and sequence-specific DNA columns [26]. Like other DNA-binding proteins, involved in transcription or translation, the sequence-specific DNA affinity chromatography would be a key technique for isolation

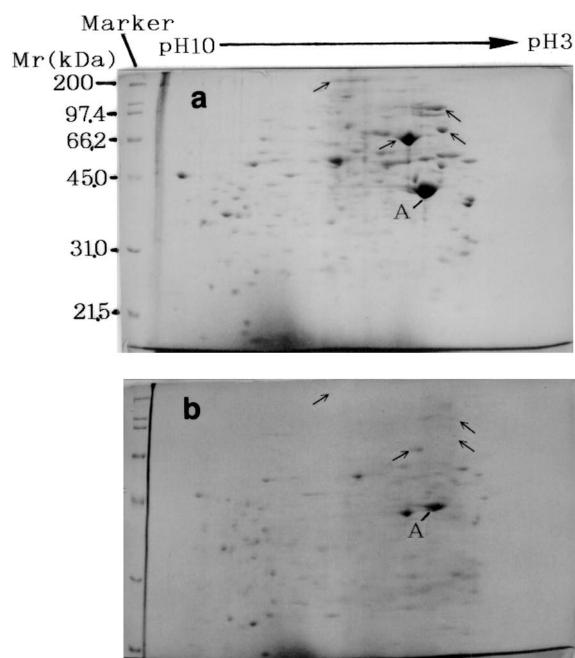

Fig. 4. Protein degradation by intrinsic proteases of intestine. Mouse intestine proteins were extracted with an extraction medium containing 1 *M* thiourea and 6 *M* urea with protease inhibitors. (a) After centrifugation, a 100-μl protein sample solution was charged onto an agarose IEF gel and then IEF was conducted. (b) Same as (a) except that the protein sample solution was kept in an ice-bath for 3 h before the IEF. A: actin. High-molecular-mass proteins indicated by arrows were preferentially degraded in panel (b). The gels were stained with PhastGel Blue R.

of a DNA-binding protein, even if it is a rare protein (see Subsection 3.4: DNA-binding proteins).

Since the choice of a liquid chromatography depends on each HMM protein's physical and chemical properties, separation techniques by liquid chromatography are shown collectively in Table 1.

3.2. Myosin and other muscle HMM proteins

Myosin (MW 450–480 kDa), the most abundant structural protein in muscle, has been separated by either or in combination with DEAE–cellulose [27], Sephadex G-200 [28], phosphocellulose [29], DEAE–Sephadex A-50 [30] or hydroxylapatite [31]. Since myosin tends to make aggregates at low ionic strength (<0.2 *M* KCl), GdnHCl is occasionally incorporated into the buffer for gel filtration. Digested peptides of myosin could be isolated by gel filtration on a Sephadex G-100 column in the presence of 5 *M* GdnHCl, followed by anion-exchange chromatography on a QAE-Sephadex A-50 column in the presence of 8 *M* urea [32].

Skeletal muscle contains many HMM proteins, such as myosin heavy chain (MHC), C-protein, M-protein, titin and dystrophin, most of which are integrated into muscle fiber. Solubilizing and separating these muscle proteins by liquid chromatography is still difficult, but affinity chromatography with a specific antibody is often used for purification of a muscle HMM protein.

For example, the cardiac myosin–actin complex from guinea pig was purified in one step using a salicylate–silica affinity column. The affinity of cardiac myosin for the immobilized salicylate was unique as MHC from guinea pig leg muscle extracts could not be purified by this procedure, suggesting that the column is specific for cardiac myosin [33].

Cardiac muscle has at least four isoforms of MHC. Affinity chromatography, using monoclonal antibodies specific for respective MHC isoforms, is a key technique for separate these isoforms. Chizzonite et al. [34] prepared monoclonal antibodies specific for ventricular MHC. They used these antibodies for separation and characterization of the molecular variants of MHC present in the rabbit heart. Two molecular forms of ventricular MHC, ventricular MHC α and MHC β, were, then, isolated from the rabbit heart by affinity chromatography. On the other hand, Komuro et al. [35,36] fractionated two isoforms of atrial MHC, atrial MHC alpha (A-MHC α) and MHC beta (A-MHC β), from the canine heart by affinity chromatography, using monoclonal antibodies specific for MHC α and MHC β, respectively.

