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Comparative studies of four protein preparation methods for proteomic study of the dinoflagellate *Alexandrium* sp. using two-dimensional electrophoresis

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ABSTRACT

Alexandrium is a wide-spread genus of dinoflagellate causing harmful algal blooms and paralytic shellfish poisoning around the world. Proteomics has been introduced to the study of *Alexandrium*, but the protein preparation method is still unsatisfactory with respect to protein spot number, separation and resolution, and this has limited the application of a proteomic approach to the study of dinoflagellates. In this study we compared four protein preparation methods for the two-dimensional electrophoresis (2DE) analysis of *A. tamarense*: (1) urea/Triton X-100 buffer extraction with trichloroacetic acid (TCA)/acetone precipitation; (2) direct precipitation with TCA/acetone; (3) 40 mM Tris (hydroxymethyl) aminomethane (Tris) buffer extraction; and (4) 50 mM Tris/5% glycerol buffer extraction. The results showed that, among the four protein preparation methods, the method combining the urea/Triton X-100 buffer extraction and TCA/acetone precipitation allowed detection of the highest number and quality of protein spots with a clear background. Although the direct TCA/acetone precipitation method also detected a high number of protein spots with a clear background, the spot number, separation and intensity were not as good as those obtained from the urea/Triton X-100 buffer extraction with TCA/acetone precipitation method. The 40 mM Tris buffer and 50 mM Tris/5% glycerol buffer methods allowed the detection of fewer protein spots and a pH range only from 4 to 7. Subsequently, the urea/Triton X-100 buffer extraction with TCA/acetone precipitation method was successfully applied to profiling protein expression in *A. catenella* under light stress conditions and the differential expression proteins were identified using MALDI TOF–TOF mass spectrometry. The method developed here appears to be promising for further proteomic studies of this organism and related species.

1. Introduction

Alexandrium is a widely distributed dinoflagellate genus in many coastal regions around the world. It is well known that many species from this genus can produce potent neurotoxins which cause paralytic shellfish poisonings (PSPs) through the consumption of shellfish contaminated by toxins (Anderson and Garrison, 1997; Cembella, 1998). Within this genus, *A. tamarense* is among the most toxic species, and also is one of the major causative agents of harmful algal blooms (HABs) in many coastal waters (Anderson et al., 1994; Kodama, 2000). In the past few years, HABs caused by *A. tamarense* appear to have been increasing in frequency, intensity and distribution, and this has resulted in serious environmental and public health concerns (Hallegraeff, 2005).

In the past few decades, considerable efforts have been devoted to studying the biogeography, ecology, physiology and toxicology of *A. tamarense* (Anderson et al., 1998). However, many basic scientific questions are still unresolved, e.g. knowledge concerning the molecular mechanisms involved in blooming and toxin biosynthesis is completely lacking due to the lack of available information on the genomics of *A. tamarense*, and this has hampered study of this organism from a genetic perspective. Traditional biochemical methods and molecular technology are also facing challenges in the study of *A. tamarense* due to its unique characteristics particularly its huge genome size (up to 250 pg DNA/cell), permanently condensed chromosomes, as well as the cell mobility. The application of new approaches and techniques to address these biological questions is urgently needed.

An alternative approach to address these biological questions is proteomics, a systematic study of an organism's complete set of proteins (Wasinger and Bjellqist, 1997; Wilkins et al., 1996). In contrast to conventional biochemical approaches which address

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only one or a few specific proteins at a time, proteomic techniques allow simultaneous isolation and identification of hundreds to thousands of proteins in one sample. In the past few years, proteins of several dinoflagellate species have been profiled and a few proteins have been identified and characterized using proteomic approaches (Chan et al., 2004a, 2005, 2006). However, the protein preparation method is still unsatisfactory with respect to protein spot number, separation and resolution although several protein extraction methods (e.g. 40 mM Tris buffer or Trizol extraction) have been reported for various dinoflagellate species (Chan et al., 2002; Lee and Lo, 2008) which limits the application of the proteomic approach to the study of dinoflagellates.

In this study, four protein preparation methods for the two-dimensional electrophoretic analysis of *A. tamarense* were compared in terms of resolution, protein spot number and intensity, high and low molecular weight proteins as well as alkaline proteins, and the protocol was also applied to profiling protein expression of *A. catenella*, a conspecific species of *A. tamarense* (Wang et al., 2008) under light stress conditions. The aim of this study is to develop an optimal protein preparation method for proteomic study using 2DE techniques when dealing with the dinoflagellate *Alexandrium* sp.

2. Materials and methods

2.1. Algal cultures

Unialgal cultures of *A. tamarense* CI01 and *A. catenella* DH01 were kindly provided by the Collection Center of Marine Bacteria and Algae (CCMBA), Xiamen University, China. The two isolates are routinely maintained in K medium (Keller et al., 1987) at 20 °C under a 12:12 h light: dark photoperiod at a light intensity of approximately 100 μmol photons m^{-2} s^{-1} provided by fluorescent lamps.

