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RESEARCH ARTICLE

Identification and characterization of a "biomarker of toxicity" from the proteome of the paralytic shellfish toxin-producing dinoflagellate *Alexandrium tamarense* (Dinophyceae)

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The objective of this study was to identify and characterize a "biomarker of toxicity" from the proteome of *Alexandrium tamarense*, a paralytic shellfish toxin (PST)-producing dinoflagellate. A combination of 2-DE and MS approaches was employed to identify proteins of interest in the vegetative cells of several strains of *A. tamarense* with different toxin compositions and from different geographical locations. The electrophoretic analysis of the total water-soluble proteins from these toxic strains by 2-DE showed that several abundant proteins, namely AT-T1, AT-T2 and AT-T3, differing slightly in apparent M_r and p*I*s, were consistently present in all toxic strains of *A. tamarense*. Further analysis by MALDI-TOF MS and N-terminal amino acid sequencing revealed that they are isoforms of the same protein. Even more intriguing is that these proteins in *A. tamarense* have similar amino acid sequences and are closely related to a "biomarker of toxicity" previously reported in *A. minutum*. Unambiguous and highly species-specific identification was later achieved by comparing the PMFs of proteins in these two species. An initial attempt to characterize these proteins by generation of murine polyclonal antibodies against the AT-T1 protein was successful. Western blot analysis using the murine AT-T1-polycolonal antibodies identified all the toxic strains of *A. tamarense* and *A. minutum*, but not the nontoxic strain of *A. tamarense*. These results indicate that these protein characteristics for toxic strains are species-specific and that they are stable properties of the tested algae which are clearly distinguishable irrespective of geographical location and toxin composition. To our knowledge, this is the first

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Abbreviations: GTX, gonyautoxin; **HAB**, harmful algal bloom; **PSP**, paralytic shellfish poisoning; **PST**, paralytic shellfish toxin; **STX**, saxitoxin

study to demonstrate the use of polyclonal antibodies against marker proteins purified from 2-DE gels to distinguish different strains and species of the PST-producing dinoflagellate *Alexandrium*. It provides the basis for the production of monoclonal antibody probes against the "biomarkers of toxicity" for those dinoflagellates whose genome is incompletely characterized. Potentially, immunoassays could be developed to detect the presence of toxic algae in routine monitoring programs as well as to predict bloom development and movement.

Keywords:
Alexandrium tamarense / Biomarker of toxicity / Harmful algal bloom species / Paralytic shellfish poisoning toxin / Polyclonal antibodies

1 Introduction

Paralytic shellfish poisoning (PSP) in humans is caused by the consumption of shellfish (*e.g.*, mussels, clams, and oysters) contaminated with potent neurotoxins produced by various microalgae of the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium* [1]. Paralytic shellfish toxins (PSTs) have a common tricyclic skeleton with varying structures that result from N-1 (=R1) hydroxylation, C11 O-sulfation (=R2, R3), C-13 carbamoylation (=R4), and N-21 sulfation (Fig. 1) [1, 2]. Saxitoxin (STX) is the "parent compound" in a family of over 20 derivatives differing in their specific toxicities. These derivatives include saxitoxin (STX), neosaxitoxin, gonyautoxins 2 and 3 (GTX2, 3), gonyautoxins 1 and 4 (GTX1, 4), decarbamoyl saxitoxin (dcSTX), B-1 (GTX5), C-1 and C-2 (C1, 2), C-3 and C-4 (C3, 4), and B-2 (GTX6) toxin. They are all involved in PSP [3] and are responsible for respiratory paralysis by blocking the sodium ion channels in nerve and muscle membranes [4].

Contamination of shellfish with PSTs can occur in the absence of observed harmful algal blooms (HABs), since toxin accumulation in shellfish results from exposure to low concentrations of toxic species over an extended period [5]. Accordingly, identification of microalgae to species level is vital so that shellfish farmers, and health and industry officials, can make safe and economically sound harvesting decisions. Monitoring of toxic microalgae is usually conducted by using reactive measurements such as periodic water sampling, identification, enumeration and separation of toxic marine microalgae. Considerable time and effort are required to identify all species present, especially if some exist at low concentration. In addition, some species are morphologically similar and/or toxic and nontoxic varieties of the same species exist and sometimes even co-occur [6]. These facts present serious problems in monitoring programs. An alternative to microscopic identification is the use of molecular probes and cellular probes that can bind to sites on the target species [7]. In this regard ribosomal RNA sequences [8] and FITC-conjugated lectins [9] appear to be promising techniques. Immunochemical procedures present another alternative for detecting nuisance and toxic marine phytoplankton cells [10–15] and Bates *et al.* [16] have used immunofluorescence to distinguish between domoic acid-producing and nontoxic forms of the diatom *Pseudonitzschia pungens*. The emergence of these molecular techniques has great potential to advance the pace of research involving the monitoring of harmful algae. However, a valid judgment of whether toxic species are present or not still relies on instrumental analysis. Quantifying the content of PSTs in dinoflagellates by instrumental HPLC [10] involves very time-consuming and expensive processes. A well-equipped analytical laboratory is also required in order to validate the precision and accuracy of the results. Such instrumental analysis requires pure PST standards that are not all available commercially for all saxitoxin derivatives. Furthermore, it is not well suited for field use, and is difficult to develop into a practical and commercially viable technique for routine monitoring. The search for new markers allowing precise and rapid species identification is thus essential for routine monitoring of toxic species.

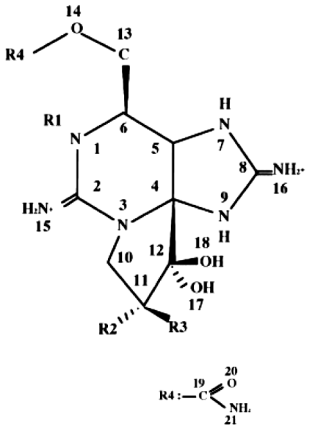

Figure 1. Structure of paralytic shellfish poisoning toxins (PSTs).

Previously, we described the use of 2-DE-based proteomic approaches as alternatives for species recognition in several HAB-related dinoflagellates [11] and a "biomarker of toxicity" was found in toxic *A. minutum* (Fig. 2A) [12]. In the

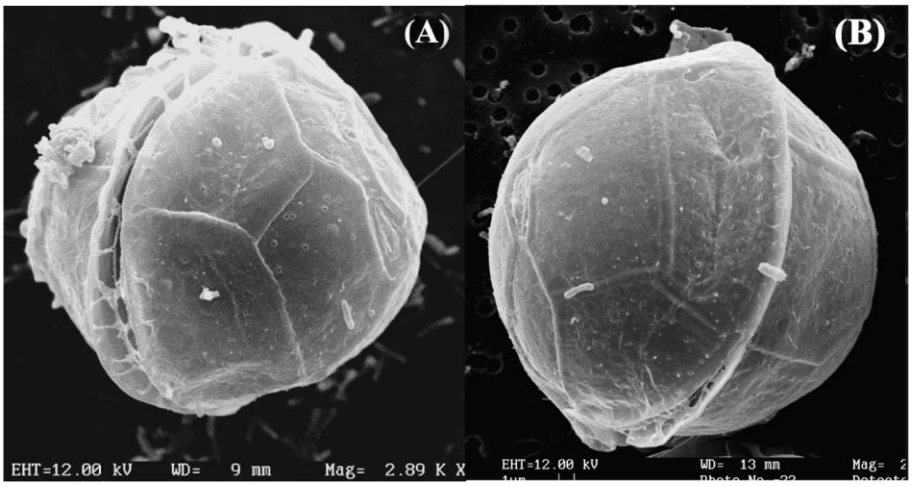

Figure 2. Scanning electron micrographs of: (A) *A. minutum*; (B) *A. tamarense*. Magnification: 2890 ×.

