

Xuchu Wang
Xiaofang Li
Xin Deng
Heping Han
Wuliang Shi
Yinxin Li

Key Laboratory of Photosynthesis
and Environmental Molecular
Physiology,
Institute of Botany,
Chinese Academy of Sciences
Beijing, P.R. China

Received December 9, 2006
Revised April 14, 2007
Accepted April 26, 2007

Research Article

A protein extraction method compatible with proteomic analysis for the euhalophyte *Salicornia europaea*

Protein extraction from plants like the halophyte *Salicornia europaea* has been problematic using standard protocols due to high concentrations of salt ions in their cells. We have developed an improved method for protein extraction from *S. europaea*, which allowed us to remove interfering compounds and salt ions by including the chemicals borax, polyvinylpyrrolidone, and phenol. The comparative study of this method with several other protocols using NaCl-treated *S. europaea* shoots demonstrated that this method gave the best distinction of proteins on 2-DE gels. This protocol had a wide range of applications as high yields and good distinction of 1-DE gels for proteins isolated from twelve other plants were rendered. In addition, we reported results of 2-DE using the recalcitrant tissue of the *S. europaea* roots. We also demonstrated that this protocol is compatible with proteomic analysis as eight specific proteins generated by this method have been identified by MS. In conclusion, our newly developed protein extraction protocol is expected to have excellent applications in proteomic studies of halophytes.

Keywords:

2-DE / Halophyte / Plant proteomics / Protein extraction method / Salt ions

DOI 10.1002/elps.200600805



1 Introduction

2-DE is often challenged by complications of protein sample preparation due to the differences among plant species and tissues [1–3]. Several protein extraction methods have been previously improved to obtain well-resolved 2-DE maps for glycophyte plants [3–5]. Among them, the most commonly used is the TCA/acetone precipitation procedure [3–10], which was developed initially by Damerval and co-workers to extract wheat-seedling proteins [11]. Recently, an improved TCA protocol was used to isolate rice leaf sheath proteins with addition of EGTA *via* homogenization (termed as E-TCA method in this paper) [12]. Hurkman and Tanaka developed the phenol (Phe) method to isolate plant membrane proteins [13]; this method was widely used for total

protein extraction from plant tissues thereafter [3, 5, 10, 14–16]. Using this method, proteins are solubilized in Phe and subsequently precipitated with methanol and ammonium acetate [3, 13, 15–17]. Recently, Phe-based methods were modified specifically for 2-DE when dealing with various recalcitrant plant tissues, such as olive leaf [17], cotton fibers [18], mature grape berry clusters [19], banana meristem cultures, meristems of apple plantlets, and leaves of potato plantlets [15].

Several salt response proteomic analyses for glycophytic plants, in particular thale cress [9, 20], tobacco [21] and rice [22], have been carried out [23]. Using the TCA-based method, Askari and co-workers recently reported a proteome pattern for proteins extracted from halophyte under salt stress [24]. However, to our best knowledge, there was no documented method specifically devised for halophytes. The application of 2-DE for halophytes is still limited largely due to the absence of an efficient protein extraction method. This is because the halophyte cells contain high concentration of salt ions [25], which can interfere with the IEF process of protein samples over IPGs [1, 4, 26, 27].

Salicornia europaea L. is a succulent and leafless euhalophyte that belongs to Chenopodiaceae [25, 28]. It is one of the most salt-tolerant plant species in the world, and requires adequate concentration of NaCl for its optimal growth and

Correspondence: Dr. Yinxin Li, Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, P.R. China
E-mail: yxli@ibcas.ac.cn
Fax: +86-10-825-96139

Abbreviations: BPP, borax/PVPP/Phe method; DW, dry weight; FW, fresh weight; Phe, phenol; PVPP, polyvinylpyrrolidone; RLU, rubisco large subunit

development [25, 28, 29]. It was reported that *S. europaea* could maintain low water potential in its tissues by accumulating high concentrations of inorganic ions, mainly sodium and chloride, and predominantly in the cell vacuoles [25, 28]. In order to investigate the proteins responsible for salt tolerance in this species using proteomic approaches, it is necessary to establish a protocol to isolate high-quality proteins with good yields, which would also allow us to remove ions during protein preparation. The widely used TCA method minimizes the protein degradation by eliminating the activity of enzymes instantly [6, 15, 30], but does not allow salt ions to be removed efficiently [4], and the proteins recovered by this method are difficult to redissolve [3, 5, 6, 15, 18]. Although the E-TCA method eliminates some salt ions during protein precipitation with TCA, it produces distinct horizontal streaks when dealing with the halophyte *S. europaea* [31]. The Phe method can produce better resolved protein spots [3, 16, 31], but the pellet is also hard to resolubilize [3, 5].

In order to overcome aforementioned difficulties, we have established a method combining the usage of ammonium sulfate saturated-methanol and Borax/PVPP/Phe, referred as the BPP method hereafter. Here, we present results of protein analysis in *S. europaea* by both 1-DE and 2-DE using this newly developed protocol.

2 Materials and methods

2.1 Plant materials

Plant species and tissues used in this research are listed in Table 1 and Table 2. The herbaceous plants were grown in a greenhouse maintained at a thermo period of 25/20°C of day/night temperature, photoperiod 16 h, and a relative humidity $50 \pm 10\%$. The woody plants were grown in Beijing Botanical Garden under natural condition. The tissues were collected, and frozen immediately in liquid nitrogen. The frozen tissues were subsequently ground into fine powders in liquid nitrogen using a mortar and pestle, and stored at -80°C .

The halophyte *S. europaea* was treated as follows. Seeds of *S. europaea* were sown on vermiculite damped with tap water. After germination, seedlings were irrigated weekly with half-strength Hoagland nutrient solution. Thirty days after sowing, the plants were divided into three groups, and sequentially watered in the morning with half-strength Hoagland solution containing 0 (control), 200 and 800 mM NaCl with two-day intervals, respectively. Three weeks later, the plant tissues were collected and ground into fine powders in liquid nitrogen plus 1% polyvinylpyrrolidone (PVPP) w/w with a mortar and pestle, then stored at -80°C until use.

2.2 Chemicals

Acrylamide, bisacrylamide, PVPP, standard molecular weight and carrier ampholyte were purchased from Sigma (St. Louis, MO, USA). SDS, TEMED, ammonium sulfate and

β -mercaptoethanol were from Amresco (Solon, OH, USA), and all other chemicals from GE Healthcare (GE Healthcare, Uppsala, Sweden). Double-distilled water was used to prepare all solutions.

2.3 Protein extraction methods

2.3.1 BPP protocol

In this BPP protocol, the Phe extraction procedure was modified from a published protocol [8]. The protein extraction buffer was optimized from the published ones [32, 33]. In short, 1 g of frozen lyophilized tissue powders was re-suspended in 3 mL ice-cold extraction buffer of 100 mM Tris (pH 8.0) containing 100 mM EDTA, 50 mM borax, 50 mM vitamin C, 1% PVPP w/v, 1% Triton X-100 v/v, 2% β -mercaptoethanol v/v and 30% sucrose w/v. After the sample was vortexed for 5 min at room temperature, two volumes of Tris-saturated Phe (pH 8.0) were added and then the mixture was further vortexed for 10 min. After centrifugation (4°C , 15 min, $15\,000 \times g$), the upper phase was transferred to a new centrifuge tube. Equal volume of extraction buffer was added into the new tube, the mixture was then vortexed for 10 min, followed by centrifugation at the same condition. The upper phase was then transferred to a new centrifuge tube. Proteins were precipitated by adding five volumes of ammonium sulfate saturated-methanol, and incubating at -20°C for at least 6 h. After centrifugation as described above, the protein pellet was re-suspended and rinsed with ice-cold methanol followed by ice-cold acetone twice, and spun down at $15\,000 \times g$ for 5 min at 4°C after each washing, and then the mixture was carefully decanted. Finally, the washed pellet was air-dried, then recovered with lysis buffer (9 M urea, 2% CHAPS, 13 mM DTT, 1% IPG buffer) or stored at -80°C .