The cells for 2DE comparison experiments were grown in 5 L flasks containing 4 L of K medium, and the culture conditions were as above. When the cells entered the late exponential phase, they were harvested for protein extraction and 2DE. For the light stress experiment, three light conditions were designed: normal light/dark cycle (14:10 h), continuous light illumination for 48 h and continuous darkness for 48 h. At the end of the experiment, cells were harvested for 2DE analysis.

2.2. Protein extraction

For each method, approximately 1×10^6 vegetative cells of *A. tamarense* CI01 in the late exponential growth phase were collected by centrifugation at 5000 × *g* for 10 min at 20 °C. The pellet was subsequently transferred to a 1.5 mL microcentrifuge tube, rinsed twice with sterile seawater, and centrifuged again at 5000 × *g* for 30 min at 20 °C. The pelleted cells were used for protein extraction using the four preparation methods.

2.3. Urea/Triton X-100 buffer extraction with trichloroacetic acid/acetone precipitation

0.5 mL of pre-chilled urea/Triton X-100 buffer containing 7 M urea, 2 M thiourea, 2% CHAPS (w/v); 2% Triton X-100 (v/v); 1% DTT (w/v) and 2% carrier ampholytes was added to the cell pellet. The pellet was lysed using an ultrasonic disrupter (Model 450, Branson Ultrasonics, Danbury, CT, USA), and the sample was chilled on ice between bursts of less than 10 s of sonication. Cell debris was removed by centrifugation at 15,000 × *g* for 30 min at 4 °C, and then 0.5 mL pre-chilled 20% trichloroacetic acid (TCA)/acetone (w/v) was added to the supernatant to precipitate the protein for 30 min at 4 °C. Next the supernatant was removed by centrifugation at 15,000 × *g* for 30 min at 4 °C. The pellet was washed three times with ice-cold acetone with 20 mM DTT. The pellet was recovered by centrifugation at 15,000 × *g* for 30 min at 4 °C each time. Residual acetone was removed in a speed Vac for about 5 min, and, finally, the powder was dissolved in 50 μL rehydration buffer containing 8 M urea, 2%CHAPS, 2.8 mg/mL DTT, 0.5% IPG buffer and a trace amount of bromophenol blue.

2.4. TCA/acetone precipitation

10% TCA/acetone (w/v) solution was added to the cell pellet and it was lysed using an ultrasonic disrupter. The supernatant was removed by centrifugation at 15,000 × *g* for 30 min at 4 °C, and the pellet was washed once with 80% acetone (v/v) and three times with ice-cold acetone with 20 mM DTT. The pellet was treated as described above.

2.5. 40 mM Tris (hydroxymethyl) aminomethane buffer extraction

Briefly, the cell pellet was lysed in 0.5 mL pre-chilled (4 °C) 40 mM Tris (hydroxymethyl) aminomethane (Tris) buffer (pH 8.7) using an ultrasonic disrupter. Cell debris was removed by centrifugation at 15,000 × *g* for 30 min at 4 °C. The supernatant was washed three times with 40 mM Tris buffer at pH 8.7 (4 °C) and concentrated to 50 μL using ultrafiltration and passing it through a 1.5 mL Microcon tube with the cut-off of 3 kD. The supernatant was collected and stored at −80 °C.

2.6. 50 mM Tris/5% glycerol buffer extraction

The cell pellet was lysed in 0.5 mL extraction buffer containing 50 mM Tris, 0.5 mM EDTA, 50 mM NaCl and 5% glycerol (v/v) using an ultrasonic disrupter. The other procedures were the same as described in 2.3.

2.7. Two-dimensional electrophoresis

Protein quantification in each sample was performed using PlusOne™ 2D Quant kit (GE Healthcare Life Sciences). 40 μg of each protein sample was mixed with a rehydration buffer then loaded onto IPG strips of linear pH gradient 3–10 (for *A. tamarense* CI01) or 4–7 (for *A. catenella* DH01). Rehydration and subsequent isoelectric focusing were conducted using the Ettan IPGphor III Isoelectric Focusing System (Amersham Biosciences, USA). Rehydration was performed overnight in the strip holder with 340 μL of rehydration buffer. After rehydration, isoelectric focusing was performed in the following manner: 2 h at 100 V, 2 h at 200 V, 1 h at 500 V, 2 h at 1000 V, 2 h at 4000 V and 6 h at 8000 V. After the first dimension run, each strip was equilibrated with about 10 mL equilibration buffer containing 50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT and trace amount of bromophenol blue for 20 min. The strip was then placed in fresh equilibration buffer containing 2.5% iodoacetamide (instead of DTT) for another 20 min. Subsequently a 12.5% SDS-PAGE second dimension was performed.

