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Preliminary Study on Puffer Fish Proteome—Species Identification of Puffer Fish by Two-Dimensional Electrophoresis

TAI-YUAN CHEN,† CHYUAN-YUAN SHIAU,† CHENG-I WEI,‡ AND DENG-FWU HWANG*,†

Department of Food Science, National Taiwan Ocean University, Keelung 202, Taiwan, Republic of China, and College of Human Environmental Sciences, Oklahoma State University, Stillwater, Oklahoma 74078-6113

The aims of this work were to determine the differential characterization of the urea soluble protein components of puffer fish species and to establish a preliminary proteomic database using an immobilized pH gradient two-dimensional electrophoresis (2DE) technique. The puffer fish muscle proteins resolved into 171−260 spots in the 2DE gels, with a pI range of 3−10 and molecular mass range of 7.4−205.0 kDa, following Comassie blue staining. Puffer fish muscle proteins fell in the region with pI values of 3.5−7.0, and molecular masses of 7.4−45.0 kDa were well-resolved and were good for species comparison. The more acidic proteins of lower molecular masses showed species specific characteristics. Therefore, the species of puffer fish can be differentiated from the comparison of the characteristic 2DE protein patterns.

KEYWORDS: Two-dimensional electrophoresis; puffer fish; species identification; proteome; protein

INTRODUCTION

Species identification of seafood products is important for implementation of the labeling regulations as set by many countries (*1*). These regulations to prevent substitution of some commercially important fish can be effectively achieved when species specific data of all fish species become available.

The annual yield of Taiwanese puffer fish is about 3000−5000 tons, and most of the puffer fish species possess tetrodotoxin (TTX) in the viscera and/or other tissues (*2*). *Lagocephalus gloveri* is the most abundant and harmless puffer fish species that has been used for preparation of dried dressed fish fillet, a favorite Taiwanese traditional snack food. However, because of morphological similarities, the manufacturers may have difficulty in distinguishing *L. gloveri* from *Lagocephalus lunaris*, a species that accumulates a lethal level of TTX in its muscle (*3*). Therefore, serious food poisoning incidents due to ingestion of toxic puffer fish or toxic dried dressed fish fillets have occasionally occurred in Taiwan (*4*, *5*). From the viewpoint of food protection and public safety, the development of methodologies for effective identification of puffer fish species is therefore critically needed.

In the effort to prevent adulteration of fish species and to ensure consumers' rights, classical electrophoretic and immunological techniques such as sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (*6*, *7*), native or urea isoelectric focusing electrophoresis (IEF) (*7*−*9*), two-dimensional electrophoresis (2DE) (*10*, *11*), and enzyme-linked immunosorbent assay (ELISA) (*12*) have been successfully applied in identifying fish species. High-performance liquid chromatography (HPLC) (*13*) and capillary electrophoresis (*14*) of muscle proteins to differentiate fish species have also been employed for this purpose. The standard operation procedures (SOP) of urea IEF (*8*) and SDS−PAGE (*15*) have been optimized, respectively, for species identification of raw and heat-processed fish. Recently, many researchers applied these SOPs to successfully characterize processed fish species in their collaborative studies (*7*, *9*, *16*). DNA-based techniques, such as the restriction fragment length polymorphism (RFLP) (*17*), the single strand conformation polymorphism (*18*), and the sequence analysis of part of species specific gene (*19*−*21*), provided a superior discriminatory power for identification of processed fish products.

Besides the human genome, several other vertebrate genomes are being investigated to gain insight into their biology and also as models to interpret the functions of human genome. Among them, the Japanese puffer fish *Takifugu rubripes*, which has the smallest vertebrate genome but has a similar repertoire of human genes, is a good complement to genetic studies in other vertebrates (*22*, *23*). However, because genomic sequencing will not be able to define the activities of all physiologically active protein components, it is necessary to systematically identify the complete protein complements of the genome, the proteome, of the puffer fish genes. Two-dimensional electrophoresis has

* To whom correspondence should be addressed. Tel: +886-2-2462-2192. Fax: +886-2-2462-6602. E-mail: dfhwang@mail.ntou.edu.tw.
† National Taiwan Ocean University.
‡ Oklahoma State University.