present study, a combination of 2-DE, MALDI-TOF MS, N-terminal amino acid sequencing by Edman degradation and Western blot analyses have been employed to identify and characterize a "biomarker of toxicity" in phylogenetically close toxic and nontoxic strains of *A. tamarense* (Lebour) Balech & Taylor from geographically diverse populations (Fig. 2B). Toxic and nontoxic strains of *A. tamarense* often co-occur in nature [13], highlighting the need for discrimination between them. Ribosomal RNA gene sequences cannot separate these strains and reliable molecular markers for total characterization of *Alexandrium* have not yet been defined. Therefore, the identification of specific protein or peptide markers would be very useful for the efficient diagnosis of these species as well as other toxic phytoplankton species.

2 Materials and methods

Unless stated otherwise, all chemicals were purchased from Sigma (USA). All solvents were at least of AR grade while most were of HPLC grade.

2.1 Cultivation of *A. tamarense* and *A. minutum*

Cultures of *A. tamarense* AT-CI01 and *A. tamarense* AT-HK9301 (responsible for the 1990 and 1991 PSP outbreaks in Daya Bay [14]) were established from germinated cysts, respectively, from Daya Bay and Dapeng Bay sediments of the South China Sea near Hong Kong. *A. tamarense* AT-HKJB was isolated from Junk Bay, Hong Kong, and *A. tamarense* AT-Polar was isolated from the Southern Ocean by Prof. K. C. Ho (The Open University of Hong Kong). The American strain of *A. tamarense* AT-WHOI was kindly donated by Dr. D. M. Anderson (Biology Department, Woods Hole Oceanographic Institution, USA). AM-TK and AM-KS of *A. minutum* strains were isolated from the Tung Kang (TK) and Kaohsiung (KS) areas of Taiwan, respectively, and *A. minutum* AM-TK4 and *A. minutum* AM-KS2 were kindly donated by Prof. H. N. Chou (Institute of Fisheries Science, National Taiwan University, Taipei, Taiwan). These unialgal isolates were batch cultured in K medium [15] at 20°C under a 12:12 h light:dark photoperiod at a light intensity of approximately 100–150 μmol photons $m^{-2}\,s^{-1}$ provided by fluorescent lamps in a Conviron growth chamber (Model S10H, Conviron Controlled Environments, Winnipeg, Canada) for 14 days until the mid-exponential growth phase was reached.

2.2 Preparation of extracts for proteomic analysis and HPLC analysis with fluorescence detection

Approximately 1×10^6 cells were collected by centrifugation at 5000 × *g* for 20 min at 22°C (himac CR 22f, Hitachi High-Speed Refrigerated Centrifuges, Japan) and the pellets were rinsed twice with sterilized seawater to avoid any carry-over of culture medium. The pelleted cells were then kept in a −80°C freezer for subsequent analysis. No sample was stored for more than 3 months. Water-soluble proteins were isolated as previously described [19].

Approximately 1×10^4 cells were collected by centrifugation at 5000 × *g* for 20 min at 22°C (himac CR 22f, Hitachi High-Speed Refrigerated Centrifuges, Japan) for toxin analysis. The pelleted cells were homogenized in 0.5 mL of 0.05 M glacial acetic acid with three successive sonications using a Microtip-probe sonifier (Model 250, Branson Ultrasonics, Danbury, CT, USA). Samples were chilled on ice between bursts of less than 10 s. Cell debris and unbroken cells were removed by centrifugation at 22 220 × *g* for 15 min at 4°C (Mikro 22R, Hettich, Germany). The supernatants were filtered with a molecular-sieve membrane with a 10 000-Da cut-off (YM-10 membrane, Amicon, Bedford, MA, USA) fol-

lowing the manufacturer's instructions. Toxin analysis was performed by HPLC with fluorescence detection (Waters Corporation, USA) using reverse phase (Inertsil C8 column, 3u, 150 mm × 4.6 mm and Inertsil C8, 5u, 7.5 mm × 4.6 mm all-guard cartridge, Alltech, USA) chromatography with post-column derivatization as described by Oshima [16]. The following three mobile phases were used for separation of different toxin groups: (1) 2 mM tetrabutyl ammonium phosphate solution adjusted to pH 6.0 with acetic acid for C toxins; (2) 2 mM 1-heptanesulfonic acid in 10 mM ammonium phosphate buffer (pH 11) for the GTX group; (3) 2 mM 1-heptanesulfonic acid in a mixture of 30 mM ammonium phosphate buffer (pH 7.1) and ACN (100:5) for the STX group.

2.3 IPG 2-DE

Forty microgram and 400 μg of each sample were applied for analytical run (pattern comparison) and preparative gel analysis (mass spectrometric analysis or *N*-terminal amino acid sequencing), respectively. Rehydration, IEF, and equilibration were performed as previously described [17]. Briefly, the sample was subjected to IEF (first dimension) with an 18-cm IPG strip (Amersham Biosciences, Hong Kong, China) over a pH gradient of 4.0–7.0 and resolved on a 12.5% slab gel SDS-PAGE (second dimension). Subsequently, proteins on the 2-DE gels were (1) visualized by silver staining for pattern comparisons; (2) electrotransferred onto an Immobilon-P membrane (PVDF, Millipore, Bedford, MA, USA) for *N*-terminal amino acid sequencing and Western blot analysis; and (3) stained with CBB R-250 for MALDI-TOF MS. Three 2-DE gels were run for each condition. Unless stated otherwise, the gels shown are representative of the three gels run. Protein spots were selected for quantitative analysis if they showed the potential to serve as a "taxonomic biomarker" or "biomarker of toxicity" and were consistently visible in all samples from one condition. The density of each spot was measured using an ImageScanner (Amersham Biosciences) equipped with ImageMaster software from Amersham Biosciences. The abundance of each spot was calculated as a percentage of the total density of all spots measured on each gel.

2.4 MALDI-TOF MS and *N*-terminal amino acid sequencing by Edman degradation

Protein spots were selected to determine the PMFs by a MALDI-TOF MS (Autoflex, Bruker Daltonics, Germany) if they showed the potential to serve as a "taxonomic biomarker" or "biomarker of toxicity" and were consistently visible in all samples from one condition. Selected protein spots were digested in gels according to the method described by Shevchenko and coworkers [18]. The digests were desalted with ZipTip (Millipore) and subjected to analysis by MALDI-TOF MS and the PMFs obtained for each protein of interest were searched against the NCBI nonredundant database using the search engine MASCOT available at http://www/matrixscience.com as previously described [19]. Unidentified proteins separated by 2-D PAGE were electrotransferred onto a PVDF membrane and selected protein spots were excised and subjected to *N*-terminal amino acid sequencing using a Procise 492 cLC Model 610A Protein sequencer (Applied Biosystems, Hong Kong, China). Amino acid sequences obtained were searched against either the Protein DataBank (PDB) or Swiss-Prot by BLAST. Settings for querying short sequences for nearly exact matches of peptides were used.