2.8. Silver staining

Silver staining was performed following the method of Chan et al. (2004a). Briefly, the gel was fixed for 2 h initially in a fixation solution containing 40% (v/v) ethanol and 10% (v/v) acetic acid. It was then sensitized in a solution containing 30% (v/v) ethanol, 0.2% (w/v) sodium thiosulphate, 6.8% (w/v) sodium acetate and 0.125% (v/v) glutaraldehyde, followed by washing with distilled water (three times for 5 min each). Then the gel was stained for 20 min in 0.25% (w/v) silver nitrate with 0.015% (v/v) formaldehyde before washing with distilled water again (twice for 1 min each). The gel

was developed in 2.5% (w/v) sodium carbonate containing 0.0074% (v/v) formaldehyde. The reaction was stopped with 1.5% (w/v) ethylenediaminetetraacetic acid, disodium salt.

2.9. Image capture and analysis

Images were made using a Gel-documentation system on a GS-670 Imaging Densitometer from Bio-Rad (USA) and 2DE electrophoretogram matching software. Images were saved in TIFF format before analysis with ImageMaster 2D Elite (a 2D Gel electrophoresis image analysis software from Pharmacia Biotech). Computerized 2D gel analysis (spot detection, spot editing, pattern matching, database construction) was performed using the Image Master 2D Elite and Melanie IV.

2.10. Protein identification

Differentially expressed protein spots under various light conditions were manually excised from 2DE gels. The gel pieces were washed twice with 200 mM ammonium bicarbonate in 50% acetonitrile/water (20 min at 30 °C), then dehydrated using acetonitrile, and spun dry. In gel trypsin digestion was performed by adding 20 ng/μL trypsin (Promega,) in 25 mM ammonium bicarbonate overnight. For MALDI TOF–TOF MS analysis, 1 μL of the peptide mixture was mixed with 1 μL matrix solution (CHCA, saturated solution in ACN: 0.1% TFA (1:1)) on the target plate before being dried and analyzed with a MALDI TOF–TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems). MALDI TOF MS and TOF/TOF tandem MS were performed and data were acquired in positive MS reflector mode with a scan range from 900 to 4000 Da, and five monoisotopic precursors (S/N > 200) were selected for MS/MS analysis. For interpretation of the mass spectra, a combination of peptide mass fingerprints and peptide fragmentation patterns were used for protein identification in an NCBI nonredundant database using the Mascot search engine (www.matrixscience.com). All mass values were considered monoisotopic, and the mass tolerance was set at 75 ppm. One missed cleavage site was allowed for trypsin digestion; cysteine carbamidomethylation was assumed as a fixed modification, and methionine was assumed to be partially oxidized. Results with C.I. % (confidence interval %) values greater than 95% were considered to give a positive identification. The identified proteins were then matched to specific processes or functions by searching Gene Ontology (http://www.geneontology.org/).

3. Results

3.1. Comparison of 2DE protein profiles obtained using different protein preparation methods

The protein content (μg/μL) of 1.0×10^6 cells extracted with each method is shown in Table 1. There was no significant difference in protein content between urea/Triton X-100 buffer extraction with TCA/acetone precipitation and TCA/acetone precipitation, and the lowest result was found in the sample extracted with 40 mM Tris buffer.

The 2DE protein profiles of vegetative cells of *A. tamarense* CI01 harvested in the late exponential growth phase are shown in Fig. 1. Compared with 2DE protein profiles obtained using protein samples prepared with the other three protein preparation methods, those obtained using the urea/Triton X-100 buffer extraction with TCA/acetone precipitation method presented the best resolution of stained spots with a clear background. The majority of proteins were separated in the apparent molecular mass range of 20–98 kDa and had a p*I* range of 4.5–7.0. The number of high and low molecular weight proteins as well as alkaline proteins were higher than those obtained using the other three methods (Fig. 1 and Table 1).

As for the TCA/acetone precipitation method, the pattern of 2DE protein profiles was similar to that obtained using the urea/Triton X-100 buffer extraction with TCA/acetone precipitation method, and the majority of proteins were separated in the molecular mass range of 20–98 kDa and had a p*I* range of 4.5–7.0. However, the separation and intensity of protein spots was lower than those obtained using the urea/Triton X-100 buffer extraction with TCA/acetone precipitation method. For example, protein spots in the rectangular and circular regions in Fig. 1A and B showed a greater intensity on the 2DE gel obtained using urea/Triton X-100 buffer extraction with TCA/acetone precipitation method than that obtained using the TCA/acetone precipitation method. Although some spots could be seen in TCA/acetone precipitation prepared 2DE, the spots were much more focused and with a higher resolution in the urea/Triton X-100 buffer extraction with the TCA/acetone precipitation prepared 2DE gel. In addition, some spots were not seen in the TCA/acetone precipitation prepared 2DE. There were fewer protein spots in 2DE gels generated from 40 mM Tris buffer (Fig. 1C) and 50 mM Tris/5% glycrol buffer (Fig. 1D), and protein spots were separated in the apparent molecular mass range of 20–98 kDa and had a p*I* range of 4–6.6. Many high and low molecular weight and/or alkaline proteins were lost.

