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Improvement of Plant Protein Solubilization and 2-DE Gel Resolution through Optimization of the Concentration of Tris in the Solubilization Buffer

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It is important to solubilize acetone-precipitated proteins before isoelectric focusing (IEF) to achieve high resolution 2-DE gels. To resolve the maximum possible number of plant protein spots, we developed an improved solubilization buffer for plant proteins. We demonstrated that the resolution of 2-DE gels increased dramatically as the concentration of Tris-base increased, with maximum solubilization obtained at 200 mM Tris-base (Ly200T). The Ly200T buffer was more effective than the commonly used solubilization buffer containing 40 mM Tris at solubilizing acetone–precipitated plant proteins. Use of the Ly200T buffer to solubilize proteins resulted in an increase in intensity of approximately 30% of plant protein spots in the larger-than-40 kDa region of the gel. The Ly200T buffer also improved the resolution of abundant and basic proteins. Thus, the Ly200T buffer can be used to achieve greater resolution of protein spots in plant proteomics research.

INTRODUCTION

High-resolution 2-D gel electrophoresis (2-DE) is a core component of proteomic applications (Herbert, 1999). Even though thousands of proteins can now be separated on a 2-D gel, certain proteins are still not well resolved with commonly used solubilization buffers, which restricts the analytical capabilities of proteomics research (Méchin et al., 2003; Rosenkrands et al., 2000). Protein solubilization prior to isoelectric focusing (IEF) is important to maximize the number of protein spots detected on the 2-DE gel (Hirano et al., 2006; Méchin et al., 2003). Protein solubility, in turn, depends on the nature of the reagents used in the solubilization buffer, such as chaotropes, detergents, and reducing reagents (Herbert, 1999; Hopkinson et al., 2005; Shaw and Reiderer, 2003). Although an established solubilization buffer (8 M urea, 4% CHAPS, 50-100 mM DTT and 40 mM Tris-base) is commonly used for 2-DE analyses, it is not always suitable for diverse biological samples (Herbert, 1999; Méchin et al., 2003). Hence, numerous attempts have been made to develop new buffers to enhance protein solubilization.

Protein denaturation is typically achieved by chaotropes, such as urea. These chaotropes facilitate protein unfolding and subsequent exposure of the hydrophobic cores of proteins (Herbert, 1999; Shaw et al., 2003). The chaotropic power of urea (7 M) can be enhanced by the addition of 2 M thiourea (an effective chaotrope) (Cho et al., 2007; Lanne et al., 2001; Rabilloud et al., 1997). Detergents, such as the zwitterionic sulfobetaine and CHAPS, enhance the effects of chaotropes and stabilize exposed hydrophobic residues by forming soluble complexes during IEF (Herbert, 1999; Hopkinson et al., 2005; Shaw et al., 2003). Sulfobetaine detergents with longer linear tails are usually more efficient than CHAPS, but their poor solubility at higher urea concentrations decreases the overall chaotropic activity of the solubilization buffer (Dunn and Burghes, 1983; Gianazza et al., 1987; Rabilloud et al., 1990).

Reduction of disulfide bonds is necessary for complete protein unfolding and linearity (Herbert, 1999; Hopkinson et al., 2005; Rabilloud, 1996). The classic reducing reagent, dithiothreitol (DTT), contains free thiols, becomes charged at basic pH, and migrates out of the immobilized pH gradient (IPG) strip during IEF (Herbert, 1999). This depletion of the reducing reagent causes poor 2-DE gel resolution and horizontal streaking (Görg et al., 2000; Herbert, 1999). Replacement of DTT with uncharged reducing reagents, such as tributylphosphine (TBP) or hydroxyethyl disulphide (HED), enhances the resolution of basic proteins (Herbert et al., 1998; Olsson et al., 2002). Another source of horizontal streaking in the basic pH region of a gel is the transport of water towards the anode, called reverse electroendosmosis (Görg et al., 1997). The addition of isopropanol or/and glycerol to the solubilization buffer improves separation of proteins in the basic region by preventing this water transport (Hoving et al., 2002; Görg et al., 1997).