2.5 Production of murine anti-AT-T1 polyclonal antibody

Approximately 1 mg of total proteins from the toxic strain of *A. tamarense* was separated by 2-D PAGE as described in Section 2.3. The resultant 2-DE gels were stained with CBB R-250 and the gel spot containing AT-T1 was excised. After several washes with PBS, the gel piece was emulsified with an equal volume of Freund's complete adjuvant and injected into a BALB/c mouse at four different sites (two subcutaneously and two intramuscularly). A schedule of two boosters of AT-T1 in Freund's incomplete adjuvant was carried out at monthly intervals and test bleeds were obtained 7 days after the second booster.

2.6 Western blot analysis of T1 proteins

For 2-D PAGE, proteins were electrotransferred onto PVDF membranes after the second dimension and processed for Western blot analysis. For 1-D SDS-PAGE, electrophoresis was carried out with 12.5% w/v polyacrylamide slab gels [19]. In total, 40 μg of each sample was mixed with an equal volume of SDS-containing treatment buffer (0.125 M Tris-HCl, 4% SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8). The mixture was reduced and denatured by heating for 3 min at 100°C. Equal amounts of total protein were separated by electrophoresis on two separate gels. One gel was stained with CBB R-250. The other gel was electrotransferred to a PVDF membrane at a constant current of 100 mA overnight at 4°C in a transfer buffer (0.025 M Tris-HCl, 0.192 glycine, 20% methanol, pH 8.3).

After blotting, the membranes were blocked with PBS containing 5% w/v nonfat dry milk powder at room temperature for 4 h. After five 10-min washes in PBS-T (PBS with 0.05% v/v Tween 20), the blots were incubated with murine anti-AT-T1 immune serum (1:4000 dilution) for 2 h at room temperature. The blots were then incubated with horse radish peroxidase–goat anti-mouse (Santa Cruz) (1:2000 dilutions) for 2 h at room temperature. The protein bands/spots were detected by incubating with ECL (Amersham) and exposing to film.

3 Results

3.1 HPLC toxin composition profiles and 2-DE protein patterns of different isolates of *A. tamarense* under optimal conditions

The composition of the PSTs of four toxic strains of *A. tamarense* were compared: one isolated from the Southern Ocean; one from the USA; and the other two from the coastal waters of Southern China; together with two toxic strains of *A. minutum* from Taiwan (Table 1). The HPLC profile of major toxins of each species is shown in Fig. 3. *A. tamarense* AT-CI01 and *A. tamarense* AT-Polar produced almost exclusively C2 toxin with only trace amounts of C1 toxin, GTX2 and GTX3; *A. tamarense* AT-WHOI produced almost exclusively C2 toxin with only trace amounts of C1 toxin, GTX5, and STX (Fig. 3A–C); and *A. tamarense* AT-HK9301 produced C1–4 toxins, GTX1, 4, 5 and 6, no GTX2/3 amounts were detected (Fig. 3D). The Junk Bay strain of *A. tamarense* (AT-HKJB) did not produce any PST-like compounds. *A. minutum* AM-TK4 produced predominantly GTX-1 and GTX-4 with trace amounts of GTX-2 and GTX-3, while in the case of AM-KS2 they were GTX-2 and GTX-3 with a small amount of GTX-1 and GTX-4, but C toxins were not detected (Fig. 3E, F).

Table 1. Location and toxin compositions of *A. tamarense* and *A. minutum*

No.	Dinoflagellates	Location	Toxin compositions
1	*A. tamarense* AT-CI01	Daya Bay, PR China	C2 (over 90%), C1, GTX2, and 3
2	*A. tamarense* AT-HK9301	Dapeng Bay, PR China	C1–C4, GTX1, 4, 5, and 6
3	*A. tamarense* AT-Polar	Southern Ocean	C2 (Over 90%), C1, GTX2, and 3
4	*A. tamarense* AT-WHOI	USA	C2 (Over 90%), C1, GTX5, and STX
5	*A. tamarense* AT-HKJB	Junk Bay, PR China	Nil
6	*A. minutum* AM-TK4	Tung Kang, Taiwan	GTX1 and GTX4 (over 90%) GTX2 and GTX3
7	*A. minutum* AM-KS2	Kaohsiung, Taiwan	GTX2 and 3 (over 90%) GTX1 and GTX4

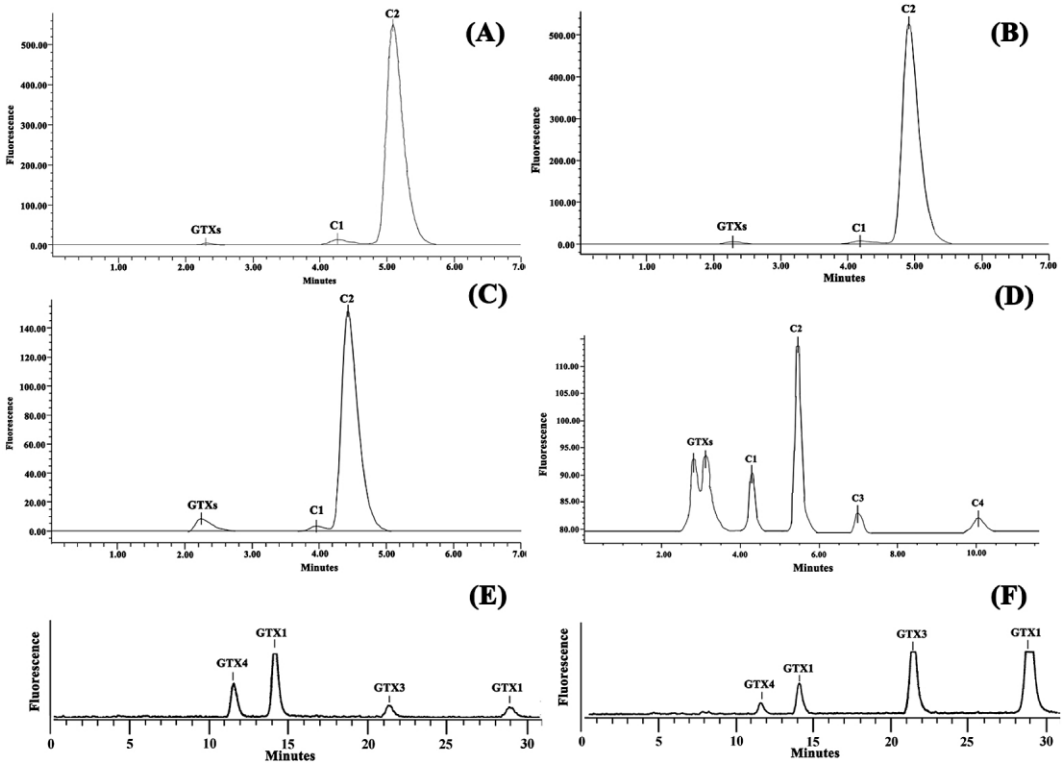

Figure 3. HPLC of paralytic shellfish poisoning (PSP) toxin profiles of: (A) *A. tamarense* AT-CI01, with C mobile phase; (B) *A. tamarense* AT-Polar, with C mobile phase; (C) *A. tamarense* AT-WHOI, with C mobile phase; (D) *A. tamarense* AT-HK9301, with C mobile phase; (E) *A. minutum* AM-TK4, with GTX mobile phase; (F) *A. minutum* AM-KS2, with GTX mobile phase.