Using the ImageMaster 2D Elite software, protein spot numbers of 2DE gels generated using different protein preparation methods from *A. tamarense* CI01 are shown in Table 1. The highest protein spots were found in 2DE gel obtained using the urea/Triton X-100 buffer extraction with TCA/acetone precipitation method, up to 1228 protein spots. The TCA/Acetone precipitation method also yielded 826 protein spots. However, there were fewer protein spots in 2DE gels generated from 40 mM Tris buffer and 50 mM Tris/5% glycrol buffer, only 353 and 382 spots, respectively.

Table 1
Comparison of protein contents, spot numbers, high and low molecular weight proteins, alkaline proteins and resolution from 2DE protein profiles of samples extracted using four buffers.

	A	B	C	D
Protein content (μg/μl)	6.41	6.73	2.82	5.58
Total number of spots	1228	826	353	382
High molecular weight	++	+	–	–
Low molecular weight	++	+	–	+
Alkaline proteins	++	++	–	–
Protein separation	++	+	–	–

Note: (A) Urea/Triton X-100 buffer; (B) TCA/acetone buffer; (C) 40 mM Tris buffer; (D) 50 mM Tris/5% glycerol buffer. "++", best; "+", good; "–" bad.

3.2. Proteomic analysis of A. catenella DH01 under light stress

As with the above comparison, the urea/Triton X-100 buffer with TCA/acetone precipitation method presented the highest quality in terms of resolution, spot number, spot intensity as well as gel background. Therefore, this method was applied to compare the protein profiles of another *Alexandrium* species, *A. catenella* DH01 grown in normal light/dark cycle (14/10 h), continuous darkness (48 h), and continuous light illumination (48 h) cultures. Representative 2DE gels are shown in Fig. 2. Protein spots were separated individually and distributed evenly in 2DE gels, and no horizontal or vertical streaks were found in 2DE gels with a clearer background. These 2DE gels shared a majority of protein spots. However, significant differences of two groups of proteins were found among various light conditions. Proteins of group 1 with apparent *M*r of 76 kDa and p*I* ranging from 5.1 to 5.9 were highly expressed in the continuous light culture (Fig. 2C) compared to those in the normal light/dark cycle and in continuous darkness