3.3. Thyroglobulin

Thyroglobulin (Tg) (MW 330 kDa), a precursor of thyroid hormones, has been used as a marker protein for separation techniques of chromatography. Since Tg is an abundant thyroid protein and forms a

Table 1
Examples of high-molecular-mass proteins purified by liquid chromatography

Protein (M_r of monomer: kDa)	Purification methods	References
Muscle proteins		
Myosin heavy chain (200)	DEAE–cellulose	[27]
	Sephadex G-200	[28]
	Phosphocellulose	[29]
	DEAE–Sephadex A-50	[30]
	DEAE–cellulose, gel filtration, and hydroxylapatite	[31]
α-Actinin (100)	DEAE–cellulose, hydroxyapatite	[70]
M-protein (165)	DEAE–cellulose	[71]
Filamin (250)	Agarose gel filtration and DEAE–cellulose	[72]
Synemin (230)	Hydroxyapatite, DEAE–Sephacel, and phosphocellulose	[73]
Dystrophin (427)	Affinity chromatography with anti-dystrophin	[74]
Molecular chaperones		
HSP100 (100)	DEAE–cellulose, hydroxylapatite, Sephacryl S-300 and Mono Q	[75]
Endoplasmin (99)	Concanavalin A–Sepharose and Sephadex G150	[76]
DNA-binding proteins		
Sp1 (80)	DNA affinity	[44]
CAAT-binding protein (114)	DNA affinity	[41]

globular dimer in aqueous solution, Tg could be isolated in one step in a very pure form by gel filtration with a Sephacryl S-300 Superfine [37].

Since Tg is a glycoprotein, affinity chromatography on a concanavalin A (con A)–Sepharose column is useful for isolation of Tg from normal thyroid tissue [38].

Itoh et al. [39] separated dimeric Tg (MW 660 kDa) by a fast-separation system with a reversed-phase high-performance liquid chromatography (RP-HPLC) on octadecylsilyl (C_{18}) silica gel. Using steep gradient elution with a 0.1% aqueous trifluoroacetic acid–acetonitrile system at a constant flow-rate of 4 ml/min, recoveries improved under fast separation, since the protein sample suffered only a slight amount of irreversible denaturation on the hydrophobic surface of the stationary phase.

3.4. DNA-binding proteins

For isolation of DNA-binding proteins, DNA affinity chromatography have been proven fruitful for preparative isolation of the proteins, which bind preferentially to specific DNA sequences. Various kinds of solid matrix, to which DNA are bound, such as Sephadex beads [40], Sepharose CL-2B beads [41], latex particles composed of polyglycidyl methacrylate [42] and a Teflon fiber support [43] were reported.

A protein fraction that is enriched for transcription factor Sp1 (MW 80 kDa) can be further purified 500- to 1000-fold by two sequential affinity chromatography steps to give Sp1 of an estimated 90% homogeneity with 30% yield [44]. This method provides a means for the purification of rare se-

quence-specific DNA binding proteins, such as Sp1 and CAAT-binding transcription factor (114 kDa) [41].

4. Gel electrophoresis

4.1. SDS–PAGE

Since the stacking gel system developed by Laemmli [45], sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (SDS–PAGE) has been widely used for analyzing protein mixture. When concentration of acrylamide is reduce to 3–5%, SDS–PAGE is effective for analyzing HMM proteins. For example, two MHC isoforms in vascular smooth muscles were isolated with SDS–PAGE; MHC1 (204 kDa), MHC2 (200 kDa) and MHC3 (196 kDa) [46].

SDS–PAGE could analyze giant proteins in the megadalton range (> 0.5 MDa) in muscle tissues [47]. Among the giant proteins, titin (~3 MDa), is involved in the generation of resting tension and the assembly and stability of the sarcomere in skeletal and cardiac muscle tissues, while nebulin (~0.7MDa) is thought to regulate thin filament length in skeletal muscle. These giant proteins, playing important structural and functional roles in the striated muscle, could enter the SDS–acrylamide gel, but the resolution of molecular weights in megadalton range on a SDS-gel is relatively poor compared with in the low-molecular range (MW < 100 kDa).

A pulse SDS–PAGE for separating HMM proteins has developed recently [48]. The key feature of the method involves the application of current to slab SDS gels in a pulsatile, repetitive manner rather than continuously as in standard gel systems. Pulse SDS–PAGE increased the resolution of myosin heavy chain isoforms compared with the conventional SDS–PAGE and reduced common artifacts associated with long running time.