Complete protein solubilization requires the reduction of protein-protein interactions (Herbert, 1999; Méchin et al., 2003; Shaw et al., 2003). Carrier ampholytes at a concentration of 0.5-2% are used widely to inhibit these associations (Berkelman and Stenstedt, 2001; Jacobs et al., 2001; Shaw et al., 2003). In some cases, buffers or salts, such as 40 mM Tris-base are required for full protein solubilization (Berkelman and Stenstedt, 2001). However, a high concentration of salts causes higher strip conductivity, as well as electroendosmosis,

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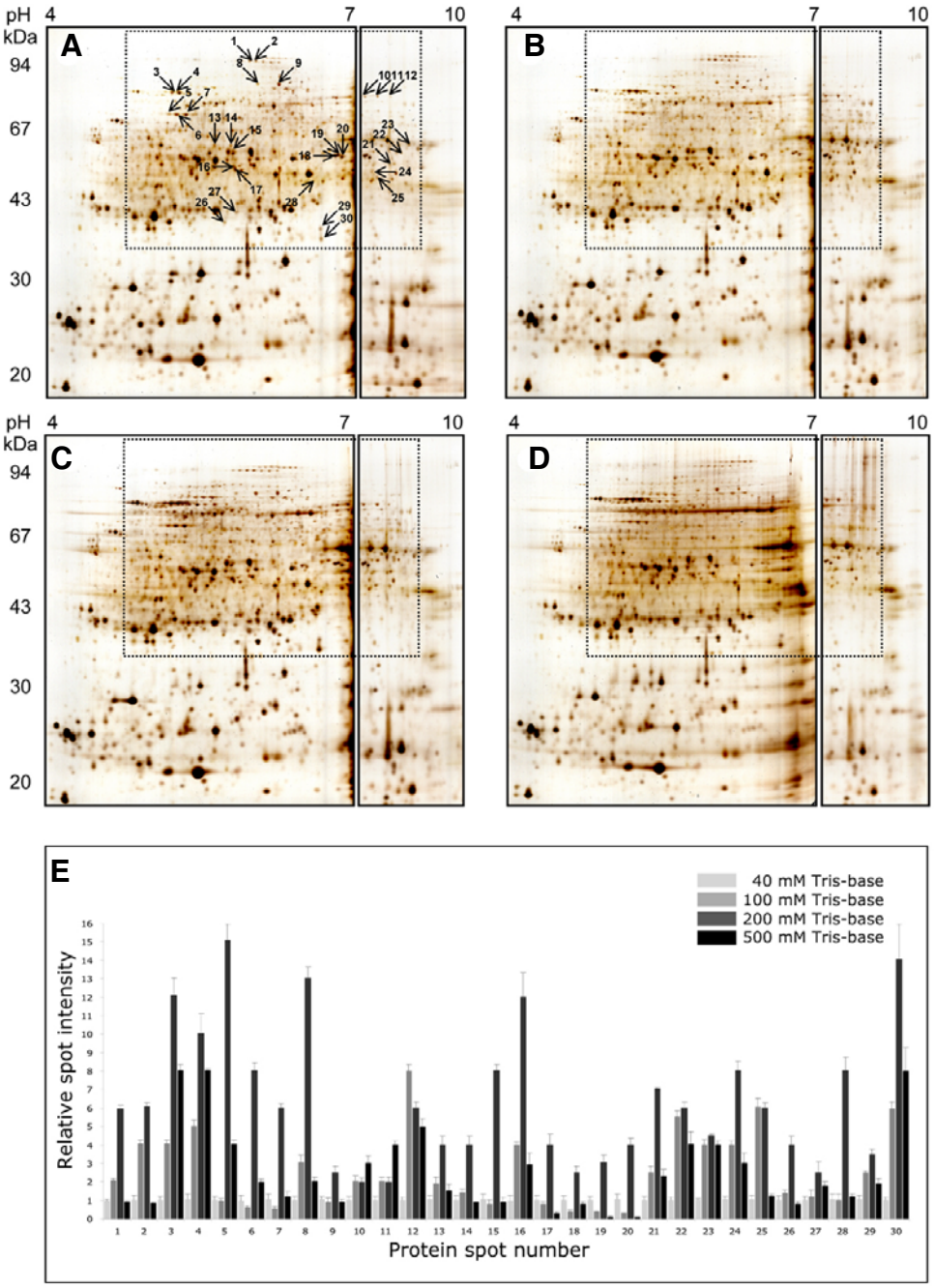