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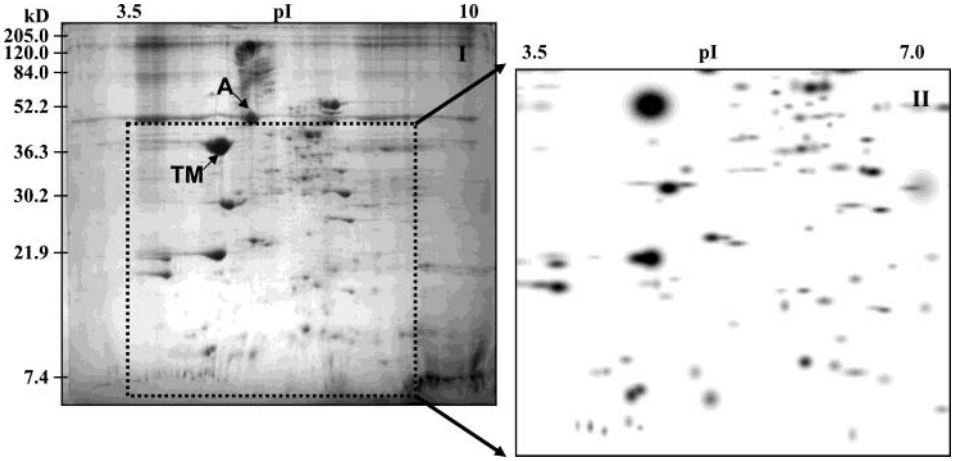

Figure 1. Two-dimensional electrophoresis pattern of the urea soluble puffer fish muscle proteins. (**I**) The whole 2DE pattern of *L. gloveri* was first separated by pH 3–10 IPG dry strip, followed by 15% separating/4% stacking SDS polyacrylamide gel. The marked proteins were tentatively identified as actin (A) and tropomyosin (TM) by comparison to the results found in salmon (*27*), sea bass (*28*), and cod (*29*). (**II**) The computer-synthesized partial proteome of *L. gloveri* with protein spots distributed in the pI range of 3.5–7.0 and molecular mass range of 7.4–45.0 kDa.

been successfully applied in many targeted organisms; however, studies concentrated on marine species are still limited (*24*). The 2DE patterns of human and mouse tissues, yeast, and bacteria can be easily accessed from the databases, but almost no fish reference patterns could be found (*25*). Reports concentrated on postmortem changes in shrimp (*26*), salmon (*27*), sea bass (*28*), and cod (*29*) were available. Species identification of marine species consisting of hake (*11*) and mussel (*24*) has also been reported.

The objectives of this work were to achieve the differential characterization of the urea soluble protein components of puffer fish species and to establish a preliminary proteomic study to reflect puffer fish genome using immobilized pH gradient (IPG)-2DE technique.

MATERIALS AND METHODS

Fish Materials. Authentic samples of five puffer fish species, *Lagocephalus wheeleri*, *L. gloveri*, *L. lunaris*, *Lagocephalus inermis*, and *Lagocephalus sceleratus*, were caught at the coast of Taipei and Keelung Counties, northern Taiwan, R.O.C. Three specimens of each species were used. They were shipped in ice during the 2−3 h of delivery to the laboratory. The specimens were kept frozen until analysis. Except for the species of *L. lunaris*, the muscles of the other species are nontoxic (*3*). Authentic puffer fish species were identified by a marine biology expert, Dr. Kwang-Tsao Shao of the Institute of Zoology, Academia Sinica, Taipei, Taiwan, as determined by their external anatomical characteristics and morphological appearances.

Protein Extraction and Determination. Extracts of urea soluble fish proteins were prepared according to the previously published procedures (*6*). The protein concentration in the extract was determined by Coomassie protein assay following the instructions provided by the manufacturer (Pierce Co., Rockford, U.S.A.) and measured at 595 nm.