4.2. Two-dimensional gel electrophoresis (2-DE)

4.2.1. O'Farrell's 2-DE

O'Farrell [49] was the first to devise a 2-DE technique which could detect more than 1000 protein spots in a gel. Though it is evident that this method was quite powerful in his days, the method is not particularly suitable in analyzing HMM proteins larger than 200 kDa [13,50,51].

Myosin, composed of two heavy chains (200 kDa) and four light chains (~20 kDa), most of which integrate into polymers in vivo, tends to be aggregates in a low ionic solution. If a protein mixture including myosin is charged on an IEF gel, the ionic strength in the mixture should be low. When myosin is denatured in a solution with high concentrations of urea and thiourea, MHC tends to aggregate in a polyacrylamide gel for first-dimensional IEF. As a result, MHC is seldom resolved on conventional 2-DE systems [52]. For 2-DE, MHC should be reduced to the smaller fragments required for electrophoresis by proteolytic degradation [53].

According to the draft human genome sequence, myosin has a total of 40 known or predicted genes including heavy and light chains [54,55], and is expected there will be more than 100 myosin isoforms in human tissues because of: (i) the alternative splicing of MHC [56,57] and light chains [58,59], and (ii) the post-translational modifications [60]. The complexity of myosin molecule requires high resolution separation techniques to isolate these isoforms.

4.2.2. 2-DE with immobilized pH gradients (IPG-Dalt)

Immobilized pH gradients (IPGs) for IEF were first introduced in 1982 [61]. A pH gradient formed by mixtures of immobiline acrylamide buffers is covalently fixed to the acrylamide matrix during gel polymerization and can neither drift nor distort during IEF. As a result of the improved at ability and reproducibility of the pH gradient and commercial availability of precast gradient gels, the IPG-Dalt enjoys wide acceptance in the proteome project. As for the upper limit of protein molecular mass that can be analyzed by the IPG-Dalt, however, the use of polyacrylamide gel in the first-dimensional IEF puts the IPG-Dalt behind the agarose 2-DE.

4.2.3. 2-DE with agarose gels in the first dimension (Agarose 2-DE)

Hirabayashi [13] was the first to develop a 2-DE method which could analyze HMM proteins, includ-

ing myosin heavy chain (200 kDa). His trick was in the use of agarose gel instead of polyacrylamide gel for the first dimensional IEF. The agarose gel, when used for IEF, can analyze much larger proteins than polyacrylamide gel. With the 2-DE method, Hori et al. [64] reported that dystrophin (350 kDa) and other HMM proteins (up to 500 kDa) from mouse skeletal muscle were focused at the first dimension. Oh-Ishi and Hirabayashi [65] further improved the method by adding 1 *M* thiourea and 5 *M* urea in an agarose IEF medium. Thiourea being a very potent protein-solubilizing reagent, especially effective for HMM proteins, they therefore increased the amount of HMM proteins that could enter the first dimensional agarose IEF gel.

It should also be mentioned that, as a side effect of thiourea, 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 even when the gel is returned to room temperature. From the practical point of view of an experimental scientist, the 1-*M* thiourea–5-*M* urea agarose IEF gel was a tremendous improvement over the 7-*M* urea agarose IEF gel originally used [13], because we no longer need to keep the agarose solution temperature above 40 °C when preparing agarose IEF gels.

4.2.4. Preparation of agarose gels for IEF

We prepared the first-dimensional agarose IEF gels following basically the same procedure as described in our former reports [65,16], to which we added several modifications [6].

Three agarose IEF gels of 180 mm in length and 3.4 mm in diameter containing 1 *M* thiourea and 6 *M* urea were prepared by the present protocol.

Agarose IEF 0.10 g and D-sorbitol 1.20 g were put into a 50-ml beaker, and were dissolved in 5.8 ml distilled water at room temperature (solution A).

When detergent containing IEF gels were to be prepared, one of the three detergents, Triton X-100, Nonidet P-40, or CHAPS, was mixed into solution A and stirred overnight. Solution A was boiled in a microwave oven for 15 s $\times$ 10 times until the solution became clear and was then kept in a 70 °C water bath for 5 min. Mixed powder of urea 3.60 g and thiourea 0.76 g was put into solution A at 70 °C, which we shall call solution B hereafter.