2.3.2 Other protocols

The BPP protocol was compared with three previously published methods: the TCA method [8], the E-TCA procedure [12], and the Phe extraction method [15].

2.4 Quantification of proteins

Protein concentration was determined using the UV-160 spectrophotometer (Shimadzu, Kyoto, Japan) by the Bradford method [34]. BSA was used as the standard.

2.5 Quantification of Na^+ content

Fresh plant tissues were washed with distilled water immediately after harvest, dried at 60°C for 72 h in an oven. Dried tissues were subsequently ground into fine powders with a mortar and pestle. Three hundred milligram powders were mixed with 10 mL of 500 mM HNO_3 , and incubated at 80°C for 1 h. After filtering the extracts, Na^+ content was assayed by the flame emission method as published [35].

Using different methods described in Section 2.3, the air-dried protein pellet extracted from 0.5 g of fresh tissues was dissolved in 200 μ L lysis buffer. Once protein concentration was determined, 700 μ g total proteins were mixed with lysis buffer in a final volume of 450 μ L (called loading sample in IEF). The Na⁺ content in the loading sample was also determined by the flame emission method.

2.6 2-DE

2.6.1 IEF

The protein pellets were resuspended with the aforementioned lysis buffer (9 M urea, 2% CHAPS, 13 mM DTT, 1% IPG buffer, pH 3–10 or pH 4–7), and incubated for 2 h at room temperature. After centrifugation at 20 000 \times g for 30 min at 21°C, proteins in the supernatants were quantified. The sample containing 700 μ g total proteins was subsequently loaded onto an IPG strip holder with 24 cm, pH 3–10 or pH 4–7 linear gradient IPG strips (GE Healthcare), and rehydrated for 24 h at room temperature. Then the strips were subject to IEF in an Ettan IPGphor system according to the manufacturer's instruction (2-DE Manual, GE Healthcare). After IEF, these strips were transferred to perform the SDS-PAGE or stored at –20°C.

2.6.2 SDS-PAGE

The strips were equilibrated with equilibration solution (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) containing 1% DTT, and subsequently 4% iodoacetamide 15 min for each equilibration solution. The separation of proteins in the second dimension was performed with SDS polyacrylamide gels (12.5%) according to the manufacturer's recommendation (2-DE Manual, GE Healthcare).

2.7 Gel staining, imaging and data analysis

The gels were visualized by CBB staining as published [36]. The analytical gels were scanned at a resolution of 600 dpi, and image analysis was performed with Image Master 2D Platinum Software Version 5.0 (GE Healthcare) following user's manual. The apparent M_r of each protein in gel was determined by referencing to protein markers.

2.8 Protein identification by MALDI-TOF MS

Proteins were identified according to a method described by Shen and colleagues [12]. Briefly, individual protein spot was excised from the gel, and was washed with a solution of 25% v/v methanol and 7% v/v acetic acid for 12 h, followed by destaining with 200 μ L of 50 mM NH₄HCO₃ in 50% v/v methanol for 1 h at room temperature. The proteins in the gel slices were reduced with 10 mM DTT in 100 mM NH₄HCO₃ for 1 h, and incubated in the solution containing

40 mM iodoacetamide and 100 mM NH₄HCO₃ for 30 min at room temperature. Each gel slice was minced, lyophilized, and rehydrated in 100 mM NH₄HCO₃ containing 5 pmol trypsin (trypsin, modified, sequencing grade, Roche Applied Science, Penzberg, Germany) overnight at 37°C. After trypsin digestion, the protein peptides were collected, and the minced gel was extracted with 0.1% TFA in 50% v/v ACN three times. After each extraction, samples were centrifuged at 1000 \times g for 30 s, and all supernatants were combined, vacuum-dried, and stored at –80°C until MS analysis.

Matrix was prepared by dissolving α -cyano-4-hydroxycinnamic acid (Bruker Daltonics, Billerica, MA, USA) in 50% ACN and 0.1% TFA. Ten microliters of matrix solutions were added into the dried digests, and the mixture was vortexed for 30 min. One and a half microliters reconstituted in-gel digested sample was spotted initially on an Anchorchip target plate (600/384F, Bruker Daltonics), followed by 1 μ L of matrix solution. The dried sample on the target plate was washed with 1 μ L of 0.1% TFA twice, left for 30 s before solvent removal, and dried for MALDI-TOF MS analysis.

Mass spectra were obtained on an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics) equipped with a pulsed N₂ laser (337 nm). Operating conditions at the positive reflectron mode were as following: ion source 1 = 19.00 kV, ion source 2 = 16.50 kV, reflector voltage = 20.00 kV, lens voltage = 8.80 kV, pulsed ion extraction time = 80 ns, matrix suppression = 400 Da. The laser strength and pulse ion extraction time were initially optimized. Calibrations were carried out using a standard peptide mixture provided by the manufacturer (Bruker Daltonics). The samples were clustered around calibrating peptide mixtures on a MALDI target plate for optimal calibration. At least three spectra were collected from five random points per sample. The spectra were analyzed with the XTOF/XMASS software. The PMF were matched against the NCBIInr database using MASCOT software (Matrix Science, London, UK). The identification was based on the combination of a high MASCOT score (higher than 67), maximum peptide coverage and additional experimental confirmation of the protein spots on the gels.

3 Results and discussion

3.1 Establishment of the BPP protein extraction method

The extraction buffer used in the BPP protocol was modified based on the recipe for extracting soluble proteins from trees by Tian and co-workers [32, 33], with modifications as described below. Triton X-100, a nonionic detergent that is efficient in breaking lipid-lipid and lipid-protein interactions [1, 19, 30], was included in the BPP buffer to isolate membrane proteins. Compared to the method described by Tian and colleagues [32, 33], the concentrations of Triton X-100

and β -mercaptoethanol were increased from 0.1 and 1% to 1 and 2%, respectively. The optimized concentrations of EDTA, PVPP and sucrose were based on the Phe method described by Saravanan and Rose [8]. PVPP is a strong H-receptor and can adsorb polyphenols efficiently [4, 15, 18], and it has been used in several methods of proteins extraction [8, 15, 18, 33, 37]. In this BPP protocol, interfering compounds such as polysaccharides, polyquinones, and phenolic compounds were removed by borax and PVPP. Ascorbic acid (vitamin C), as well as β -mercaptoethanol [5], are strong reducing agents and can inhibit the phenolic oxidation efficiently (2-DE Manual, GE Healthcare). Ascorbic acid and sodium borate, originally introduced to extract storage proteins from barks of poplar tree [38], were used to isolate proteins from the recalcitrant tissues of olive leaf [39], and vegetative storage proteins from tropical trees [32, 33]. The oxidization of polyphenol to polyquinones and the activity of many enzymes were inhibited under the deoxidized condition generated by β -mercaptoethanol, vitamin C, and PVPP. Thus, in the BPP buffer, 1% PVPP w/v, 50 mM vitamin C and 50 mM borax were also included. Phe serves as a strong protein solvent while having little activity in dissolving nucleic acids and polysaccharides [15]. Thus, in the BPP protocol, the Phe extraction step allows us to efficiently solubilize proteins while removing salt ions to certain degrees [8, 31].