2-DE analysis was performed using protein extracts from four toxic strains of *A. tamarense* (namely, AT-CIO1, AT-HK9301, AT-Polar, and AT-WHOI) as well as one nontoxic local strain of *A. tamarense* (AT-HKJB). In order to evaluate the correlation between toxin compositions and the expression pattern of marker proteins, we classified them into three distinct groups based on the suite of toxins produced by each isolate as revealed by HPLC analysis: the C1–C4 toxin-dominated group (AT-HK9301), the C1–C2 toxin-dominated group (AT-CIO1, AT-Polar and AT-WHOI) and the nontoxic group (AT-HKJB). Proteome reference maps were established for all these three groups (Fig. 4). In general, we found strong similarities in gel patterns of the arrayed proteins between the strains of the C1–C2 toxin-dominated group (AT-CIO1, AT-Polar, and AT-WHOI) (Fig. 4A–4C). 2-DE gels of different strains of the same group grown under the same conditions were superimposable. The proteome of the C1–C4 toxin-dominated group (AT-HK9301) (Fig. 4D) is slightly different from the C1–C2 toxin-dominated group. The distinct 2-DE protein patterns of toxic strains were readily discernable from those of the nontoxic strain (AT-HKJB) (Fig. 4E). The objective of this research was to characterize specific protein(s) from *A. tamarense* to evaluate its use as "taxonomic marker" or "toxin indicator." To identify such protein(s) we concentrated our efforts on characterizing proteins with a high level of expression in vegetative cells of *A. tamarense* and characteristic for toxic and nontoxic strains irrespective of the various growth conditions. The electrophoretic analysis of a total protein cell extract showed the presence of several abundant protein spots, AT-T1 (with p*I* 4.8 and an apparent M_r of 18.0 kDa) and AT-T2 (with p*I* 4.9 and an apparent M_r of 17.5 kDa), which were consistently found in all toxic strains of *A. tamarense* (Fig. 4A–D), while AT-T3 (with p*I* 4.6 and an apparent M_r of 20 kDa) was detected only in AT-CIO1, AT-Polar, and AT-WHOI strains of *A. tamarense* (Fig. 4A–C). All these proteins were absent in the nontoxic strain of *A. tamarense* (AT-HKJB) (Fig. 4E). On the other hand, a series of highly abundant protein spots of apparent M_r of 32 kDa, namely PCBP, were detected in all *Alexandrium* species tested and therefore its potential use as a "taxonomic marker" in the identification of *A. tamarense* is not feasible. Further comparison of the 2-DE protein profiles of toxic strains of *A. tamarense* with a toxic strain AM-TK4 of *A. minutum* (Fig. 4F) revealed that AT-T1 and AM-T1 were most similar, judged in terms of their relative position and shapes in gels. In order to determine whether these proteins were consistently expressed in high abundance in vegetative cells of *A. tamarense,* AT-Polar (toxic) and AT-HKJB (nontoxic) strains of *A. tamarense* were selected as representative strains for further investigation. One milligram of total protein of these two strains was separated by 2-D PAGE analysis followed by CBB R-250 staining (Fig. 5). Such analysis showed the presence of several predominant protein spots, AT-T1, AT-T2 and AT-T3, which were stained sharply on the CBB-stained 2-DE gel of the AT-Polar strain (Fig. 5A), whereas none of these proteins was found in the CBB-stained 2-DE gel of the AT-HKJB strain despite the large amount of protein loaded into the gel (Fig. 5B). Approximately 130 spots were detected in the CBB-stained 2-DE gel involving 1 mg of total protein from the AT-Polar strain of *A. tamarense* (Fig. 5A). The abundance of each spot was calculated as a percentage of the total density of all 130 spots measured on the CCB-stained gel. The relative abundance of AT-T1 was about 4.0% of the total quantified protein (*ca.* 40 μg per spot) and was the most abundant protein among the spots characteristic for toxic strains of *A. tamarense.* Since growth of *A. tamarense* is relatively slow and it is not possible to obtain high cell densities in culture, these abundant protein spots appeared as a good potential molecule to be used as a marker to study protein expression in this toxic dinoflagellate. All these proteins have the potential to serve as a "biomarker of toxicity" and thus were further investigated by a combination of MALDI-TOF MS, enzyme digestion, and Edman sequencing for internal sequences to determine whether species-specific characteristics could be found at the sequence level. The amino terminus of the PCBP proteins was also recovered from 2-D SDS-PAGE and processed for Edman sequencing.

3.2 Protein identification by MALDI-TOF MS and *N*-terminal amino acid sequencing by Edman degradation

About 14 prominent spots derived from the 2-DE analysis of the AT-Polar strain of *A. tamarense* (Fig. 5A) were selected for MS analysis after trypsin digestion. Bioinformatic searches using PMFs obtained from these spots against the NCBI nonredundant, Swiss-Prot, and TrEMBL databases revealed no similar protein in the database. We have consistently identified several abundant proteins, namely, AT-T1, AT-T2, and AT-T3, in the AT-Polar strain of *A. tamarense*. Their peptide maps were highly reproducible, and upon careful comparison, it was discovered that they produced identical PMFs in all the individuals analyzed (Fig. 6A–C). Tryptic digestion of 2-DE gel spots corresponding to AT-T1, AT-T2, and AT-T3 produced several peaks. The four masses that were shared among all spots were 1200.70, 1439.78, 1507.75, and 2615.50 *m/z*. Detailed comparison between AT-T1 in *A. tamarense* and its counterpart, AM-T1, in *A. minutum* (Fig. 6D) revealed that unambiguous and highly species-specific PMFs were generated from these proteins (Fig. 6), which are clearly distinguishable between these two different species. Tryptic digestion of 2-DE gel spots corresponding to AT-T1 and AM-T1 produced several peaks. One mass that was shared between these two spots was 2615.50 *m/z*. The three masses that differed among spots were 1200.70, 1439.78, and 1507.75 *m/z* produced by digestion of the AT-T1 spot, and 1259.00, 1493.98, and 2197.50 *m/z* produced by the digestion of AM-T1. Further investigation by *N*-terminal amino acid sequencing analysis suggested that these proteins are isoforms of the same proteins with exactly the same first 30 *N*-terminal amino acid sequences (Table 2). Bioin-