Fig. 1. 2-DE gel patterns of 7-day-old rice seedlings showing the effects of different concentrations of Tris-base on protein solubilization buffer. Acetone-precipitated protein pellets were resolubilized with different solubilization buffers containing 40 mM (A), 100 mM (B), 200 mM (C), or 500 mM (D) Tris-base. Representative regions of the gels that showed resolution changes are indicated with dotted boxes. Thirty representative protein spots with a > 1.5-fold increase in intensity due to the use of 200 mM Tris-base are indicated with arrows (A). The relative intensities of the indicated spots were quantified using the image analysis software, ProteomWeaver (E). Data are represented as means (± SD) from three replicate gels.

resulting in uneven water distribution in the IPG strip gel (Hopkinson et al., 2005; Shaw et al., 2003). Thus, such buffers or salts should be diluted to 10 mM or lower prior for IEF (Berkelman and Stenstedt, 2001; Shaw et al., 2003).

In addition to the inherent difficulties in obtaining good quality 2-DE data, plant materials have several attributes that make attaining high protein resolution in 2-DE gels challenging. In general, both protein content and concentration are lower in plants than in microorganisms or animal cells (Jacobs et al., 2001). Moreover, plant cells are rich in substances that affect the resolving power of 2-DE, such as salts, phenolic compounds, and pigments (Jacobs et al., 2001). These non-protein contaminants are highly soluble and, therefore, co-precipitate with proteins during acetone precipitation, which results in poor solubilization (Jacobs et al., 2001). Plant proteins need to be better solubilized to further proteomics research in plants. In this study, we describe the development of an effective solubilization buffer for plant proteins; this buffer has a Tris-base concentration of 200 mM.

MATERIALS AND METHODS

Plant proteins were extracted from rice seedlings, rice leaves, and *Arabidopsis* seedlings. Using a crude extraction procedure with some minor modifications (Cho et al., 2008). Briefly, all plant samples were snap frozen in liquid nitrogen and ground