In addition, ammonium sulfate saturated-methanol, rather than ammonium acetate saturated-methanol, was used as a new protein precipitation reagent in the BPP method. Ammonium sulfate precipitation of proteins was widely used in protein purification [40–42], whereas ammonium acetate saturated-methanol was used in Phe-based methods for glycophytes [8, 13, 14, 17–19, 43]. As illustrated in the following Section 3.5, when ammonium sulfate saturated-methanol, compared to ammonium acetate saturated-methanol, was used to precipitate proteins for shoots of *S. europaea*, more protein spots on the 2-DE maps were visualized.

3.2 Comparison of protein quality extracted by BPP and other methods

Using the BPP method, proteins extracted from *S. europaea* tissues treated with 0, 200 and 800 mM NaCl (Fig. 1) were compared to those extracted by TCA, E-TCA and Phe methods in 1-DE (Fig. 2). Proteins bands were distinct at both higher and lower molecular weight (M_r) regions of the gels (Fig. 2, gels A and B, lanes 1–14). Except for few bands indicated by arrows on the gels, proteins extracted by different methods from different NaCl-treated *S. europaea* shoots showed no obvious differences (Fig. 2A, lanes 1–14). However, the root extracts obtained by different methods had small but significant differences in 1-DE patterns (Fig. 2B, lanes 1–14). More protein bands in the high M_r regions on the BPP (Fig. 2B, lanes 1–3) and Phe (Fig. 2B, lanes 4–6) gels were found, whereas more bands were visible around the low-

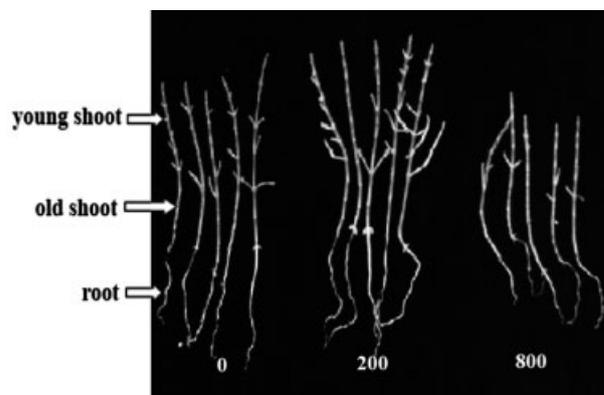


Figure 1. *S. europaea* grown under NaCl treatment conditions. The plants were grown in vermiculite and irrigated with half-strength Hoagland nutrient solution containing 0, 200 and 800 mM NaCl for three weeks.

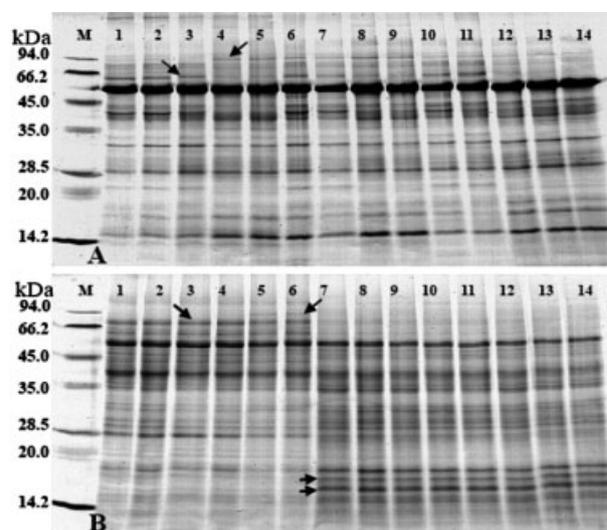


Figure 2. Proteins extracted from tissues of *S. europaea* by different methods. (A) 1-DE result of proteins extracted from shoots of NaCl-treated *S. europaea* using different methods. Lanes 1–5, proteins extracted from young shoots of 0 mM NaCl-treated plants by BPP-A, BPP, Phe, E-TCA and TCA, respectively. Lanes 6–9 and lanes 10–13, proteins from young shoots of 200 and 800 mM NaCl-treated plants extracted by BPP, Phe, E-TCA and TCA, respectively. Lane 14, proteins from old shoots of 800 mM NaCl treated *S. europaea* by TCA method. (B) 1-DE results of proteins extracted from roots of NaCl treated *S. europaea* using different methods. Lanes 1–12, proteins extracted from roots of 0, 200 and 800 mM NaCl treated plants by BPP (lanes 1–3), Phe (lanes 4–6), E-TCA (lanes 7–9) and TCA (lanes 10–12), respectively. Lanes 13 and 14, proteins from main roots of 800 mM NaCl treated plants extracted by E-TCA (lane 13) and TCA (lane 14), respectively. Twenty micrograms proteins were loaded per lane. The arrows indicated the differential bands of the protein extracts on the gel. M, molecular weight markers.

M_r regions of the E-TCA (Fig. 2B, lanes 7–9) and TCA extracts (Fig. 2B, lanes 10–14). These results were consistent with the previous findings indicating that some large proteins were

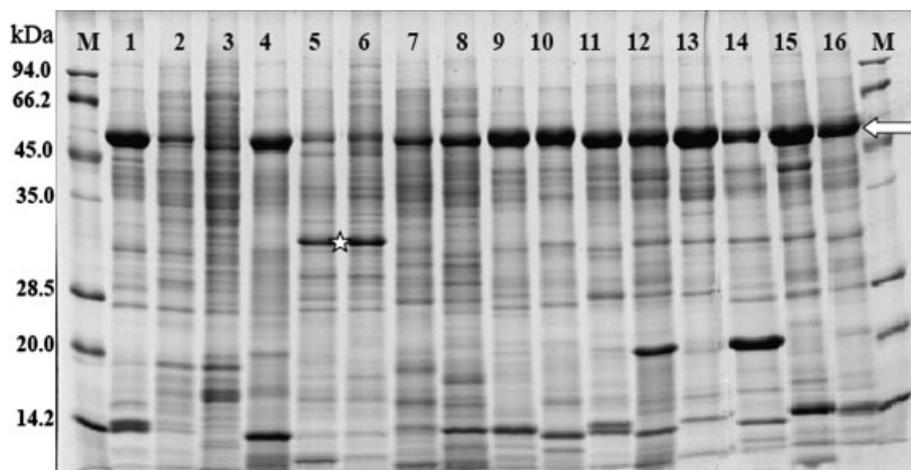


Figure 3. SDS-PAGE gels of proteins extracted from tissues of different plant species by BPP. Lanes 1–3, proteins extracted from the needle (lane 1), phloem (lane 2) and xylem (lane 3) of *Pinus bungeana* Zucc. Lanes 4–6, proteins extracted from the leaf (lane 4), phloem (lane 5) and xylem (lane 6) of *Populus canadensis* Moench. Lanes 7–16, proteins isolated from leaves of *Carya illinoensis* K. (lane 7), *Prunus persica* Rehd (lane 8), *Musa nana* Lour (lane 9), *Gossypium hirsutum* L. (lane 10), *Arabidopsis thaliana* L. (lane 11), *Nicotiana tabacum* L. (lane 12), *Oryza sativa* L. (lane 13), *Lycopersicon esculentum* Mill. (lane 14), *Tetragonia tetragonioides* O. (lane 15) and *Taraxacum officinale* Weber. (lane 16), respectively. Twenty micrograms proteins were loaded per lane. The star represented the position of 32 kDa protein in the phloem and xylem of poplar. The arrows indicate the positions of the main bands in the protein extracts. M, molecular weight markers.