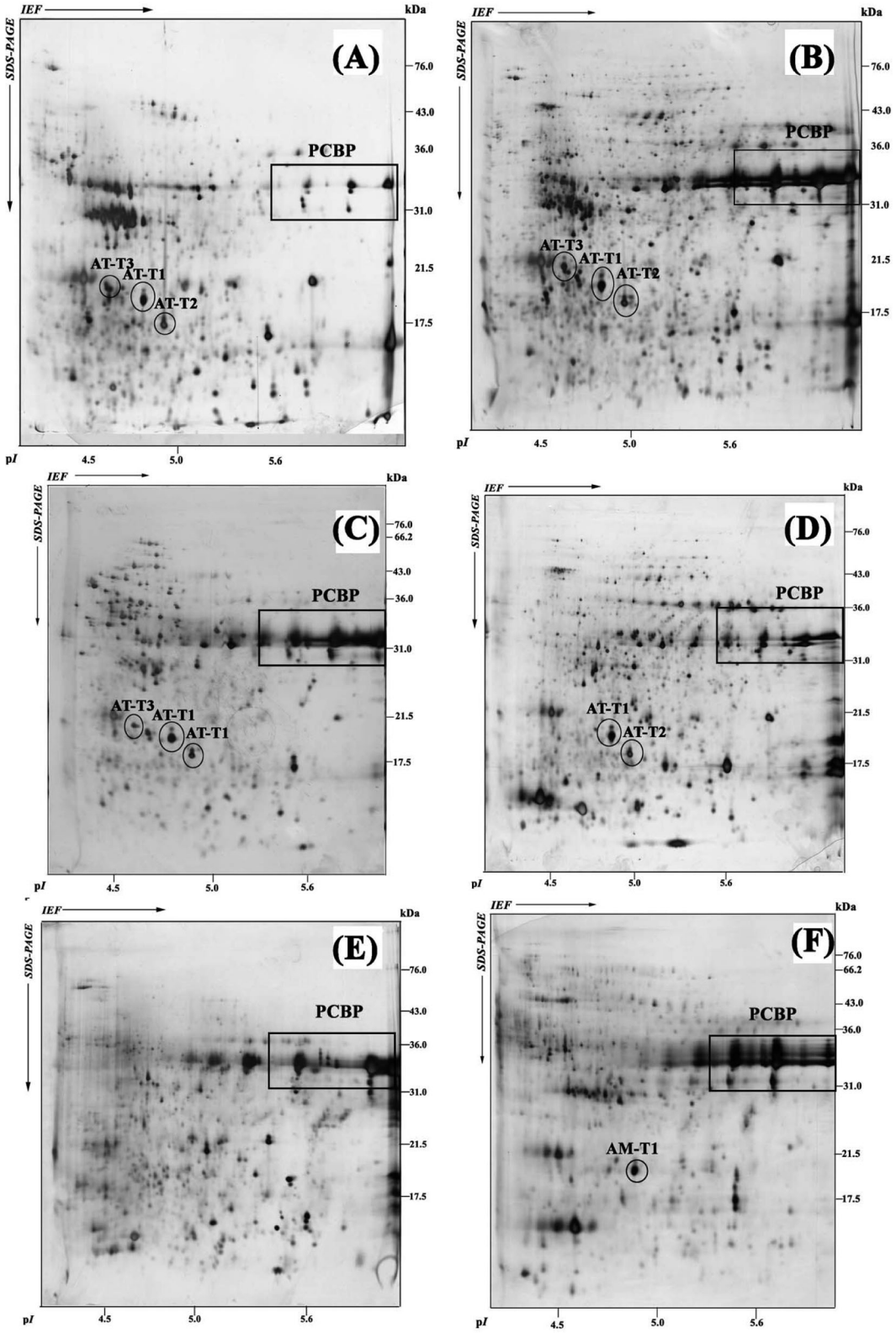

Figure 4. 2-DE protein profiles of 40 μg of soluble proteins extracted with 40 mM Tris base from: (A) *A. tamarense* AT-CI01; (B) *A. tamarense* AT-WHOI; (C) *A. tamarense* AT-Polar; (D) *A. tamarense* AT-HK9301; (E) *A. tamarense* AT-HKJB; (F) *A. minutum* AM-TK4.

Table 2. Comparison of amino acid sequences of proteins AT-T1, AT-T2, and AT-T3 from toxic strains of *A. tamarense* and AM-T1 from toxic strains of *A. minutum* by *N*-terminal sequence analysis

Proteins spots	*N*-terminal sequence	Matching protein in database
AT-T1 AT-T2 AT-T3 AM-T1	VSAEY LERLG PKDAD VPFTA APGGA EHPVT	No
PCBP	DEIGD AAKKL GDASY AFAKE VDWKN GIFLQ	Peridinin-chlorophyll a-binding protein precursor

formatic searches using partial amino acid sequences against PDB and Swiss-Prot by BLAST revealed no similar protein in the database to these novel proteins; however, PCBP was identified as peridinin-chlorophyll a-binding protein precursor, a dinoflagellate light-harvesting protein precursor.

3.3 Western blot analysis of different strains of *A. tamarense*

A murine polyclonal antibody was raised against the gel spot containing AT-T1 (*ca.* 40 μg per spot) excised from the 2-DE gel of different toxic strains of *A. tamarense* by the procedure described in Section 2.5. An antiserum dilution of 1:4000 was the lowest concentration that produced positive reaction with AT-T1 and AM-T1 from *A. tamarense* and *A. minutum*, respectively. The specificity of this murine anti-AT-T1 immune serum was tested against the total proteins of the AT-Polar strain of *A. tamarense*, which was separated by 2-D PAGE, by Western blot analysis. The murine anti-AT-T1 immune serum recognized all isoforms of AT-T1, *i.e.*, AT-T1, AT-T2, and AT-T3, in the resultant 2-DE map, showing no cross-reaction with other proteins (Fig. 7A). Furthermore, this murine anti-AT-T1 immune serum was used to detect the presence of T1 protein and/or its homologues in different strains of *A. tamarense* and *A. minutum*. Equal amounts of total proteins from four toxic strains and one nontoxic strain of *A. tamarense* as well as one toxic strain of *A. minutum* were resolved by 1-D SDS-PAGE on two separate gels. One gel was stained with CBB R-250 (Fig. 7B) and the other gel was electrotransferred onto a PVDF membrane and processed for Western blot analysis using the murine anti-AT-T1 immune serum (Fig. 7C). Different toxic strains of *A. tamarense* showed similar protein profiles in CBB-stained 1-D SDS-PAGE (Fig. 7B, lane 1 to lane 4). The protein profiles of the nontoxic strain of *A. tamarense* differed substantially (Fig. 7B, lane 5) from the toxic strains. although they shared some common protein bands. A strikingly different protein profile was found in the toxic strain of *A. minutum* when compared with *A. tamarense*. An abundant 32-kDa protein, namely PCBP, was found in all of these species (as indicated by an arrow in Fig. 7B). As shown in Fig. 7C, the murine anti-AT-T1 immune serum reacted with one band corresponding to a M_r of 18.0 kDa in all toxic strains of *A. tamarense* and 18.5 kDa in all toxic strains of *A. minutum* and one band corresponding to a M_r of 20 kDa in AT-CI01, AT-Polar, and AT-WHOI. No protein included in the total homogenate of AT-HKJB (nontoxic strain of *A. tamarense*) was recognized by murine anti-AT-T1 immune serum.