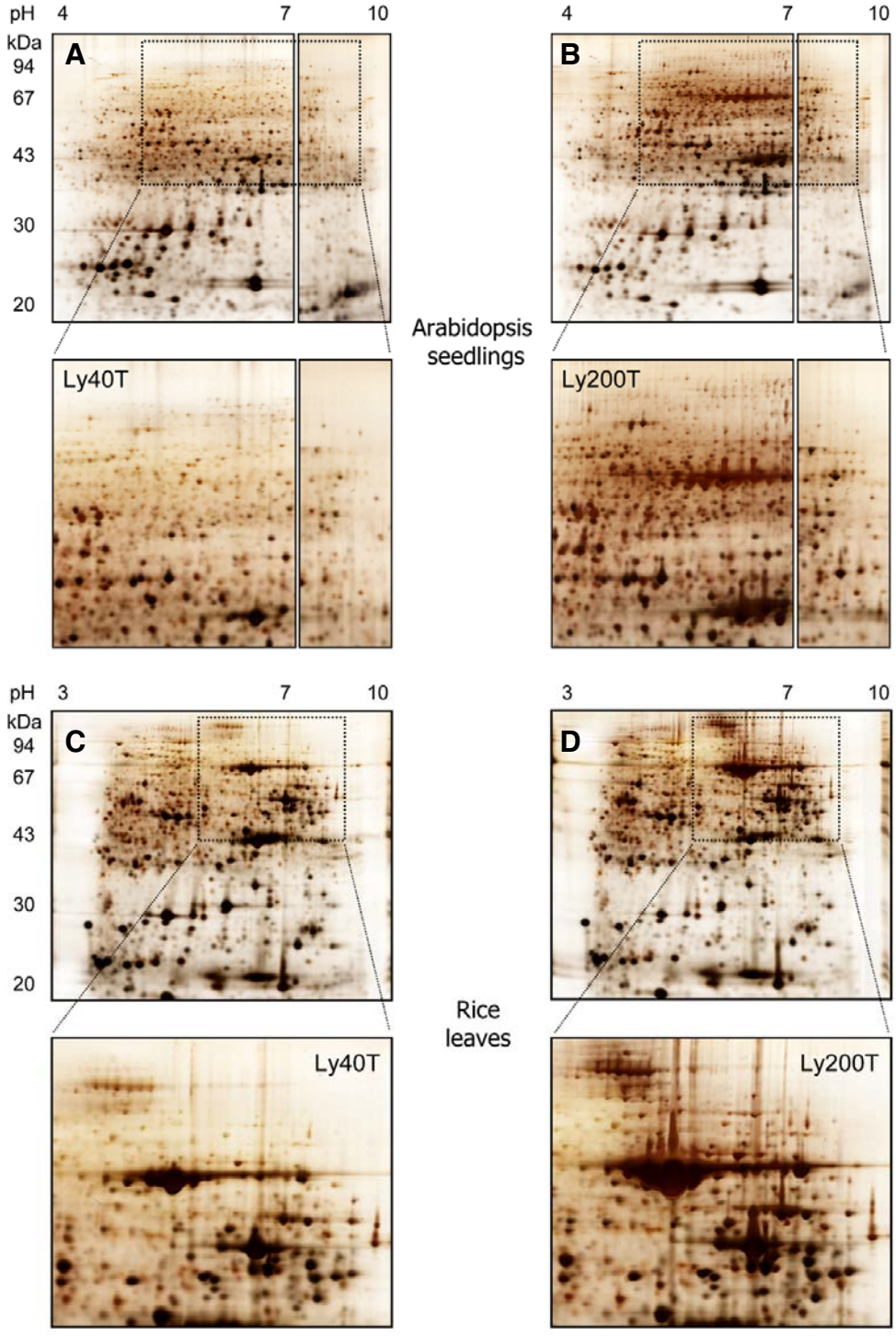

Fig. 2. 2-DE gel patterns of 5 day-old *Arabidopsis* seedlings (A, B) and adult rice leaves (C, D) showing the increased protein solubilization achieved using the Ly200T buffer. Acetone-precipitated protein pellets were resolubilized using the Ly40T (A, C) or Ly200T buffers (B, D). Representative regions of the gels that showed resolution changes are indicated with dotted boxes and are shown at higher magnification in the lower panels. The Ly200T buffer is especially effective in resolving basic proteins extracted from *Arabidopsis* seedlings and rice leaves.

into very fine powder with a mortar and pestle. The ground powder was then homogenized by adding two volumes of crude extraction buffer (20 mM HEPES/KOH, pH 7.5, 40 mM KCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10% v/v glycerol). The homogenate was then centrifuged at 20,000 × g for 30 min at 4°C. The supernatant was centrifuged a further five times at the same speed to completely remove the cell debris. The protein concentrations of the final extracts were determined using a 2-D Quant Kit (GE Healthcare, USA). For 2-DE, the crude extract containing 200 μg soluble proteins was precipitated with four volumes of acetone containing 65 mM DTT at -20°C for 20 min. After centrifugation at 20,000 × g for 15 min at 4°C, the resulting pellets were air-dried for 30 min at room temperature.

In this study, the effect of various concentrations of Tris-base on the solubilization of acetone–precipitated protein pellets was investigated. The precipitated pellets were solubilized in 20 μl solubilization buffer (9.8 M urea, 4% w/v CHAPS, and 65 mM DTT) containing different concentrations of Tris-base or 2% v/v