lost while lots of small proteins were enriched in the TCA extracts [15]. In addition, the proteins precipitated with either ammonium sulfate saturated-methanol (Fig. 2A, lane 2) or ammonium acetate saturated-methanol (termed as BPP-A method, Fig. 2A, lane 1) showed no significant difference on 1-DE. Furthermore, the proteins extracted from young shoots (Fig. 2A, lane 13) or old shoots (Fig. 2A, lane 14), as well as total roots (Fig. 2B, lanes 11 and 12) or main roots (Fig. 2B, lanes 13 and 14), presented similar protein profiles on 1-DE. There was also similar 1-DE patterns obtained from the extracts prepared by the same methods from the same tissues of different NaCl-treated *S. europaea* (Fig. 2A, lanes 2–13; B, lanes 1–12).

Proteins extracted by the BPP method from twelve plant species (Fig. 3, lanes 1–16) including five woody plants (Fig. 3, lanes 1–8 and 10) were separated by 1-DE. Protein bands were resolved clearly without smearing for all these species (Fig. 3, lanes 1–16), suggesting this method was applicable to a wide range of plant species. Bands containing the abundant proteins were observed in all the species, indicating that common proteins are shared among these plants. However, sizes of bands with low M_r varied, revealing the difference of protein contents among different species (Fig. 3, lanes 1–16).

3.3 Quantification of protein extracted from plant species by the BPP method

Protein yields of the BPP products from tissues of thirteen species, including five woody plants and three model herbaceous plants, are listed in Table 1. The average protein

yield is approximately 2.01 mg/g fresh weight (FW). The average protein yield of leaves (215 mg/g FW) is higher than that in other tissues (1.82 mg/g FW), which is consistent with the previously report [8]. There are two possible reasons: One is that the abundance of Rubisco (ribulose-1, 5-bisphosphate carboxylase/oxygenase) in leaf tissues makes the proteins facile to be isolated by the BPP method. The other is that many plant tissues, particularly phloems and xylems in woody plants, contain large amounts of polysaccharides, polyquinones, and polyphenoles, which hinders protein isolation and results in reduced yield [18].

The average protein yield of *S. europaea* was about 2.03 mg/g FW, less than that of leaves in other plants (2.13 mg/g FW) (Table 1). We also compared the protein yields for tissues of different NaCl-treated *S. europaea* by the BPP, Phe, E-TCA and TCA methods. The average yield of all the tissues was 2.01, 2.11, 1.58, and 1.74 mg/g FW for each method, respectively (Table 2). The results indicated that the Phe and BPP methods gave significantly greater yields than that obtained using other methods for both shoot and root tissues (Table 2). This might be attributed to the use of Phe as an efficient protein solvent, which can minimize molecular interactions between proteins and other compounds [17]. Recent reports also demonstrated similar observations indicating that Phe-based methods could generate higher protein yield than that produced using TCA methods [8, 19]. However, the protein yield dropped when *S. europaea* was treated with a higher concentration of NaCl (Table 2). This could be attributed to the accumulation of large amounts of salt ions and other secondary metabolites in the NaCl-treated *S. europaea* plants.

Table 1. Evaluation of protein yield from different plant species and tissues extracted by BPP^{a)}

Plant species	Tissue	Protein yield (µg/g FW)	Note
<i>Salicornia europaea</i> L.	Shoots, young	1820 ± 334	Euhalophyte
	Shoots, old	1980 ± 283	Succulent
	Root, total	2289 ± 203	Recalcitrant tissue
<i>Arabidopsis thaliana</i> L.	Leaf, fully expanded	2250 ± 321	Model plant
<i>Nicotiana tabacum</i> L.	Leaf, fully expanded	2480 ± 334	Model plant
<i>Oryza sativa</i> L.	Leaf, young	2260 ± 312	Model plant
<i>Gossypium hirsutum</i> L.	Leaf, young	1880 ± 272	Main crop
<i>Lycopersicon esculentum</i> L.	Leaf, fully expanded	2242 ± 302	
<i>Taraxacum officinale</i> Weber	Leaf, fully expanded	2250 ± 372	
<i>Tetragonia tetragonioides</i> ,	Leaf, mature	1900 ± 275	Halophyte
<i>Carya pecan</i> Graebn	Leaf, young	1969 ± 212	Woody plant
<i>Musa nana</i> Lour.	Leaf, young	2120 ± 301	
<i>Prunus persica</i> Rehd	Leaf, young	2176 ± 288	Woody plant
<i>Pinus bungeana</i> L.	Needle, mature	1960 ± 352	Recalcitrant plant
	Phloem	1550 ± 169	Terminal branch, 2–4 years old
	Xylem	1250 ± 89	Woody plant
<i>Populus canadensis</i> Moench	Leaf, young	2024 ± 232	Terminal branch, 2–4 years old
	Phloem	1850 ± 199	
	Xylem	2012 ± 305	

a) Each sample was extracted at least three times, the value of OD₅₉₅ was detected by UV-160, and the statistic analysis was performed by the software SPSS 10.0. The values were mean ± SD.

Table 2. Comparison of protein yields from *S. europaea* tissues treated with different concentrations of NaCl by BPP, Phe, E-TCA and TCA methods^{a)}

Tissue	NaCl (mM)	Protein yield (µg/g FW)			
		BPP	Phe	E-TCA	TCA
Shoots	0	1918 ± 203	2202 ± 225	1666 ± 213	1698 ± 322
	200	1923 ± 119	1989 ± 312	1591 ± 89	1677 ± 189
	800	1835 ± 319	1911 ± 351	1521 ± 198	1588 ± 229
Roots	0	2299 ± 454	2269 ± 308	1817 ± 219	1952 ± 412
	200	2108 ± 254	2236 ± 358	1423 ± 109	1859 ± 333
	800	1983 ± 316	1998 ± 189	1455 ± 258	1677 ± 123

a) *S. europaea* plants for this test were treated with different concentrations of NaCl for one month. Each sample was extracted at least three times. The values were mean ± SD.