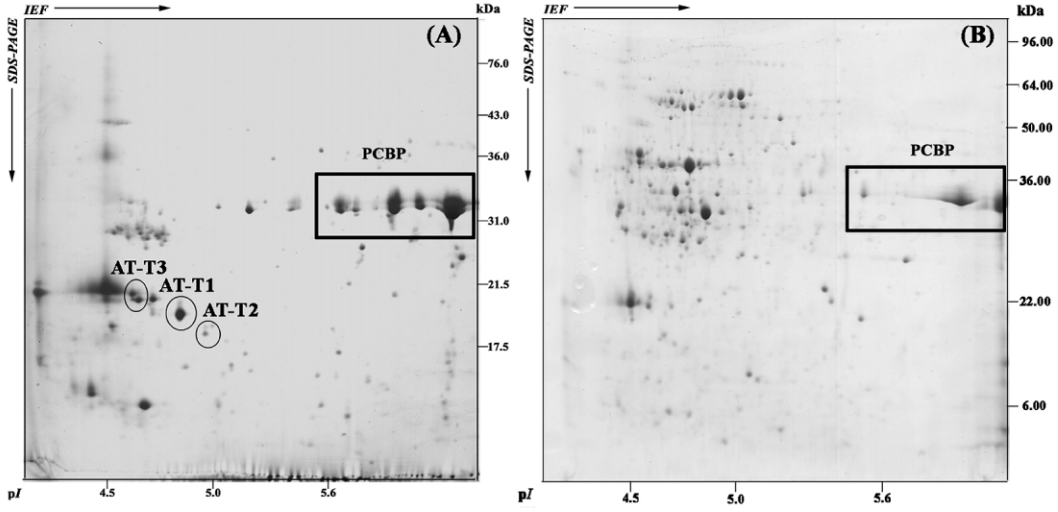

Figure 5. 2-DE protein profiles of 1.0 mg of soluble proteins extracted with 40 mM Tris base from: (A) *A. tamarense* AT-Polar; (B) *A. tamarense* AT-HKJB. The IEF of the first dimension was over a pH range of 4.0–7.0. The second dimension was a SDS-PAGE in a 12.5% polyacrylamide gel. The gels were stained with CBB R-250.

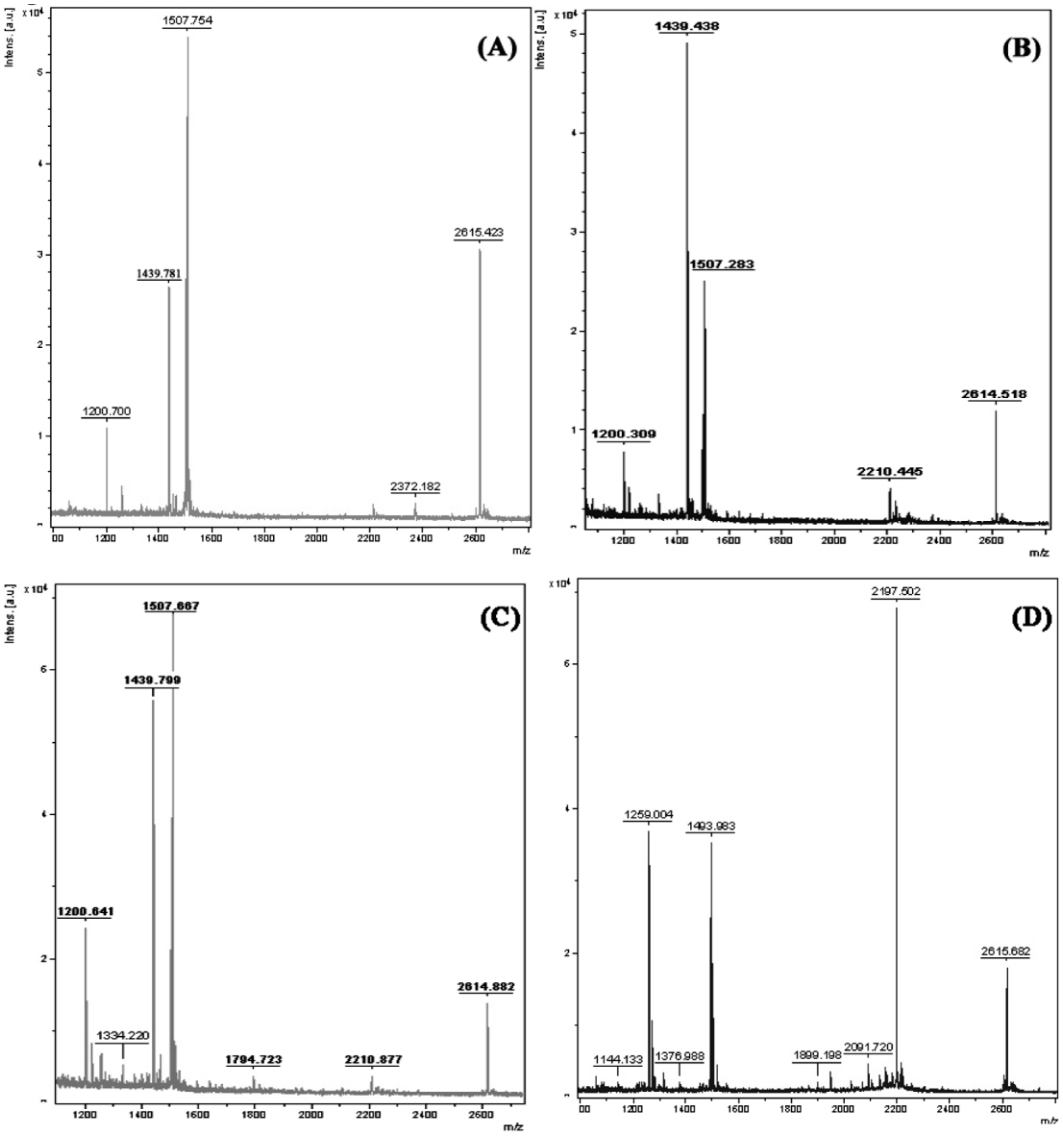

Figure 6. MALDI-TOF peptide mass map of the peptide mixture obtained from in-gel tryptic digestion of: (A) AT-T1; (B) AT-T2; (C) AT-T3 obtained from toxic strains of *A. tamarense* and (D) AM-T1 obtained from toxic strains of *A. minutum*.