Solution B was transferred into an water bath at room temperature, stirred with a magnetic stirrer until urea and thiourea were completely dissolved. The volume of solution B was adjusted finally to 9.0 ml with distilled water.

Solution B was divided into three test tubes: 1.0 ml for acidic, 4.5 ml for neutral and 2.5 ml for basic solutions. Four kinds of Pharmalyte (pH 2.5–5 for acidic, pH 3–10 and pH 4–6.5 for neutral and pH 8–10.5 for basic solutions) were added to the respective tubes according to the protocol shown in Table 2.

A glass tube (standard size: 260 mm length $\times$ 3.4 mm I.D.) was prepared beforehand, the bottom of which was covered with a piece of dialysis membrane and tied with a rubber band. The glass tube was set to AE-6300 electrophoresis unit (an apparatus for agarose IEF made by ATTO). The acidic, neutral and basic solutions were, respectively, sucked in with three syringes through 30-cm long thin polyethylene tubing. At first, the acidic solution was softly injected into the glass tube until the meniscus reached 20 mm high from the bottom of the tube. Next, the neutral solution was slowly injected until the meniscus reached 135 mm high from the tube bottom, and lastly the basic solution was carefully injected until the meniscus reached 180 mm from the tube bottom. A 10-μl overlaying solution containing 4 *M* urea and 1 *M* thiourea was gently laid on top of the agarose solution, which would facilitate for proteins to enter the agarose IEF gel.

The glass tube filled with agarose solutions was moved without jolting into a 4 °C chamber and kept there at least 6 h until the agarose solution gelled.

4.2.5. Sample application on agarose IEF gels

A 10–200-μl protein sample solution was applied at the cathodic end of the gel and the overlaying solution was gently filled above the sample solution to the top of the glass tube. The first-dimensional IEF was conducted at 600 V for 18 h at 4 °C.

After 18 h of isoelectric focusing, the agarose gel was transferred onto the top of the second-dimensional SDS-gel either directly or after proteins in the gel were fixed.

When proteins in the agarose IEF gel were fixed prior to the second-dimensional SDS–PAGE, the gel was extruded into a 300-mm long and 5-mm diameter glass tube filled with a protein-fixing solution

Table 2
Protocols for making agarose IEF gels

	Agarose IEF		0.10 g
	D-Sorbitol		1.20 g
	Distilled water		5.80 ml
	Dissolved at 100 °C	→	Solution A
		↓	
		Set at 70 °C	
	Urea		3.60 g
	Thiourea		0.76 g
	Dissolved at 50 °C	→	Solution B
		↓	
		Addition of distilled water until solution B comes to 9.0 ml	
Solution B			
Pharmalyte	1.0 ml	4.5 ml	2.5 ml
pH 2.5–5	100 μl	–	–
pH 3–10	–	300 μl	–
pH 4–6.5	–	150 μl	–
pH 8–10.5	–	–	250 μl
	↓	↓	↓
	Acidic solution	Neutral solution	Basic solution

containing 10% trichloroacetic acid and 5% sulfosalicylic acid. Both ends of the 5-mm diameter tube were connected to a peristaltic pump with polyethylene tubing to form a closed circuit filled with the fixing solution. When more than two gels were to be fixed, each of the gels was, respectively, extruded in a 5-mm diameter glass tube, which was serially connected to one another to form a bigger closed circuit filled with the fixing solution.

The proteins in the gel were fixed by a 1-h circulation of the fixing solution in the 5-mm diameter glass tube with a peristaltic pump, followed by another 1-h circulation of the 500-ml of distilled water. The fixed agarose IEF gel should be used immediately after the wash. When the fixed proteins on the agarose gel are kept at 4 °C overnight, the fixed HMM proteins, such as MHC and C-protein, would make aggregates occasionally on the agarose gel, and most of the HMM proteins could not enter the second-dimensional SDS–polyacrylamide gel.

Slab gels for the second dimensional electrophoresis were 12% polyacrylamide gel and were 195 mm in width, 120 mm in height, and 1.4 mm in thickness. The second-dimensional SDS–PAGE was carried out according to the stacking system of Laemmli [45] with a slight modification of adding 1% SDS both in the stacking and separation gels. When HMM proteins are to be separated in molecular size, the use of 3–5% gels is recommended to remove all small proteins so that HMM proteins have enough space to separate out.