3.4 The BPP method efficiently removes salt ions from *S. europaea* tissues

Salt ions can cause high conductivity due to the electroendosmosis in the IPG strips, which interferes with the IEF process by limiting voltage climb. In the presence of high salt ions, focusing of the proteins would not occur until the ions moved to the end of the strip, consequently the time required for IEF would be prolonged [1, 4]. In some extreme cases, the IEF could virtually stop for salt fronts [4]. High salt concentrations could also lead to uneven water distribution in the gel, which could cause the formation of zones of dehy-

dratation and over hydration [1]. Therefore, to obtain the satisfactory results in 2-DE, salt concentration in protein samples should be reduced to less than 10 mM [1]. When the samples were applied *via* rehydration loading, salt ions may interfere with the IEF more severely [4]. The salt concentration should be kept less than 50 mM [26, 27]. Although a salt concentration of 50 mM can be tolerated if samples are loaded using the cup, proteins may precipitate at the sample-loading site, which makes the subsequent gel running in the second dimension problematic [1, 4]. Under any circumstance, the salt ions must be removed when their concentration exceeds 100 mM [4].

The euhalophyte *S. europaea* accumulates large amounts of salt ions in its succulent shoots [25, 29]. Using the flame emission method, we detected Na⁺ contents at 75.73, 199.59 and 295.19 mg/g dry weight (DW) in its shoots treated with 0, 200 and 800 mM NaCl, respectively (Table 3). These concentrations were much higher than those of NaCl-treated halophyte *S. aegyptiaca*, which contains less than 70 mg/g DW salt ions in its leaves [24]. The *S. europaea* roots contained less Na⁺ than that in the shoots; the Na⁺ concentrations were 7.98, 17.62 and 53.61 mg/g DW in the roots of plants treated with 0, 200 and 800 mM NaCl, respectively (Table 3). In contrast to halophytes, the glycophytes accumulated far less salt ions under salinity [29]. For example, in shoots of barley, the Na⁺ content was about 10 mg/g DW [44]. When a vacuolar Na⁺/H⁺ antiporter was ectopically over-expressed, leaves of transgenic *Brassica juncea* [45] and *Oryza sativa* [46] exhibited Na⁺ content at about 6 and 10 mg/g DW, respectively.

In order to achieve optimal IEF focusing prior to 2-DE, salts in the protein extracts are often removed using desalting techniques of gel filtration, precipitation and resuspension [1, 4]. Such a practice often has the disadvantage of protein loss (2-DE Manual, GE Healthcare). An alternative desalting method has been performed by using columns [1], which was more practical for the analysis of halophilic proteins [26]. But in addition to being costly, such a desalting process also led to the loss of proteins. Recently, it has been reported that Trizol reagent was effective in desalting proteins isolated from the haloarchaeon *Haloferax volcanii* [27]. The TCA method has also been used to extract proteins from halophyte *S. aegyptiaca* [24]. However, both methods have resulted in horizontal streaking on both dimensions in 2-DE gels [24, 27].

Compared to aforementioned methods, the BPP method could generate protein extracts with low Na⁺ content (Table 3). Overall the Phe-based methods (BPP and Phe) were superior to the TCA-based methods (E-TCA and TCA) for

achieving low salt concentration in proteins extracts from normal or salt-treated shoot or root tissues (Table 3). For example, the Na⁺ content in the shoots of 200 mM NaCl-treated *S. europaea* obtained by the BPP methods was 3.24 mM, compared to 16.79, 68.15 and 134.89 mM in the protein extracts obtained by Phe, E-TCA and TCA methods, respectively (Table 3). The 800 mM NaCl-treated *S. europaea* contained very high concentrations of Na⁺ (295.19 mg/g DW in shoots and 53.61 mg/g DW in roots), which hampered 2-DE analyses by traditional protein isolation methods. Using the BPP method, proteins can be isolated from these salt-rich tissues with a permissible low Na⁺ content (Table 3). In contrast, the TCA method resulted in high Na⁺ content in the protein extracts (Table 3). Although the Phe method could produce better result than the TCA-based methods, the Na⁺ content is still much higher than that obtained from the BPP method (Table 3).

3.5 The BPP protocol produces more protein spots

Rubisco, similar to salt ions, causes problems in the protein sample preparation from halophytic plants like *S. europaea* for 2-DE. Conventionally, the Rubisco large subunit (RLU) is considered as one of the criteria to evaluate the efficiency of protein extraction from green tissues [7, 17]. As the most abundant protein in plants, Rubisco accounts for up to 50% of the soluble protein in green tissues [5, 8, 20, 22, 47], which would severely limit the amount of target proteins loaded on the IPG strips for IEF [1, 3–5, 8, 22, 23]. In addition, the Rubisco-binding proteins were difficult to recover [47]. The enlarged spot of RLU on the 2-DE gels often smears to some extent on both horizontal and vertical dimensions, which may cloud spots of other proteins, leading to the inaccurate spot positions or failure to detect other proteins at similar positions [1]. This problem can be exacerbated when a large amount of proteins are loaded onto the IPG strips.

Table 3. Comparison of Na⁺ content in the protein extracts from *S. europaea* tissues treated with different concentrations of NaCl by BPP, Phe, E-TCA and TCA methods^{a)}

Tissue	NaCl (mM)	Na ⁺ content (mg/g DW)	Na ⁺ in protein extracts (mM)			
			BPP	Phe	E-TCA	TCA
Shoots	0	75.73 ± 2.51	0.99 ± 0.31	3.04 ± 0.88	8.35 ± 1.68	26.74 ± 6.89
	200	199.59 ± 5.69	3.24 ± 1.13	16.79 ± 2.12	68.15 ± 3.15	134.89 ± 19.67
	800	295.19 ± 14.75	10.01 ± 1.21	35.18 ± 9.37	129.13 ± 5.35	169.85 ± 17.58
Roots	0	7.98 ± 1.19	0.25 ± 0.12	0.29 ± 0.11	1.55 ± 0.28	3.21 ± 1.33
	200	17.62 ± 1.48	1.37 ± 0.43	3.89 ± 2.35	5.69 ± 2.15	31.61 ± 8.88
	800	53.61 ± 5.65	5.87 ± 1.88	6.12 ± 2.21	18.83 ± 5.69	44.44 ± 19.69

a) Each sample was measured for at least three times. The mean values ± SD were presented. The proteins extracted with different methods were adjusted to the same concentration (1.556 μg/μL) by adding lysis buffer to render loading sample. The Na⁺ content in the loading samples was determined by the flame emission method.

As seen on the 1-DE gels, the extracts of all the tissues have prominent bands near the approximate 50 kDa position (Fig. 2A; Fig. 3). We identified the proteins in the thickest band on 1-DE gels (Fig. 2, gel A, lane 2; Fig. 3, lane 1) as RLU by MALDI-TOF MS (Supporting Information, Fig. 1). The most abundant protein spot with a horizontal streak was always visible on each 2-DE gel (Fig. 4). This abundant spot (Fig. 5B) was also excised individually and further identified as RLU (Accession number: gi34576735) by MALDI-TOF MS (Supporting Information, Fig. 1). On the BPP 2-DE gel, the RLU appeared as a well-focused spot even when a great protein amount (0.7 mg) was loaded (Fig. 5B; Supporting Information, Fig. 3). In contrast to other methods, the BPP method generated better resolved RLU on the 2-DE gel (Fig. 4), probably because BPP extracts contained less interfering compounds.

In Fig. 4, the 2-DE gels of the four protocols generated hundreds of protein spots with a broad distribution on both dimensions. The Phe and BPP (including BPP-A) methods generated more spots and less streaking on both horizontal and vertical dimensions compared to the TCA and E-TCA methods. In addition, the BPP method resulted in 2-DE separation of the shoots proteins with superior resolution over other protocols (Fig. 4).