4 Discussion

4.1 HPLC toxin composition profiles and 2-DE protein patterns of different strains of *A. tamarense* and *A. minutum*

This study examined several strains of *A. tamarense* from geographically diverse populations. The strains included PST- and non-PST-producing *A. tamarense*. It is commonly accepted that the total concentration of all toxins (toxin content) in one isolate of PST-producing dinoflagellates varies with growth conditions, but that the relative abundance of each toxin (toxin composition) does not change [20]. In the absence of an appropriate molecular technique, the toxin profile "fingerprint" may be used to distinguish different strains within *Alexandrium* species [21], and can be regarded as a potential taxonomic marker [22]. Similarly, the toxin profiles in the test *A. tamarense* strains were fairly consistent when cells were grown in optimal environmental and nutritional conditions, indicating that "toxin fingerprints" could be used for chemotaxonomic analysis. For example, the toxin profile of *A. tamarense* strains from Daya Bay of Southern China (AT-CI01) was similar to both the Southern Ocean strain (AT-Polar) and the USA strain (AT-WHOI) which produced mainly C1 and C2 toxins. These similarities in toxin profiles might result from dispersion of strains by ship bal-

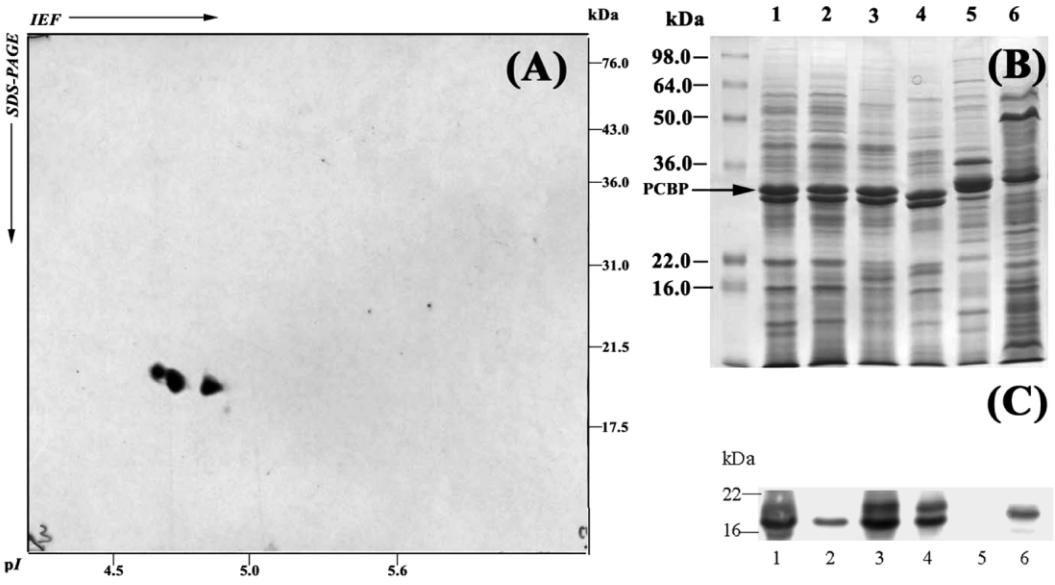

Figure 7. Detection of AT-T1 protein; 40 μg of soluble proteins from the AT-Polar strain of *A. tamarense* was separated by 2-D SDS-PAGE followed by Western blot analysis using the immune serum anti-AT-T1 (A). Total equal amount of protein (40 μg per well) of soluble proteins extracted from different strains of *A. tamarense* and *A. minutum* (Lane 1, AT-CI01; Lane 2, AT-HK9301; Lane 3, AT-Polar; Lane 4, AT-WHOI; Lane 5, AT-HKJB and Lane 6, AM-TK4) was separated by electrophoresis on two separate gels. One gel was stained with CBB R-250 (B). The other gel was electrotransferred onto a PVDF membrane and processed for Western blot analysis using the immune serum anti-AT-T1 (C).

last water from a common origin. On the other hand, C3 and C4 toxins, for the first time, were detected in the strain of *A. tamarense* AT-HK9301 [23].

This study clearly demonstrates that the 2-DE-based proteomic approach can detect a "biomarker of toxicity" in *A. tamarense* by comparing the 2-DE protein patterns of toxic and nontoxic strains. Proteome reference maps generated for various toxic strains of *A. tamarense* revealed that variations in 2-DE protein patterns among toxic strains with different toxin compositions and from different geographical locations were minimal. However, significant differences were noted in several abundant proteins, *e.g.*, AT-T1 to AT-T3, which were only found in toxic strains of *A. tamarense* (Fig. 4). As a result, morphologically similar toxic and nontoxic strains could be clearly distinguished by an examination of their characteristic 2-DE protein patterns. The proteome maps in Fig. 4 showed that AT-T1 and AT-T2 are the most dominant spots in the 2-DE protein pattern of all toxic strains of *A. tamarense*, and these toxic strains are clearly distinguishable irrespective of their actual toxin composition or geographical origins. AT-T3 was only present in AT-CI01, AT-Polar, and AT-WHOI strains of *A. tamarense* (although weakly expressed), but was not found in *A. tamarense* strain AT-HK9301. Whether the presence of this additional protein, AT-T3, accounts for the discrepancy in toxin compositions between the C1 and C2 toxin-dominated group (AT-CI01, AT-Polar, and AT-WHOI) and the C1–C4 toxin-dominated strain AT-HK9301 remains to be further investigated. It will be interesting to determine whether the various homologues of the marker proteins are specific to different toxin compositions by comparing other *A. tamarense* populations with differing toxin profiles. Although PCBP proteins appeared to be the most intensive protein spots in the 2-DE maps, they were found in all *A. tamarense* strains tested and their potential use in discriminating between toxic and nontoxic strains of *A. tamarense* is therefore not feasible.

Growth phases of *A. minutum* do not induce major changes in the expression of the "biomarker of toxicity", and they are not transient characteristics during the growth stages or a response of the cells to external stresses [20]. Results of the present study clearly demonstrated that AT-T1 and AT-T2 were diagnostic of toxic strains of *A. tamarense*, and that they are stable properties of the test algae. They therefore have the potential to serve as a "biomarker of toxicity". Although the expression of these marker proteins provides no information on their biogeography and toxin compositions, they have great potential to be developed into a commercial and practical tool for routine identification of toxic *A. tamarense* strains.

4.2 Protein identification by MALDI-TOF MS and *N*-terminal amino acid sequencing by Edman degradation

Three proteins, which are characteristic of toxic strains of *A. tamarense*, were further characterized by a combination of MALDI-TOF MS and *N*-terminal amino acid sequencing.

MS and *N*-terminal sequence analyses revealed that AT-T1, AT-T2, and AT-T3 shared identical PMFs (Fig. 6A–C) and N-terminal amino acid sequences (Table 2). The first 30 identical *N*-terminal amino acids between these three spots obtained by Edman degradation together with the spectra in Fig. 6 suggested that these proteins were likely to have resulted from expression of the same gene. Differences in their masses and p*I*s indicate that they had been proteolytically processed in a different manner, or post-translationally modified in other ways, *e.g.*, phosphorylated. These proteins had no polymorphic variations and were conserved among the strains analyzed. The process of modification in the toxic strains and their effects on toxin composition of the test algae are the subject of ongoing research. Apparently, the identical protein sequences of these marker proteins among different strains of *A. tamarense* indicate they are possibly a dispersed population from genetically similar sources [24].