The slab gels were then stained with PhastGel Blue R (Coomassie brilliant blue R 350; Amersham, Uppsala, Sweden) and destained with 30% methanol and 10% acetic acid.

4.2.6. Comparison of agarose 2-DE with IPG-Dalt

Figs. 5–7 are one-to-one comparisons of corresponding 2-DE patterns of IPG-Dalt and agarose 2-DE, respectively.

Proteins were extracted with a 100-fold volume extraction medium consisting of 6 *M* urea, 1 *M* thiourea, 0.5% 2-ME and protease inhibitors, and 100 μl each of the crude extracts was applied to both of the first-dimensional IPG-Dalt and agarose IEF gels. Total proteins of mouse skeletal muscle, rat liver, and the *rdw*/*rdw* rat thyroid applied to the gels were 130, 142, and 45 μg in weight, respectively.

By comparing Coomassie-stained 2-DE patterns, we easily notice that the protein spot densities are in general much thinner in the IPG-Dalt patterns than in the agarose 2-DE ones. The first-dimensional IPG

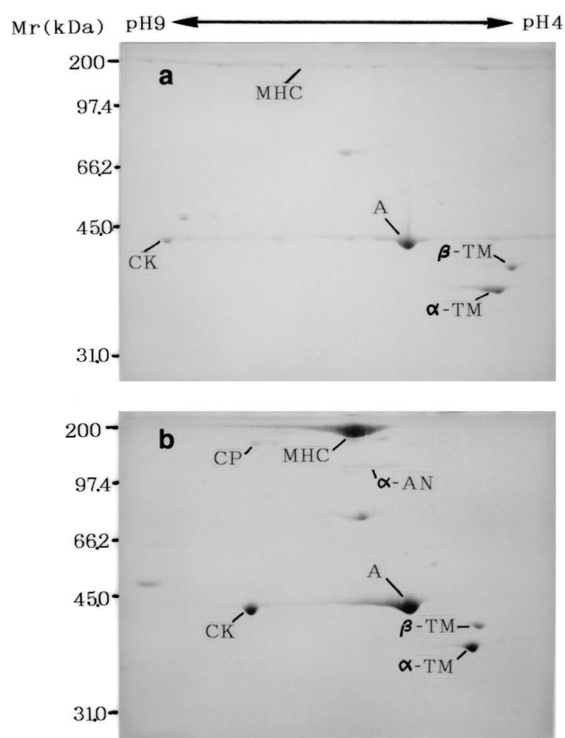

Fig. 5. Comparison of IPG-Dalt and agarose 2-DE of mouse gastrocnemius muscle. (a) 2-DE gels with IPGs for IEF; (b) 2-DE gels with agarose gels for IEF. Proteins (65 μg in weight) were loaded on an agarose IEF gel and an IPGs gel. Labels in the figures stand for protein species. MHC, myosin heavy chain; CP, C-protein; α-AN, α-actinin; A, actin; CK, creatine kinase; α-TM, α-tropomyosin; β-TM, β-tropomyosin. The gels were stained with PhastGel Blue R.

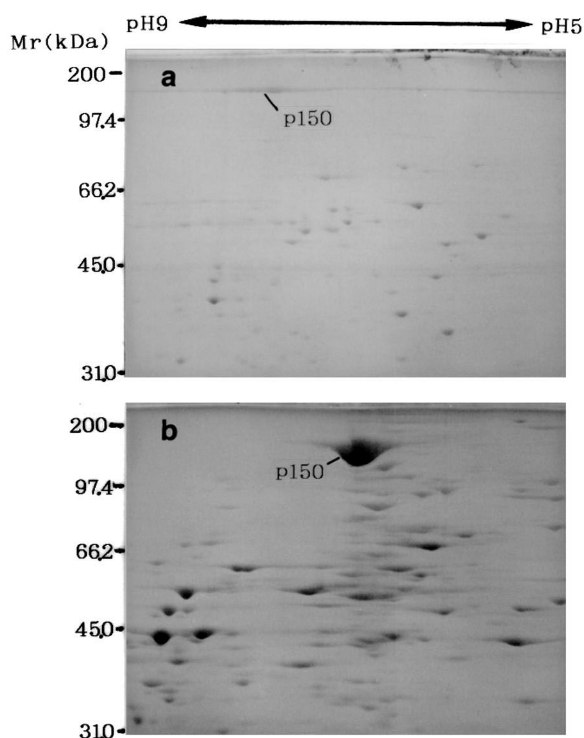

Fig. 6. Comparison of IPG-Dalt and agarose 2-DE of rat liver. (a) 2-DE gels with IPGs for IEF; (b) 2-DE gels with agarose gels for IEF. Proteins (142 μg in weight) were loaded on an agarose IEF gel and an IPGs gel. Labels in the figures stand for protein species. p150: a 150-kDa unidentified liver protein. The gels were stained with PhastGel Blue R.

gel strip is much less efficient in transferring proteins to the second-dimensional SDS–PAGE than an agarose IEF gel does.