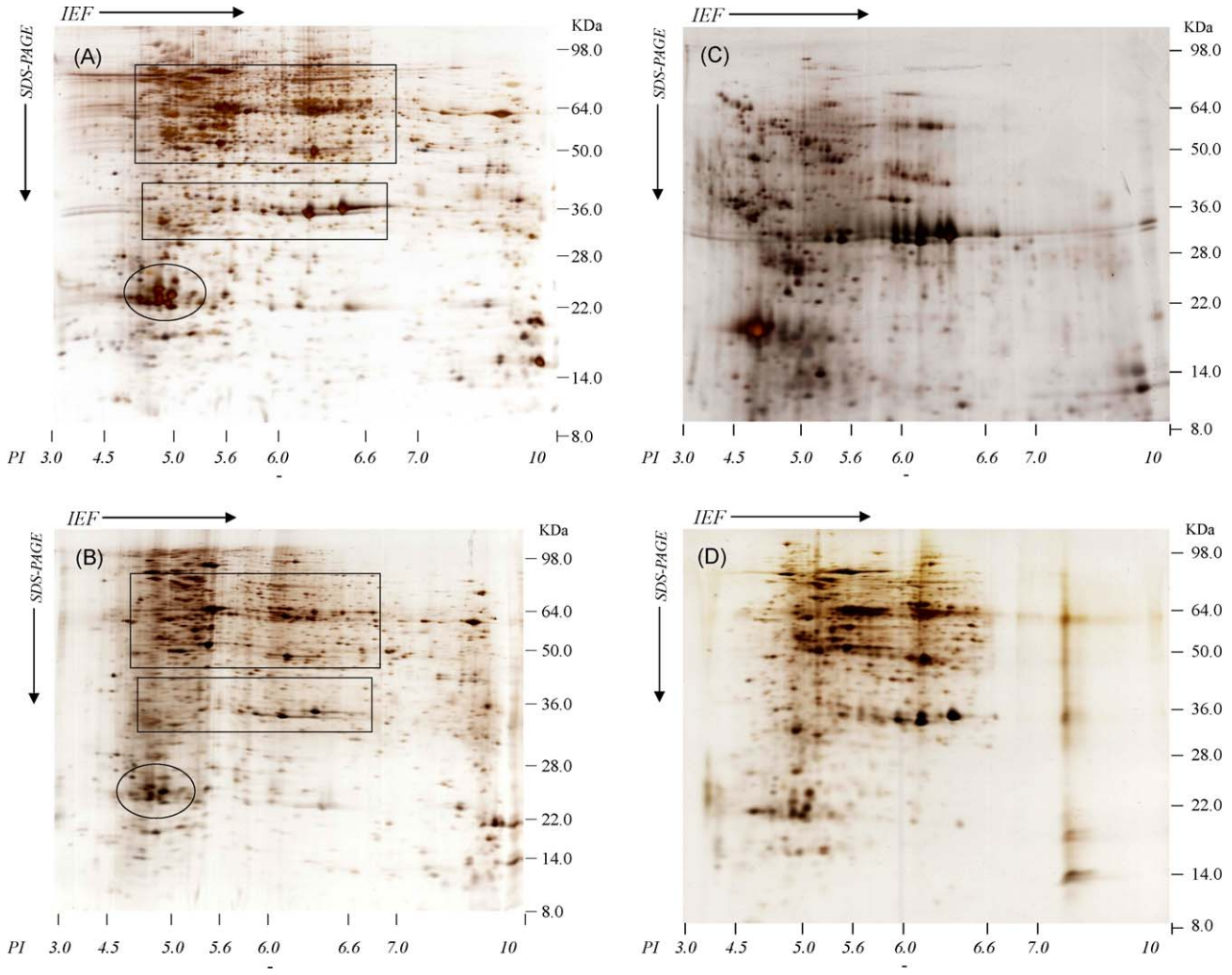

Fig. 1. 2DE protein profiles of samples extracted using four buffers (pH 3–10). (A) Urea/Triton X-100 buffer; (B) TCA/acetone buffer; (C) 40 mM Tris buffer; (D) 50 mM Tris/5% glycerol buffer.

(Fig. 2A and B). While proteins of group 2 exhibited low expression in continuous light illumination 2DE gel compared to those in normal light/dark cycle and continuous darkness (Fig. 2A and B). The results from MALDI TOF–TOF MS indicated that these differential expression proteins were related to either photosynthesis or protection from light damage (Table 2). Spots 1 and 4 were ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCO) large submit and form II, respectively, and spot 2 was glutathione synthetase, a chloroplast precursor. These proteins were highly expressed in *A. catenella* cells grown in a normal light/dark cycle and in continuous darkness. Spots 6 and 7 were crytochrome, which is a blue light photoreceptor of plants and animals. Spots 3 and 5 were heat shock proteins 90 and 83, and these proteins were highly expressed in *A. catenella* cells grown in continuous illumination, which might be a physiological response of cells to light stress.

4. Discussion

Since its description more than 30 years ago (O'Farrell, 1975), 2DE has become one of the most commonly used techniques in proteomic research. It allows a fast and relatively inexpensive overview of changes in cell processes by analyzing the entire proteome of the cells. However, to obtain high quality protein samples free from contaminated substances for subsequent 2DE, high quality sample preparatory methods are required (Chan et al., 2002, 2004a,b; Lee and Lo, 2008). The optimal protein preparation method should be efficient in removing high endogenous levels of salts, nucleic acids, polysaccharides, phenolic compounds, pigments, and other interfering compounds from samples, since all these compounds interfere with the IEF-focusing process, the first step of 2DE. Most of the limited number of publications concerning studies of dinoflagellate proteomes, rely on the high power of resolution of 2DE (Akimoto et al., 2004; Chan et al., 2004a,b, 2005, 2006; Lee and Lo, 2008).

Chan et al. (2002) extensively compare protein preparation methods for analysis with 2DE using the dinoflagellate *Prorocentrum triestinum* as the model species, and optimize the protein extraction method. They suggest that a combination of sequential extraction and desalting using BioSpin chromatography for sample treatment before first dimension 2DE gives the best results based on its simplicity and minimal protein loss. However, the resolution, protein spot numbers and spot separation of this method are not ideal. Moreover, high and low molecular weight proteins (as well as alkaline proteins) are lost during 2DE gel generation using this method. Recently, Lee and Lo (2008) compare three protein extraction methods in one of their studies and find that 2DE profiles obtained with Trizol treatment are of very high quality in terms of resolution, spot number and spot intensity in two dinoflagellates, *Alexandrium* spp. and *Scrippsiella* spp. Compared to the other two