On the TCA gel, there are approximately 420 detectable protein spots with dark background and strong vertical streaking (Fig. 4A). The E-TCA gel resolved about 630 detectable protein spots with obvious horizontal streaking, especially around the RLU region (Figs. 4A and B). The Phe method yielded 1150 detectable spots on the gel, and the horizontal streaking was similar to that generated from the E-TCA method (Fig. 4A). These streaks were caused by the interference of RLU during the electrophoresis process (Fig. 4A). In contrast, when the protein sample was prepared by the BPP method, more than 1500 visible protein spots were clearly resolved with little background and minimal streaking on the 2-DE gel (Fig. 4A). Furthermore, the shapes of spots appeared round or elliptical (Fig. 4B), even at both cathode and anode points, and around the RLU regions, suggesting that the focusing was complete (Figs. 4A and B).

The BPP-A procedure is the BPP method with minor modification. In BPP-A method, proteins were extracted by BPP procedure, but precipitated with ammonium acetate saturated-methanol. As shown in Fig. 4, the BPP-A method rendered similar focusing patterns as the Phe method prepared gel, which was better than the TCA-based methods (Fig. 4A), but not as good as the BPP method (Figs. 4A and B). This is because some proteins were lost during the precipitation process with ammonium acetate, especially those with low molecular weights (Fig. 4, region d, e and f), and those found in the region of the cathode points (Fig. 4, region c and f) of the gel. We also determined the protein yield and Na⁺ content in the BPP-A method generated extracts from the shoots of 200 mM NaCl-treated *S. europaea*, and found that lower protein yield and higher Na⁺ content were produced using the BPP-A method than the BPP method (data not

shown). Thus, our results indicated that ammonium sulfate saturated-methanol was more effective for protein precipitation than ammonium acetate saturated-methanol when applied to halophytic plant *S. europaea*. However, it is more time-consuming (6 h) than the ammonium acetate precipitation (2 h).

We have also observed substantial number of proteins that were different in the magnified regions of gels, both qualitatively and quantitatively (Fig. 4B). In the selected areas, the TCA and E-TCA methods generated gels showing fewer spots than those obtained by the Phe, BPP-A and BPP methods. And the spots on the Phe and BPP-A gels were fewer than that on the BPP gel (Fig. 4B), especially in the acidic and low-molecular-weight regions (Fig. 4B, regions c, e and f). Thus, the BPP method minimizes protein loss while efficiently removing the salt ions.

The root of halophyte *S. europaea* can tolerate exceptionally low water potentials, and exhibits anomalous secondary thickening with concentric series of collateral vascular bundles embedded in a lignified ground tissue [25]. After being treated with 800 mM NaCl for three weeks, its roots were significantly lignified, which made them more recalcitrant for protein isolation, although less salt ions were accumulated in roots than in shoots for salt treated halophyte *S. europaea* (Table 3). In Fig. 5, there were about 1200 and 1850 detectable protein spots on the gels of roots (Fig. 5A) and shoots (Fig. 5B), respectively. These results allowed us to conclude that the BPP method was suitable for salt-rich tissues (the shoots) as well as recalcitrant tissues (the roots).

3.6 The BPP protocol produces specific protein spots

Proteins extracted from the shoots of 200 mM NaCl-treated *S. europaea* using the BPP protocol revealed over one hundred additional protein spots were visible on the 2-D gel compared to those prepared by other methods including the BPP-A method (Fig. 4A). Such a result could be attributed to the different precipitation and extraction buffers used in the BPP method. Eight BPP-specific spots, indicated by the arrows and marked with numbers in the magnified regions, were excised and identified *via* MALDI-TOF MS (Fig. 4B; Supporting Information, Fig. 2). Their identities are listed in Table 4.

It was reported that salt stress could generate ROS in the chloroplast in plant cells [48]. The balance between removal and formation of ROS is a crucial factor in the severity of oxidative stress and cell damage in plants [24, 48]. Among the BPP-specific proteins, two oxidative stress response proteins (oxidoreductase, spot 5; NBS-LRR type resistance protein, spot 7) were identified. A salt-inducible protein kinase (spot 2) was also identified, which indicated that salt stress related proteins exists in *S. europaea*. In previous studies, a significant fraction of the proteins identified by proteomics analysis is involved in energy production, either in ATP production or in photosynthetic electron transport [22]. Among the identified proteins, there were three energy production-