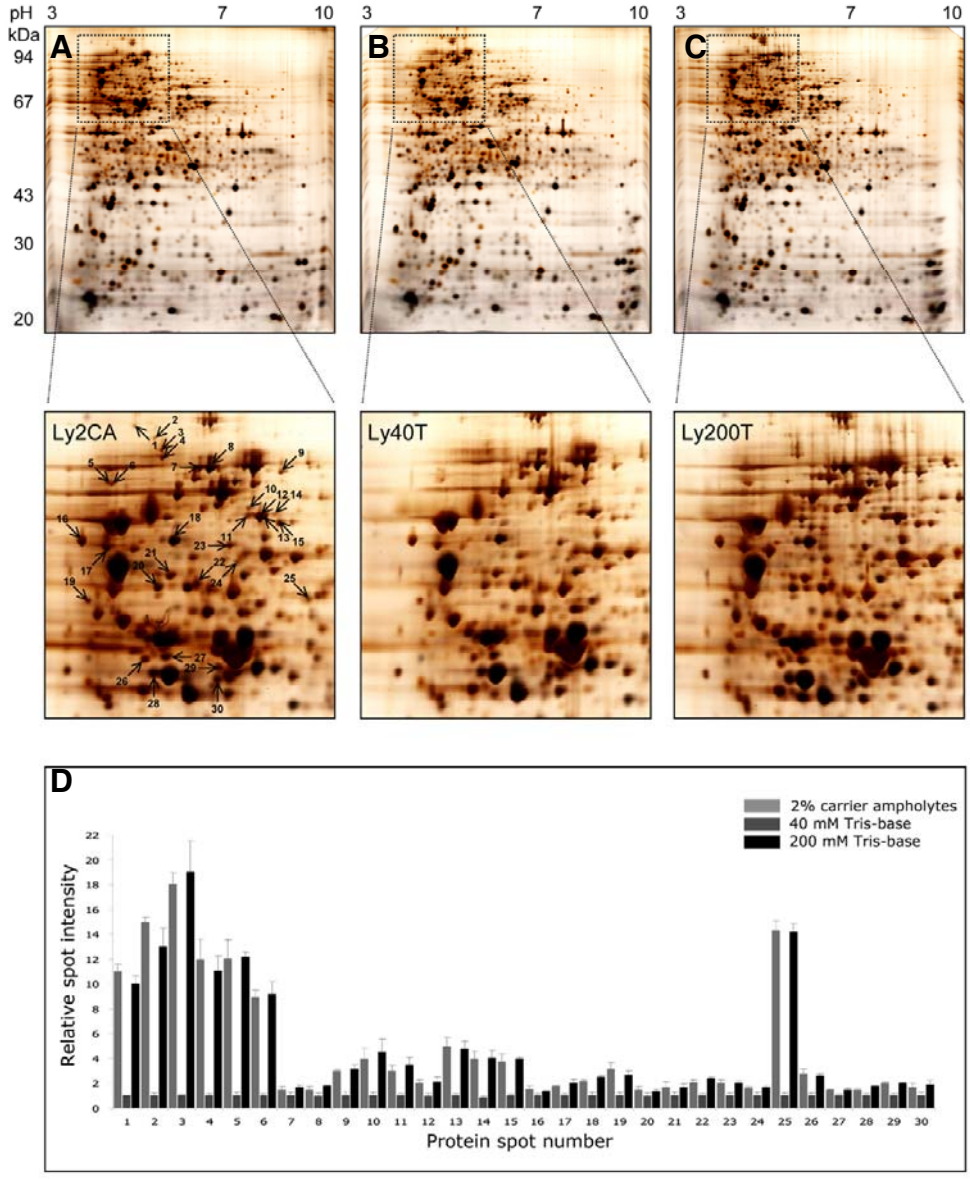

Fig. 3. 2-DE gel patterns of *E. coli* showing the effects of different concentrations of Tris-base upon protein solubilization. *E. coli* soluble proteins, extracted according to Chang et al. (21), were precipitated with acetone. The resulting acetone-precipitated protein pellets were resolubilized in different solubilization buffers, containing 2% carrier ampholytes (Ly2CA, A), 40 mM Tris-base (Ly40T, B), or 200 mM Tris-base (Ly200T, C). Representative regions of the gels that showed resolution changes are indicated with dotted boxes and are shown at higher magnification in the lower panels. Thirty representative protein spots with > 1.5-fold changes in intensity due to the use of different solubilization buffers are indicated with the arrows (A). The relative intensities of the indicated spots were quantified with the image analysis software, ProteomWeaver (E). Data are represented as means (± SD) from three replicate gels.