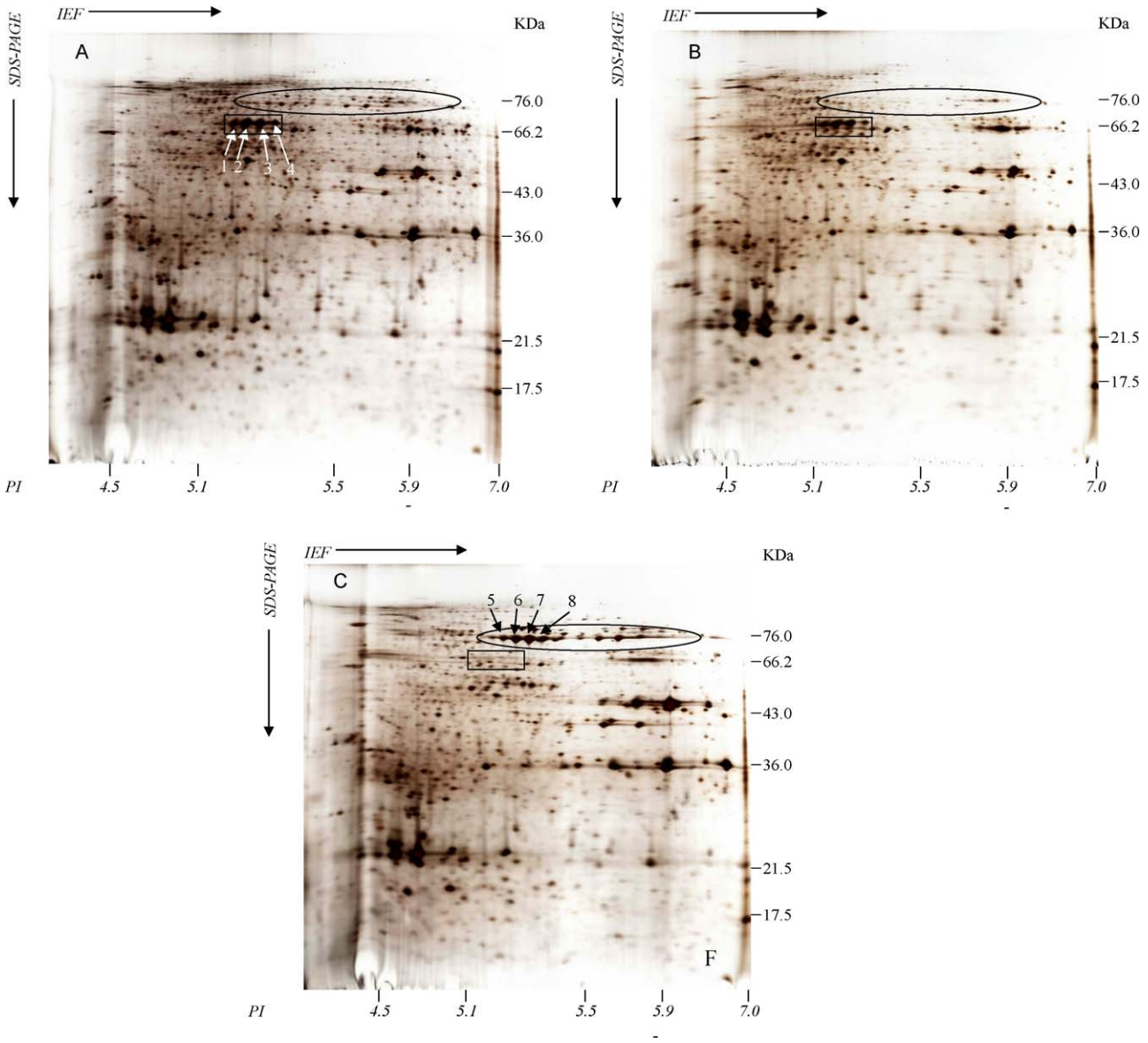

Fig. 2. 2DE protein patterns of *A. catenella* DH01 under various light conditions (pH 4–7). (A) Normal light/dark cycle (14:10 h); (B) 48 h continuous light; (C) 48 h continuous dark.

methods, namely lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris pH 8.7) and acetone precipitation, the Trizol extraction method is a simple and fast method, which can remove interfering substances efficiently as well as having a shorter time of sample preparation, resulting in the best resolution of stained spots with a clear background. However, they only compared these methods within a narrow p*I* range (4–7) which could not give the whole information of proteins in the 2DE gel. Moreover, the intensity of protein spots in the 2DE gel obtained from the Trizol extraction method was always a little vague in our study (unpublished data). In the present study, the best resolution was found in the 2DE gel obtained from the urea/Triton X-100 buffer extraction with TCA/acetone precipitation method, which not only obtained the best protein spot separation, highest spot number and density, but also presented a clear background, indicating that this method removes high endogenous levels of salts, nucleic acids (estimated to be about

Table 2
Identification of differential expression proteins of *A. catenella* DH01 grown under various light conditions.