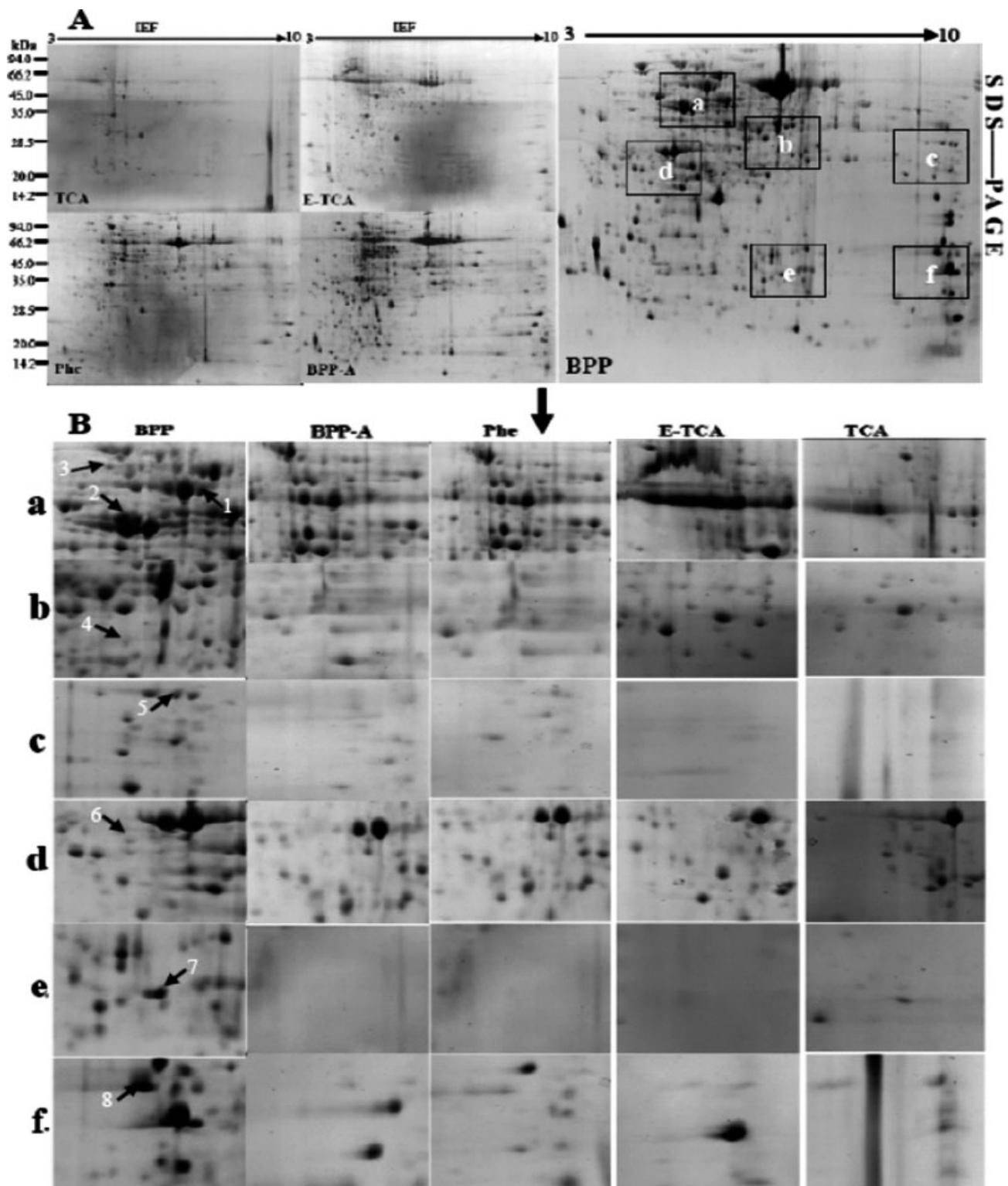


Figure 4. Comparison of 2-DE results of proteins extracted from shoots of 200 mM NaCl-treated *S. europaea* using different methods. (A) 2-DE gels of proteins extracted from the same tissues by BPP, BPP-A, Phe, E-TCA and TCA method. About 700 μ g proteins were separated on a 24 cm, pH 3–10 linear gradient IPG strip in the first dimension and stained with CBB. Regions of the gels indicating particularly different distributions of protein spots were highlighted by rectangular boxes. (B) Magnified representative regions in 2-DE maps. Regions a to f represented similar represent regions of different gels. The protein spots unique to the BPP method, which were indicated by the arrows and marked with numbers, were positively identified *via* MALDI-TOF MS and their identities were listed in Table 4.

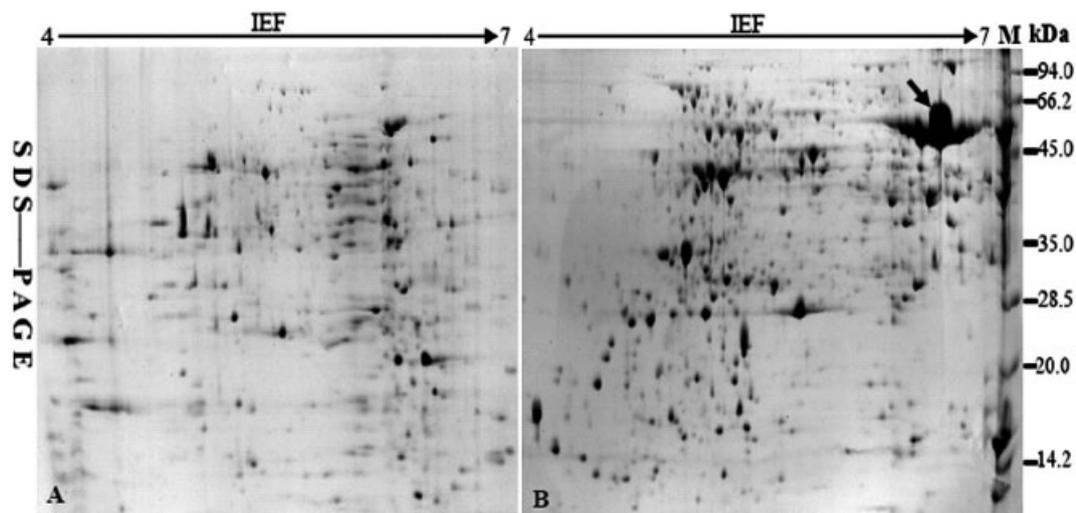


Figure 5. Representative 2-DE gel patterns of proteins extracted by BPP from tissues of 800 mM NaCl-treated *S. europaea*. Seven hundred micrograms proteins extracted from roots (A) and shoots (B) were loaded on linear gradient 24 cm IPG strips with pH 4–7. M, molecular weight marker. The arrow pointed at the most abundant protein spot identified by MALDI-TOF MS. Numbers on the right represented M_r in kDa.

Table 4. Identification of BPP-specific protein spots from shoots of *S. europaea* by MALDI-TOF MS^{a)}

Spot No.	Theor pI/ M_r	M score	M No.	SC %	Accession number	Plant species	Protein identification
1	5.03/52	154	11	43	17224743	<i>Tamus communis</i>	ATP synthase beta subunit
2	8.70/48	75	7	26	52352563	<i>Zea mays</i>	Salt-inducible protein kinase
3	5.03/74	84	9	18	3913786	<i>Spinacia oleracea</i>	Luminal -Bip precursor
4	7.58/69	93	12	25	30679610	<i>A. thaliana</i>	ATP binding/microtubule motor
5	5.58/32	84	7	23	15224100	<i>A. thaliana</i>	Oxidoreductase
6	5.84/44	91	10	25	11245655	<i>Hordeum vulgare</i>	G protein alpha subunit
7	6.12/19	85	6	62	2792250	<i>O. sativa</i>	NBS-LRR type resistance protein
8	9.58/23	98	7	32	92893306	<i>M. truncatula</i>	Photosystem I protein PsaD

a) The spot numbers were accordant with the BPP-specific protein spots shown in Fig. 4. The pI and M_r (kDa) are theoretical. Abbreviations: SC for sequence coverage, M score for MASCOT search score, and M No. for matched peptide numbers. The NCBI database accession number for the homolog was listed in the column 6. Protein identification was based on the MS spectrum data and the species from which this sequence homolog have been identified.

associated proteins: ATP synthase beta subunit (spot 1), ATP binding/microtubule motor (spot 4), and Photosystem I protein PsaD (spot 8). In addition, a HSP 70 family chaperone (spot 3), homologous to Luminal-Bip precursor, and a G protein alpha subunit (spot 6) were also recovered on the BPP gel and identified by MS (Table 4).

Proteins were also extracted from *S. europaea* shoots treated with different concentrations of NaCl using the BPP method. The comparison of different gels generated from the different BPP extracts indicated that the proteins were well resolved and patterns of protein spots on 2-DE were highly reproducible (Supporting Information, Fig. 3). More than one hundred differential protein spots had been excised from 2-DE gels and analyzed by MS to reveal protein identities (data not shown). Therefore, the BPP protocol

was proven on our hands to be one of the most suitable methods for extracting proteins from *S. europaea* under salinity stress.

4 Concluding remarks

In this study, we established a Phe-based protein extraction method, termed as BPP method, to analyze proteins extracted from halophytes. We have shown that the BPP method allowed optimized 1-D and 2-D PAGE separation, which in turn improved protein identification by subsequent MS analysis. In this optimized method, the extraction buffer contained agents of EDTA, vitamin C, borax, β -mercaptoethanol, and PVPP to inhibit activities of pro-

teolytic enzymes and to successfully remove interfering compounds. Furthermore, the Phe-buffer extraction process allowed us to remove the terpenoids, pigments, lipids, and wax-like polymers. It is worth pointing out that the BPP method allows simultaneous separation of salt and proteins in the samples by partitioning them into different phases. The removal of salt ions was further improved by protein precipitation using ammonium sulfate saturated-methanol. Compared to other published methods, the BPP protocol allowed the most protein spots to be revealed with excellent focusing patterns. This method can be applied to protein extraction from tissues rich in salt ions and recalcitrant ones. The method is suitable for plants other than halophytes. In conclusion, the method documented here allows us to efficiently extract proteins from recalcitrant tissues of plants, which is compatible with MS for proteomic research of halophytes.