Biolyte ampholyte, pH 3-10 (Bio-Rad, USA). After incubation at room temperature for 30 min, the protein solutions were mixed with 200 μl rehydration buffer (9.8 M urea, 2% CHAPS, 0.5% v/v IPG buffer at pH 4-7 or pH 3-10 NL (GE Healthcare), 65 mM DTT, and a trace of bromophenol blue) to dilute the Tris-base. IEF was performed by loading the protein samples onto immobilized strip gels (pH 4-7, pH 3-10 NL, 18 cm (Bio-Rad)) at 22°C using the IPGphor system (GE Healthcare). To achieve optimal separation, IEF was performed as following steps; rehydration at 50 V for 10 h; pre-separation at 200 V for 1 h, 500 V for 1 h, and then 1,000 V for 1 h; gradient focusing from 1,000 V to 8,000 V for 30 min; and steady-state focusing at 8,000 V for 8 h. After IEF, the strip gels were equilibrated, as described previously by Phee et al. (2004), and applied to 1.5 mm-thick 12% acrylamide gels. SDS-PAGE was performed at 15 mA overnight using a PROTEAN II xl 2-D Cell apparatus (Bio-Rad).

Analytical gels from three independent experiments were stained with silver nitrate and scanned using a Powerlook III flatbed scanner (Techville, USA). Spot detection, matching, normalization, and quantification were performed automatically using the auto-processing image analysis software, ProteomWeaver V3.1 (Bio-Rad). For high reliability, the same parameters, based on the default settings, were applied to each set of gels stained simultaneously in the same tray. After calculating the coefficient of variation (CV), the statistical significance of the variation in silver-stained spot intensity across three replicates was calculated. Through the processes of filtration by CV, spot frequency estimation, and statistical test, protein spots with at least 1.5-fold-difference (*t*-test, $p < 0.05$) in intensity between gels were selected.

RESULTS AND DISCUSSION

To optimize the solubilization of acetone-precipitated pellets of plant proteins, the concentrations of reagents in the solubilization buffer, such as urea, CHAPS, DTT, carrier ampholytes, and Tris-base, have been varied, and the resulting resolution obtained by 2-DE have been compared. Even though 0.5-2%

Table 1. Number of protein spots obtained in each 2-DE gel region following solubilization with the Ly40T or Ly200T buffers.

		Total number of spots[a]		Number of changed spots[b] (increase / decrease)	
		pH 4-7	pH 7-10	pH 4-7	pH 7-10
Rice seedlings	Ly40T buffer	1786	123	–	–
	Ly200T buffer	2079	185	408 ± 7 / 19 ± 3	118 ± 2 / 11 ± 2
Arabidopsis seedlings	Ly40T buffer	1939	294	–	–
	Ly200T buffer	2287	359	617 ± 9 / 29 ± 2	150 ± 5 / 15 ± 2
Rice leaves	Ly40T buffer	1567	119	–	–
	Ly200T buffer	1747	171	271 ± 5 / 11 ± 2	79 ± 3 / 9 ± 1

The entire area of silver-stained gels was analyzed with ProteomWeaver software.
[a]Number of reproducible protein spots among three replicate gels
[b]Number of protein spots showing a greater than 1.5-fold difference, significant at $p < 0.05$

carrier ampholytes are widely used to solubilize proteins, we found, based on preliminary experiments, that 40 mM Tris-base is more effective at solubilizing plant proteins than carrier ampholytes. To determine the optimal concentration of Tris-base for plant protein solubilization, we varied the concentration of Tris-base from 40 mM to 1000 mM. As shown in Fig. 1, high-quality 2-D gel images of rice seedlings were obtained using a solubilization buffer containing higher concentrations of Tris-base, such as 100 mM Tris-base (Ly100T buffer, Fig. 1B) or 200 mM Tris-base (Ly200T buffer, Fig. 1C), when compared with 40 mM Tris-base (Ly40T buffer, Fig. 1A). The 2-DE gel resolution increased dramatically at these elevated concentrations of Tris-base. We assumed that the enhanced resolution of 2-DE gels was due to improved protein solubilization, given that the protein concentrations in the different solubilization buffers were the same (data not shown).