Spot no.	Sequence	Sequence similarity	Accession no.
1	QFLHYHR	Ribulose 1,5-bisphosphate carboxylase oxygenase large subunit [Prorocentrum minimum]	gi|33317821
2	FLEPHLLKSSK	Glutathione synthetase, chloroplast precursor (Glutathione synthase) (GSH synthetase) (GSH-S)	gi|20138145
3	HFNVEGQLEFK	Heat shock protein 90 [Guillardia theta]	gi|59894162
4	QFLHYHR	Ribulose 1,5-bisphosphate carboxylase oxygenase form II [Prorocentrum minimum]	gi|37727276
5	DSSMAQYMVSK	Recombinant Lbhsp83 = 83 kDa heat shock protein [Leishmania braziliensis, Peptide, 656 aa]	gi|1168148
6	MEATSSVANSPVSR	Cryptochrome 2 [Oryza sativa (indica cultivar-group)]	gi|40644276
7	ETSPCALPIDQR	Cryptochrome 2 [Oryza sativa (indica cultivar-group)]	gi|40644276
8	CSPVAGCSGR	Ferrochelatase [Chlamydomonas reinhardtii]	gi|13249285

10–80 times more than in humans), polysaccharides, phenolic compounds, pigments, and other interfering compounds. Using TCA/acetone directly also obtained relatively good results regarding spot separation and background. However, the protein spot number and intensity were not ideal since many spots were lost or the intensity of spots was low in the rectangular and circular regions of 2DE gel prepared using the TCA/acetone precipitation method (Fig. 1A). Moreover, this method was affected more profoundly by cell debris, nucleic acids, polysaccharides and phenolic compounds, and when dissolving proteins using rehydration buffer, the extract always became sticky and difficult to dissolve. 40 mM Tris buffer was applied to extract water soluble proteins from various dinoflagellate species (Chan et al., 2004a,b, 2005, 2006), and this method is a non-denaturing extraction method, which maintains the natural characteristics of proteins and provides a useful method for protein functional study. The protocol of this method is simple, but it results in loss of water insoluble proteins, especially alkaline proteins. 50 mM Tris/5% glycerol buffer is widely used to extract proteins from plant samples, and this protects the natural protein characteristics (e.g. various enzymes) due to the addition of glycerol. This method is regarded as a good choice for the study of enzyme activity or enzyme identification. However, this method can only extract water soluble proteins and always causes IEF gel burn at the alkaline end due to the existence of glycerol during running the IEF gel. So these two methods are not a good choice for 2DE of dinoflagellates.

In 2DE, urea is one of the common chaotropes, but urea alone is often not sufficient to completely quench the hydrophobic interactions. Urea–thiourea mixtures are reported to exhibit a superior solubilizing power and are found to increase dramatically the solubility of membrane or nuclear proteins (Molloy et al., 1998). In the present study, 7 M urea and 2 M thiourea were introduced into the lysis buffer and this increased the solubility of membrane or nuclear proteins. Triton X-100 is a non-ion detergent while CHAPS is a zwitterionic detergent, both of which can enhance protein dissolving ability and protect the protein forming polyer due to the hydrophobic reaction. In this study, cell pellets were firstly broken in the urea/Triton X-100 buffer, and then proteins and other endogenous compounds were extracted to the supernatant. Subsequently, proteins were separated from any interfering compounds using TCA/Acetone precipitation, and high quality proteins were obtained. This protocol not only avoided the disturbance of cell debris, nucleic acids, polysaccharides and phenolic compounds during protein extraction, but also reduced the modification of TCA to proteins.

The present study identified the differentially expressed proteins of *A. catenella* under the various light conditions using partial amino acid sequence BLAST in the NCBI non-reductant database, and they all gave a positive identification of the protein orthologues in the protein database. However, given the lack of genomic sequence data available for dinoflagellates, and the fact that the amino acid sequence obtained was short, the accuracy of these proteins needs further intensive verification. For example, spots 1 and 4 were identified as RuBisCO large subunit and form II, respectively. However, only one form of RuBisCO, RuBisCO form II, is found in dinoflagellates. The difference might be caused by either protein being partially degraded during the experiment or post-translational modification of the proteins. The short amino acid sequence obtained from MALDI TOF–TOF might be the other reason. In future, other powerful identification methods, such as *de novo* protein sequence analysis combined with N-terminal sequence analysis should be introduced to the protein identification of dinoflagellates. However, this was neither the aim nor within the scope of this study.

Overall, the present study demonstrated that the urea/Triton X-100 buffer extraction with TCA/acetone precipitation method was the best method to prepare protein samples for proteomic study using 2DE in *Alexandrium* sp. This protocol was then successfully applied for profiling the protein expression of *A. catenella* under light stress conditions, and all 2DE gels presented a clear background with high quality resolution, spot separation and intensity. Using this protocol, two groups of proteins were found, which varied significantly in 2DE gels obtained under different light conditions, and the differentially expressed proteins were successfully identified using MALDI TOF–TOF MS.