Comparison of the 2-DE patterns of mouse skeletal muscle (Fig. 5a and b) reveals further differences between IPG-Dalt and the agarose 2-DE methods, i.e. spot focusing and integrated spot density depend on protein species.

We shall consider spot focusing of MHC and actin, 200 and 42 kDa major structural proteins in a skeletal muscle and labelled as MHC and A in Fig. 5a and b, respectively. The MHC in Fig. 5a looks like a long horizontal streak, but the MHC spot in Fig. 5b is a big spot with a tail on the basic side. The actin spot in Fig. 5a has long streak extending towards both acidic and basic ends; however, the spot A in Fig. 5b, on the other hand, is a rather big spot having a tail on the basic side.

Supporting data of the above suspicion are also given in Fig. 5. Such HMM proteins (MW>150 kDa) as α-actinin (lalelled as α-AN) and C-protein (CP) were not found in the IPG-Dalt gel (Fig. 5a) but in the agarose 2-DE gel (Fig. 5b).

When rat liver is analyzed either by IPG-Dalt or by agarose 2-DE, spot p150 in Fig. 6a and b appears as a long horizontal streak in the former but is a big spot in the latter.

In the case of thyroid extracts, spot Tg of thyroglobulin (MW ~330 kDa) (Tg) can not be seen in the IPG-Dalt gel (Fig. 7a) at all but is present in the agarose 2-DE gel (Fig. 7b).

The folding and assembly of Tg begins in the endoplasmic reticulum (ER) and is likely to involve a variety of reactions catalyzed by molecular

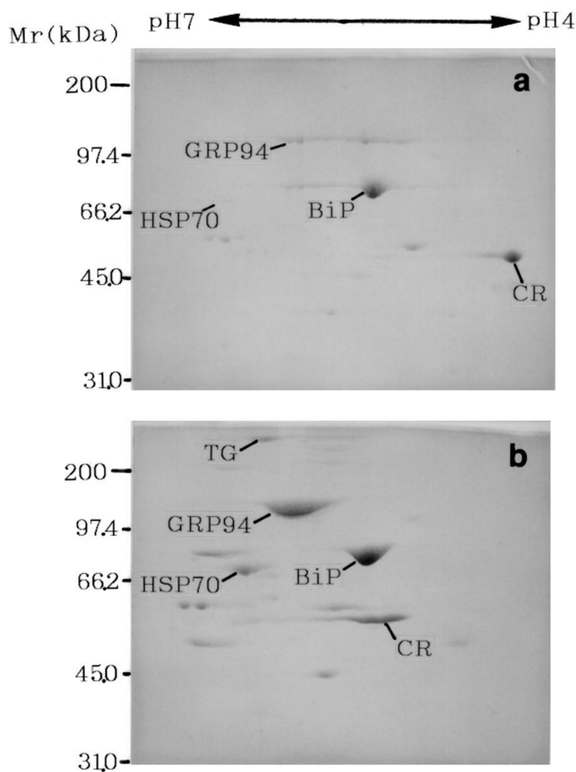

Fig. 7. Comparison of IPG-Dalt and agarose 2-DE of thyroid gland of the *rdw*/*rdw* rat with hereditary hypothyroidism. (a) 2-DE gels with IPGs for IEF; (b) 2-DE gels with agarose gels for IEF. Proteins (45 μg in weight) were loaded on an agarose IEF gel and an IPGs gel. Labels in the figures stand for protein species. TG, thyroglobulin; BiP, immunoglobulin binding protein; GRP94, glucose regulated protein 94 (or endoplasmin); HSP70, heat shock protein 70. The gels were stained with PhastGel Blue R.

chaperones [62]. In rat thyroid cells, BiP (68 kDa) and GRP94 (endoplasmin) (~100 kDa), function as specific molecular chaperones, and complex with Tg during its maturation [63]. GRP94 can be seen as an elongated streak rather than a spot in Fig. 7a, but the protein is present in the agarose 2-DE gel (Fig. 7b).