Further comparison between AT-T1, AT-T2, and AT-T3 found in *A. tamarense* in the present study and AM-T1 in *A. minutum* in our previous work revealed that they shared similar amino acid sequences. However, the PMFs generated from these biomarkers of toxicity from different species were highly species-specific, discriminating between *A. tamarense* and *A. minutum*, and strain-specific, differentiating between toxic and nontoxic strains. This demonstrates that the amino acid compositions of these biomarkers of toxicity are significantly conserved across species boundaries, although some microheterogeneity (*i.e.*, amino acid substitution) existed in certain sequences within these two species. The conserved nature of these structural motifs in two or more proteins indicates their possible involvement in toxin biosynthetic or other metabolic and regulatory pathways in different PST-producing dinoflagellates. The conservation of these proteins is only able to maintain their functional integrity through evolutionary time as long as genetic drift and mutational forces are restricted to regions of the molecule other than functional motifs. So far, the direct precursors, chemical intermediates, specific enzymes, and the full biosynthetic pathway of STX synthesis and the metabolic role of STX in toxin-producing dinoflagellates have not been defined and resolved [32–34]. Although a few enzymes were found to be related to modifications of the final toxins [35–37], the exact number of enzymatic steps and sequence of the biosynthetic reactions is still uncertain. Our previous study [19] demonstrated the possibility that these marker proteins are mechanistically involved in toxin production. However, the exact role of these biomarkers of toxicity in STX production remains unresolved until they can be successfully cloned and their DNA sequences elucidated. The identification of these biomarkers of toxicity could play a pivotal role in identification of the genes involved in STX synthesis (*i.e.*, the "STX genes"). Although these biomarkers of toxicity are not completely characterized, this study provides an important first step in elucidating features that may serve as biomarkers of toxicity, but also for studies related to protein expression and cell physiology under bloom conditions, and also toxin synthesis mechanisms. All this information could be useful in the prediction and control of blooms of toxic species. The use of these highly conserved marker proteins as molecular probes could well alleviate problems arising from the failure of other molecular probes to operate in different geographical regions due to intraspecific genetic variations [25].

4.3 Western blot analysis of different strains of *A. tamarense*

The murine anti-AT-T1 immune serum reacted specifically with the AT-T1, AT-T2, and AT-T3 proteins and showed no discernible cross-reaction with other proteins in the 2-DE maps of the AT-Polar strain of *A. tamarense* (Fig. 7A). This suggests that AT-T1, AT-T2, and AT-T3 could possibly share similar glycosylation motifs and are isoforms of the same protein. Thus, the specificity of this polyclonal antibody is established. As shown in Fig. 7B, the intensity of the PCBP protein remained more or less constant in all *Alexandrium* species indicating that equal amounts of total protein (40 μg per well) of different species were separated by electrophoresis. This polyclonal antibody consistently exhibited specificity against all toxic strains of *A. tamarense* and *A. minutum* tested and showed no cross-reactivity with a nontoxic *A. tamarense* strain (Fig. 7C). A single protein band at 18.0 kDa (in *A. tamarense*) or 18.5 kDa (in *A. minutum*) had a strong reaction with this polyclonal antibody in all toxic strains. The abundant protein band observed at 18.0 kDa in *A. tamarense* (Fig. 7C) contains AT-T1 and AT-T2 of similar size but differing in their p*I*s as resolved by 2-DE PAGE (Fig. 7A). When comparing the protein bands of different toxic strains of *A. tamarense*, a band at roughly 20 kDa (corresponding to AT-T3 protein) was found only in the C1–C2 toxin-dominated groups (*i.e.*, AT-CI01, AT-Polar, and AT-WHOI), and not in the C1–C4 toxin-dominated group (AT-HK9301). This result seems to be in good agreement with the 2-DE proteome maps of these strains (Fig. 4).

In accordance with 1-D SDS-PAGE and 2-D PAGE analyses, AT-T1 appeared to be the most intensive protein spot or protein band among the spots characteristic of toxic strains. These data indicate that murine anti-AT-T1 immune serum is likely to be specific to both AT-T1 and AM-T1, and that the nontoxic strains of *A. tamarense* have no protein antigenically similar to AT-T1. These results further confirmed that T1 proteins and their homologues are highly conserved across species boundaries. A previous study showed that the expression pattern of the biomarker of toxicity (*i.e.*, AM-T1) typically varies as a function of total cellular toxicity in the test alga [19]. However, the dose–response relationship between the STX burden and the expression of these protein markers has yet to be established. Additional experiments are required to assess more thoroughly this relationship by Western blot analysis. Assuming that expression of these marker proteins is toxin dependent, immunoassays can be

developed to quantify protein markers that will directly reflect the STX load in dinoflagellates, and can also be used to study the biosynthetic pathway of these toxins.

Future work based on the present results will involve the use of mice with anti-serum with a strong reactivity and specificity to AT-T1 to develop a monoclonal antibody. Once cloned, the antibody will be used to develop a rapid ELISA assay to provide a qualitative (positive/negative) differentiation of toxic and nontoxic strains of *A. tamarense*. While this immunoassay will not provide the same information on toxin composition obtainable from instrumental analysis, it will represent a robust and reliable means of rapidly assessing PST-like toxicity in algal samples, and will reduce the overall biotoxin testing costs and the need for animal testing. Ultimately, monoclonal antibody probes for toxin detection and routine monitoring could be incorporated into automated devices [26] so as to provide real-time *in situ* monitoring of these toxic algal species to predict bloom development and movement. This study opens the door for more detailed immunological studies of the biomarkers of toxicity in other PST-producing dinoflagellates. The next logical step will be the validation of the significance of the antibody to recognize biomarkers of toxicity in other strains of the genus *Alexandrium* as well as other genera, *i.e.*, *Gymnodinium* and *Pyrodinium*.

In conclusion, in this work we present a simple, fast, and straightforward approach to identify species-specific protein markers by a combination of 2-DE, MALDI-TOF MS, *N*-terminal amino acid sequencing and Western blot analyses in species whose genome is incompletely characterized. The "biomarker of toxicity" found in this study is a stable property and is distinguishable irrespective of geographical location and toxin composition. The highly conserved nature of the biomarker suggests that toxigenesis in *A. tamarense* and *A. minutum* has a genetic basis. Differences in the PST analogue profile might result from PTM of these toxin-related proteins in response to environmental and nutritional stresses rather than as a genetic trait. These data are consistent with the results previously reported by Anderson *et al.* [27].

The results presented here represent, to our knowledge, the first demonstration of the use of polyclonal antibodies against marker proteins purified from 2-DE gels to distinguish between different strains and species of any PST-producing dinoflagellate in the genus *Alexandrium*. Whether this polyclonal antibody would recognize biomarkers of toxicity in other genera of PST-producing dinoflagellates remains to be determined. Taken together, these results reinforce the potential importance of proteomic-based methodology for the development of antibodies against proteins in association with nonproteinaceous, low-molecular-weight toxins of biological origin, whose chemical nature renders conventional approaches to antibody development difficult at best, and whose extreme *in vivo* toxicity prevents their use as immunogens.

The high-throughput and speed of analysis of the current models of MALDI-TOF MS may allow the extension of this kind of comparative study to hundreds and even thousands of proteins from a large number of individuals, resulting in the likely identification of protein markers. Given the fact that few proteins and DNA sequences of dinoflagellates are known, the choice of MALDI-TOF MS as an identification tool is not suitable. Lower-throughput MS techniques (*e.g.*, ESI-MS/MS) or *N*-terminal amino acid sequencing may be used for more detailed characterization. Potentially, immunoassays by lateral flow immunochromatography (LFI) and antibody probes for a range of toxic microalgae could be developed to detect the presence of toxic algae in routine monitoring programs as well as for research into, and monitoring of, HABs.

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