5. Conclusions

The 2DE protein profiles of *Alexandrium* sp. showed that the urea/Triton X-100 buffer extraction with TCA/acetone precipitation method was the best method among the four protein preparation methods. It not only removed all the interfering substances and salts efficiently but also presented a high quality in terms of resolution, spot number and spot intensity with a clear background. Using the optimized conditions described above, *Alexandrium* sp. yielded highly reproducible protein expression patterns, and we were also able to identify differentially expressed proteins under different experimental conditions in both tested dinoflagellates, indicating that it is a potential powerful method for further proteomic studies of *Alexandrium* and related dinoflagellate species.

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References

Akimoto, H., Wu, C., Kinumi, T., Ohmiya, Y., 2004. Biological rhythmicity in expressed proteins of the marine dinoflagellate *Lingulodinium polyedrum* demonstrated by chronological proteomics. Biochem. Biophys. Res. Commun. 315, 306–312.

Anderson, D.M., Kulis, D.M., Doucette, G.J., Gallagher, J.C., Balech, E., 1994. Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada. Mar. Biol. 120, 467–478.

Anderson, D.M., Garrison, D.J., 1997. Ecology and oceanography of harmful algal blooms. Limnol. Oceanogr. 42 (Suppl.), 1009–1305.

Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), 1998. Physiological Ecology of Harmful Blooms. Springer-Verlag, Berlin/Heidelberg.

Cembella, A.D., 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Blooms. Springer-Verlag, Berlin/Heidelberg, pp. 381–403.

Chan, L.L., Lo, S.C.L., Hodgkiss, I.J., 2002. Proteomic study of a model causative agent of harmful red tide, *Prorocentrum triestrinum*. I. Optimization of sample preparation methodologies for analyzing with two dimensional electrophoresis. Proteomics 2, 1169–1186.

Chan, L.L., Hodgkiss, I.J., Lo, S.C.L., 2004a. Proteomic study of a model causative agent of harmful algal blooms, *Prorocentrum triestinum*. II. The use of differentially expressed protein profiles under different growth phases and growth conditions for bloom prediction. Proteomics 4, 3214–3226.

Chan, L.L., Hodgkiss, I.J., Lu, S.H., Lo, S.C.L., 2004b. Use of 2-DE proteome reference maps of dinoflagellates for species recognition of causative agents of harmful algal blooms. Proteomics 4, 180–192.

Chan, L.L., Hodgkiss, I.J., Lam, P.K.S., Wan, J.M.F., Chou, H.N., Lum, J.M.K., Lo, M.J.Y., Mak, A.S.C., Sit, W.H., Lo, S.C., 2005. Use of two-dimensional gel electrophoresis to differentiate morphospecies of *Alexandrium minutum*, a paralytic shellfish poisoning toxin-producing dinoflagellate of harmful algal blooms. Proteomics 5, 1580–1593.

Chan, L.L., Sit, W.H., Lam, P.K.S., Hsieh, D.P.H., Hodgkiss, I.J., Wan, J.M.F., Ho, A.Y.T., Choi, N.M.C., Wang, D.Z., Dudgeon, D., 2006. Identification and characterization of a "biomarker of toxicity" from the proteome of the paralytic shellfish toxin-producing dinoflagellate *Alexandrium tamarense* (Dinophyceae). Proteomics 6, 654–666.

Hallegraeff, G.M., 2005. Harmful algal blooms: a global overview. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. Imprimerie Landais, Paris, France, pp. 25–49.
Keller, M.D., Selvin, R.C., Claus, W., Guillard, R.R.L., 1987. Media for the culture of oceanic ultraphytoplankton. J. Phycol. 23, 633–638.
Kodama, M., 2000. Ecobiology, classification, and origin. In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection[M]. Marcel Dekker, Inc., New York, USA, pp. 125–151.
Lee, F.W.F., Lo, S.C.L., 2008. The use of Trizol reagent (phenol/guanidine isothiocyanate) for producing high quality two-dimensional gel electrophoretograms (2-DE) of dinoflagellates. J. Microbiol. Methods 73, 26–32.
Molloy, M.P., Herbert, B.R., Walsh, B.J., Tyler, M.I., Traini, M., Sanchez, J.C., Hochstrasser, D.F., Williams, K.L., Gooley, A.A., 1998. Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis. Electrophoresis 19, 837–844.
O'Farrell, P.H., 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250, 4007–4021.
Wang, D.Z., Lin, L., Gu, H.F., Chan, L.L., Hong, H.S., 2008. Comparative studies on morphology, ITS sequence and protein profile of *Alexandrium tamarense* and *A. catenella* isolated from the China Sea. Harmful Algae 7, 106–110.
Wasinger, V.C., Bjellqist, B., 1997. Proteomic "contig" of Ochroboctrum anthropi application of extensile pH gradients. Electrophoresis 18, 1373–1383.
Wilkins, M.R., Pasquali, C., Apple, R.D., Ou, K., Golaz, O., Sanchez, J.C., Yan, J.X., Gooley, A.A., Hughes, G., Humphery-Smith, I., Williams, K.L., Hochstrasser, D.F., 1996. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. Biotechnology 14, 61–65.