We conclude from these observations that the Immobiline DryStrip we used in this comparison would not be particularly suitable for analyzing proteins larger than 100 kDa.

As far as spot focusing of HMM proteins is concerned, protein load on the first-dimensional agarose IEF gel must be carefully adjusted, i.e. neither too much nor too small loads are recommended. Even when the protein load is optimized, spot focusing of HMM proteins is not so good as that of small proteins, but the agarose 2-DE is far better than the IPG-Dalt in which no HMM protein can focus at all.

The use of high concentration of detergents (Triton X-100, Nonidet P-40 and CHAPS) seemed to have adverse effects for focusing small proteins. If HMM proteins are to be analyzed by the Agarose 2-DE, analyzing of small proteins on the same gel is compromised.

4.2.7. Applications of agarose 2-DE for various tissues and cells

We shall examine in this section a number of applications of the agarose 2-DE method in analyzing proteins extracted from various tissues and cells.

Since 2-DE in the proteome project is expected to reveal virtually all proteins present in various cells and tissues at any given time, proteome analyses of the various tissues and cells are more or less challenging and will be of value as practical application examples of the agarose 2-DE.

To date, the tissues routinely analyzed in our laboratory are brain (cerebral cortex, cerebellum, brain trunk and hypothalamus), lens, spinal cord, pituitary, thyroid gland, salivary gland (parotid gland, sublingual gland and submandibular gland), liver, testis, spleen, thymus, pancreas, kidney, adrenal gland, oesophagus, stomach, intestine (duodenum, jejunum, blind gut and large intestine), mesentery, heart (atrium and ventricle), lung, adipose tissue (subcutaneous adipose tissue and visceral adipose tissue), blood vessel, skeletal muscle, diaphragm, skin and hair.

Since resolution of an agarose 2-DE pattern always depends on a tissue used, a troubleshooting guide for agarose 2-DE is shown in Table 3.

Proteome analysis of brain tissue is not an easy tasks, because: (1) a lot of structural proteins, including actin and tubulin, are expressed in the tissue, (2) intrinsic proteases are still active even in a 1-*M* thiouea–6-*M* urea extraction medium containing protease inhibitors, and (3) lipids, abundantly present in the tissue, can easily hinder us from obtaining good 2-DE patterns. In spite of these difficulties,

Table 3
Troubleshooting guide for agarose 2-DE various tissues

Tissue	Problem	Suggested remedy
Brain	Proteolysis	Immediate sample preparation
		GdnHCl for extraction
		Urea and thiourea for extraction
	Aggregation of proteins	Ultracentrifugation
Eye (lens)	Abundant crystallin	Reduce total proteins
Spinal cord	Proteolysis	Protease inhibitors
Pituitary gland	Abundant GH and PRL	Reduce total proteins
Thyroid gland	Abundant Tg	Reduce total proteins
	Aggregation of Tg	Ultracentrifugation
Salivary gland	Viscosity of saliva	Ultracentrifugation
	Protein overload	Reduce total proteins
Liver	Abundant 150 kDa protein	Reduce total proteins
	Hydrophobic proteins	Detergents
Testis	Abundant HSP70	Reduce total proteins
Spleen	Contamination of DNA	Ultracentrifugation
Thymus	Contamination of DNA	Ultracentrifugation
Pancreas	Proteolysis	Protease inhibitors
		Immediate sample preparation
	Abundant amylase	Reduce total proteins
Kidney	Hydrophobic proteins	Detergents (CHAPS)
Adrenal gland	Hydrophobic proteins	Detergents (CHAPS)
Oesophagus	Insoluble MHC	Use of thiourea
Stomach	Proteolysis	Protease inhibitors
		Increase pH
Intestine	Proteolysis	Protease inhibitors
		Immediate sample preparation
	Insoluble MHC	Use of thiourea
Mesentery	Abundant lipid	Ultracentrifugation
Heart	Insoluble MHC	Use of thiourea
	Lipid	Ultracentrifugation
Lung	Hydrophobic proteins	Detergents (CHAPS)
Adipose tissue	Abundant lipid	Ultracentrifugation
Blood vessel	Insoluble MHC	Use of thiourea
Skeletal muscle	Insoluble MHC	Use of thiourea
		Use of GdnHCl
Diaphragm	Insoluble MHC	Use of thiourea
Skin	Insoluble keratin	Use of thiourea
Hair	Insoluble keratin	Increase 2-ME

HMM proteins, such as neurofilament protein and spectrin, can be analyzed by the agarose 2-DE.