Tris-base is usually included in solubilization buffers as a salt (de Marqui et al., 2006). Chang and Bae (2003) reported that protein-protein interactions are governed by salt types and their concentrations. These associations can be affected by salt concentrations due to the anisotropy of the protein charge distribution (Chang and Bae, 2003; Curtis et al., 2002; Dumetz et al., 2007). Thus, we suggest that protein-protein associations were reduced when using 200 mM Tris-base in the solubilization buffer, as compared to when using 40 mM Tris-base (Ly40T buffer). In this study, the Tris-base in the solubilization buffer was diluted prior to loading the samples for IEF. However, extensive horizontal streaking and smearing, and, thus, poor resolution, occurred when the concentration of Tris-base exceeded 200 mM (Fig. 1D), possibly due to increased conductivity. Although poor focusing could be avoided by greater dilution of this salt, no improvements were observed at concentrations greater than 200 mM Tris-base (Fig. 1D).

We performed systematic comparisons of our 2-DE data using the auto-processing image analysis software, ProteomWeaver, and found that 293 protein spots were more resolved within the acidic region when using the Ly200T buffer, as compared with the Ly40T buffer (Table 1). Using a 1.5-fold difference as a threshold, solubilization of proteins in Ly200T enhanced the intensity of 408 protein spots in the acidic region, as compared to the Ly40T buffer (Fig. 1E, Table 1). Furthermore, approximately 120 basic proteins were either detected for the first time or showed a far better signal intensity above the 40 kDa region (Fig. 1E, Table 1).

To examine the effects of the Ly200T buffer on the solubilization of other plant proteins, proteins extracted from *Arabidopsis* seedlings and rice leaves were solubilized in different buffers. Remarkable increases in the numbers and intensities of protein spots larger than 40 kDa were evident on the gels (Fig. 2). The intensities of approximately 760 and 350 protein spots from *Arabidopsis* seedlings and rice leaves, respectively, increased when the Ly200T buffer rather than the Ly40T buffer was used (Table 1). In particular, the spot intensities and resolution of proteins in the basic region of the gels were significantly enhanced (Supplementary Figs. 1 and 2). When performing 2-DE, it is difficult to resolve basic proteins because of several technical limitations. In the present study, we demonstrated that the Ly200T buffer is effective at solubilizing basic proteins.

Although the 40 mM Tris-base was effective at solubilizing plant proteins, 2% carrier ampholytes in a solubilization buffer (Ly2CA) solubilized *E. coli* proteins better (Figs. 3A and 3B), in agreement with previous studies (Berkelman and Stenstedt, 2001; Jacobs et al., 2001; Shaw et al., 2003). However, the reduction in the number and intensity of *E. coli* protein spots seen when using 40 mM Tris-base was reversed by the use of 200 mM Tris-base (Figs. 3B and 3C). The resolution of the 2-DE gels containing proteins obtained by solubilization in a buffer containing either 2% carrier ampholytes or 200 mM Tris-base was indistinguishable for *E. coli* proteins (Figs. 3A and 3C), indicating that 200 mM Tris-base can also solubilize *E. coli* proteins effectively. The change in intensity of 30 representative *E. coli* protein spots due to the use of different solubilization buffers was quantified; the results are shown in Fig. 3D.

According to Berkelman and Stenstedt (2001), basic pH conditions may in some cases maximize the solubilization of proteins. However, the increased solubilization of proteins obtained using the Ly200T buffer was not due to the increased pH of the solubilization buffer (data not shown). Furthermore, various combinations of 40-200 mM Tris-base and 0.5-2% carrier ampholytes did not result in improved protein solubilization, as compared with 200 mM Tris-base alone (data not shown). Interestingly, the ability of Ly200T buffer to solubilize *E. coli* proteins was comparable to that of the widely-used solubilization buffer that contains 2% carrier ampholytes to inhibit protein associations (Fig. 3). We hypothesize that Ly200T reduces protein-protein interactions, making it an efficient solubilization buffer for plant proteins. We anticipate that the Ly200T buffer will further the research goals of plant proteome researchers.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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