This research was supported by the National High Technology and Research Development Program of China ("863" project) (Grant No. 2007AA091705) and the Key Directional Research Project of CAS (Grant No. KSCX2-YW-N-003 and 013). The authors thank Dr. Jindong Feng for technical supports of MALDI-TOF MS and Dr. Liqin Li for determining the Na⁺ content. We thank in particular Dr. Bo Liu at University of California-Davis and Dr. Qiong Liu for revisions and language polishing of this article. We also thank the two reviewers of this article for their helpful suggestions and the very detailed comments.

5 References

- [1] Shaw, M. M., Riederer, B. M., *Proteomics* 2003, 3, 1408–1417.
- [2] Cánovas, F. M., Eliane, D. G., Recorbet, G., Jorrián, J. *et al.*, *Proteomics* 2004, 4, 285–298.
- [3] Chen, S. X., Harmon, A. C., *Proteomics* 2006, 6, 5504–5516.
- [4] Görg, A., Weiss, W., Dunn, M. J., *Proteomics* 2004, 4, 3665–3685.
- [5] Kim, S. T., Cho, K. S., Jang, Y. S., Kang, K. Y., *Electrophoresis* 2001, 22, 2103–2109.
- [6] Gialvalisco, P., Nordhoff, E., Lehrach, H., Gobom, J., Klose, J., *Electrophoresis* 2003, 24, 207–216.
- [7] Islam, N., Lonsdale, M., Upadhyaya, N. M., Higgins, T. J. *et al.*, *Proteomics* 2004, 4, 1903–1908.
- [8] Saravanan, R. S., Rose, J. C., *Proteomics* 2004, 4, 2522–2532.
- [9] Ndimba, B. K., Chivasa, S., Simon, W. J., Slabas, A. R., *Proteomics* 2005, 5, 4185–4196.
- [10] Song, J., Braun, G., Bevis, E., Doncaster, K., *Electrophoresis* 2006, 27, 3144–3151.
- [11] Damerval, C., Vienne, D. D., Zivy, M., Thiellement, H., *Electrophoresis* 1986, 7, 52–54.
- [12] Shen, S. H., Jing, Y. X., Kuang, T. Y., *Proteomics* 2003, 3, 527–535.
- [13] Hurkman, W. J., Tanaka, C. K., *Plant Physiol.* 1986, 81, 802–806.
- [14] Meyer, Y., Grosset, J., Chartier, Y., Cleyet, M. J., *Electrophoresis* 1988, 9, 704–712.
- [15] Carpentier, S. C., Witters, E., Laukens, K., Deckers, P. *et al.*, *Proteomics* 2005, 5, 2497–2507.
- [16] Faurobert, M., Pelpoir, E., Chaib, J., *Methods Mol. Biol.* 2007, 355, 9–14.
- [17] Wang, W., Scali, M., Vignani, R., Spadafora, A. *et al.*, *Electrophoresis* 2003, 24, 2369–2375.
- [18] Yao, Y., Yang, Y. W., Liu, J. Y., *Electrophoresis* 2006, 27, 4559–4569.
- [19] Vincent, D., Wheatley, M. D., Cramer, G. R., *Electrophoresis* 2006, 27, 1853–1865.
- [20] Rossignol, M., Peltier, J. B., Mock, H. P., Matrons, A. *et al.*, *Proteomics* 2006, 6, 5529–5548.
- [21] Dani, V., Simon, W. J., Duranti, M., Croy, R. D., *Proteomics* 2005, 5, 737–745.
- [22] Agrawal, G. K., Rakwal, R., *Mass Spectrom. Rev.* 2006, 25, 1–53.
- [23] Lee, S., Lee, E. J., Yang, E. J., Lee, J. E. *et al.*, *Plant Cell* 2004, 16, 1378–1391.
- [24] Askari, H., Edqvist, J., Hajheidari, M., Kafi, M., Salekdeh, G. H., *Proteomics* 2006, 6, 2542–2554.
- [25] Davy, A. J., Bishop, G. F., Costa, C. B., *J. Ecol.* 2001, 89, 681–707.
- [26] Cho, C. W., Lee, S. H., Choi, J., Park, S. J. *et al.*, *Proteomics* 2003, 3, 2325–2329.
- [27] Kirkland, P. A., Busby, J., Stevens, J. S., Julie, A. M., *Anal. Biochem.* 2006, 351, 254–259.
- [28] Riehl, T. E., Ungar, I. A., *Oecologia* 1982, 54, 193–199.
- [29] Flowers, T. J., Troke, P. F., Yeo, A. R., *Ann. Rev. Plant Physiol.* 1977, 28, 89–121.
- [30] Valcu, C. M., Schlink, K., *Proteomics* 2006, 6, 4166–4175.
- [31] Li, X. F., Han, H. P., Wang, X. C., Fan, P. X., Li, Y. X., *Acta Ecologica Sinica* 2006, 26, 1848–1853.
- [32] Tian, W. M., Han, Y. Q., Wu, J. L., Hao, B. Z., *Trees* 1998, 12, 153–159.
- [33] Tian, W. M., Wu, J. L., Hao, B. Z., Hu, Z. H., *Can. J. Bot.* 2003, 81, 492–500.
- [34] Bradford, M. M., *Anal. Biochem.* 1976, 72, 248–254.
- [35] Chuang, F. S., Sarbeck, J. R., John, P. S., Winefordner, J. D., *Mikrochim. Acta* 1973, 61, 523–531.
- [36] Canadano, G., Bruschi, M., Musante, L., Santucci, L. *et al.*, *Electrophoresis* 2004, 25, 1327–1333.
- [37] Usuda, H., Shimogawara, K., *Plant Cell Physiol.* 1995, 36, 1149–1155.
- [38] Coleman, G. D., Chen, T. H., Ernst, S. G., Fuchigami, L., *Plant Physiol.* 1991, 96, 686–692.
- [39] Garcia, J. L., Avidan, N., Troncoso, A., Sarmiento, R., Lavee, S., *Sci. Hortic.* 2000, 85, 271–284.
- [40] Anna, Z. P., Agnieszka, K., Jolanta, Z. C., *Protein Expr. Purif.* 2006, 48, 126–133.
- [41] Sharma, A. D., Gill, P. K., *J. Food Eng.* 2007, 79, 1172–1178.
- [42] Zhang, H., Yao, H. Y., Chen, F., Wang, X., *Food Chem.* 2007, 101, 1670–1676.

- [43] Mijnsbrugge, K. V., Meyermans, H., Montagu, M. V., Bauw, G., Boerjan, W., *Planta* 2000, 210, 589–598.
- [44] Pakniyat, H., Handley, H. H., Thomas, W. B., Connolly, T. *et al.*, *Euphytica* 1997, 94, 7–14.
- [45] Rajagopal, D., Agarwal, P., Tyagi, W., Pareek, S. S. *et al.*, *Mol. Breeding* 2007, 19, 137–151.
- [46] Chen, H., An, R., Tang, J. H., Cui, X. H. *et al.*, *Mol. Breeding* 2007, 19, 215–225.
- [47] Schiltz, S., Gallardo, K., Huart, M., Negroni, L. *et al.*, *Plant Physiol.* 2004, 135, 2241–2260.
- [48] Borsani, O., Valpuesta, V., Botella, M. A., *Plant Physiol.* 2001, 126, 1024–1030.