Pancreas is famous for having a lot of proteolytic enzymes and it is easy to imagine that those proteases would be activated immediately after death of an animal. The activated proteases will surely degrade 2-DE patterns of a pancreas tissue extract, but the agarose 2-DE pattern of rat pancreas is good enough to clearly show at least 30 spots of major proteins [66,67]. Pancreas 2-DE patterns were quite reproducible as long as we do not waste time in homogenizing a frozen tissue piece in an ice-bath. When we intensionally left a frozen pancreas tissue piece for 2 h at room temperature before homogenization, we obtained a poor quality 2-DE pattern, in which many spots of HMM proteins disappeared and spots of half digested proteins appeared.

Duodenum (intestine), being a heterogeneous tissue with a majority of smooth muscle cells, epithelial cells and endocrine cells, is also famous for secreting

various proteases. Intestine would, therefore, be a challenging organ to be analyzed with agarose 2-DE and we obtained a 2-DE pattern of the tissue (Fig. 4) as good as that of pancreas (see Section 2.5: Inhibition of proteolysis).

Cardiac muscle, expressing MHC in large quantities, is not so easily analyzed with conventional 2-DE. The cardiac MHC is a big protein having MW ~200 kDa and tends to aggregate, even in a 1-*M* thiourea–6-*M* urea extraction medium, by itself and with such structural proteins as C-protein and creatine kinase.

Testis expresses a considerable amount of heat shock proteins, hsp70 and hsc70. Hsp70 and hsc70 change their abscissa in the 2-DE pattern even when a same testis sample was divided into half and each half was simultaneously analyzed with the agarose 2-DE. The reason for the positional variation of hsp70 and hsc70 in the agarose 2-DE pattern may be as follows. Being molecular chaperon proteins, heat shock proteins intrinsically have a binding ability to unfolded proteins in a cell. Because many proteins are denatured in a 1-*M* thiourea–6-*M* urea extraction medium, some of heat shock proteins barely survived in the extraction medium would also bind to many unfolded proteins. Proteins bound to heat shock proteins would be different from time to time, and thus the heat shock proteins would also change their positions during IEF.

4.3. Electrophoresis in non-denaturing conditions

Whereas myosin is insoluble in low ionic strength buffers normally used for electrophoretic studies, it is quite soluble in low ionic strength pyrophosphate buffers, since ATP and its analogs, including pyrophosphate, prevent the formation of myosin polymers in low ionic strength buffers [68]. From this principle, Hoh et al. [69] developed the methods and apparatus used for polyacrylamide gel electrophoresis of myosin as intact molecules in pyrophosphate buffer. Intact fast and slow muscle myosins were separated by this method, i.e. normal fast muscle myosin has a higher electrophoretic mobility than slow muscle myosin. In addition, electrophoretic analysis of intact myosin from rat heart in pyrophosphate gels revealed the presence of five distinct components, two in atrial myosin (A1 and A2) and three in ventricular myosin (V1, V2, V3). SDS–PAGE of the myosin purified by the gel electrophoresis in non-denaturing conditions showed myosin heavy chain and light chains with little or no contaminants.

5. Nomenclature

2-DE	Two-dimensional gel electrophoresis
2-ME	2-Mercaptoethanol
Agarose 2-DE	Two-dimensional gel electrophoresis with agarose gels in the first dimension
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
GdnHCl	Guanidine hydrochloride
HMM	High-molecular-mass
HPLC	High-pressure liquid chromatography, high-performance liquid chromatography
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
IPG-Dalt	Immobilized pH gradient two-dimensional gel electrophoresis
LC	Liquid chromatography
MHC	Myosin heavy chain
PAGE	Polyacrylamide gel electrophoresis
PVDF	Polyvinylidene difluoride
RP-HPLC	Reversed-phase high-performance liquid chromatography
SDS	Sodium dodecyl sulfate, sodium lauryl sulfate
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
Tg	Thyroglobulin
Tris	*N*-Tris-(hydroxymethyl)-aminomethane

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