Two-Dimensional Electrophoresis. IPG-IEF electrophoresis was carried out at 20 °C using the Protean IEF Cell (Bio-Rad, Richmond, CA) with Bio-Rad Ready Strip IPG strips. The 17 cm long strips with a linear pH gradient of 3−10 were rehydrated overnight at 50 V in a solution consisting of sample protein extracts (600 μg) in rehydration buffer supplemented with 8 M urea, 4% CHAPS, 50 mM DTT, 0.2% pH 3−10 carrier ampholytes (Bio-Rad), and bromophenol blue. IEF was performed in a gradient mode at 250 V for 30 min, followed by a gradual increase to 10 000 V for 3 h, and kept at this voltage until reaching 60 000 V hours (Vh). The current was limited to 0.05 mA per IPG strip. IPG strips were kept frozen at −80 °C immediately after focusing. Before SDS−PAGE analysis, the gel strips were equilibrated for 10 min in a reducing buffer of 130 mM DTT, 6 M urea, 2% SDS, and 20% glycerol in 0.375 M Tris-HCl (pH 8.8), followed by another 10 min in an alkylating buffer of 135 mM iodoacetamide, 6 M urea, 2% SDS, and 20% glycerol in 50 mM Tris-HCl (pH 8.8). The equilibrated strips were placed onto the SDS−PAGE apparatus and sealed with 0.5% agarose in Laemmli running buffer (*30*). Discontinuous SDS−PAGE was performed in a Protean 2D slab plate (Bio-Rad) using 15% separating/4% stacking polyacrylamide gels. Electrophoresis was performed at 16 °C at a maximum current of 50 mA per gel. Gels were stained with Coomassie blue using the PhastGel Blue (Pharmacia Biotech, Uppsala, Sweden).

Table 1. Matching of Protein Spots between *L. gloveri* and Four Other Puffer Fish Species

fish species	total spots[a]	match spots[b]	percentage (%)[c]	match rate (%)[d]
L. gloveri	260	260	100	100
L. wheeleri	235	77	32.8	29.6
L. lunaris	225	77	34.2	29.6
L. inermis	228	75	32.9	28.8
L. sceleratus	171	54	31.6	20.8

[a] Total protein spots identified on the IPG-2DE gel for each puffer fish species. [b] The number of protein spots of a member puffer fish species that are also detected on the IPG-2DE gel of *L. gloveri* (master species). [c] Percentage is the ratio of the number of matched protein spots to the total protein spots of the specific puffer fish species. [d] Match rate is the ratio of the number of matched protein spots to the 260 total protein spots of the master species *L. gloveri*.

Image Analysis. The gel images were acquired by the Image Master system (Pharmacia Biotech) and analyzed by the Discovery Series PDQuest 2D Analysis Software Version 7.1.1 (Bio-Rad). After automated spot detection, the computer-synthesized images were edited manually (adding, splitting, and removing of spots). The spot positions were manually defined for about 20 anchor spots followed by automated matching of the remaining protein spots. Gel matching was then generated. The gel matches as suggested by automated image analysis were finally individually inspected and confirmed. Determinations of experimental isoelectric points (pI) and molecular masses (Mw) of the protein spots of interest were achieved by comparison of a ready-to-use 2D SDS−PAGE standards mixture (Bio-Rad) with a sample of the fish muscle extract. The molecular masses of the protein spots were reconfirmed from coelectrophoresis with SDS−PAGE molecular mass protein standards (Bio-Rad).

RESULTS AND DISCUSSION

As shown in **Figure 1** and **Table 1**, the urea soluble proteins of puffer fish resolved into 171−260 spots in the 2DE gels with a pI range of 3−10 and a molecular mass range of 7.4−205.0

kDa. Except for proteins of higher molecular masses, the resolution of most protein spots and reproducibility of their patterns on the gels were reasonably achieved. Protein spots of lower pI values and higher molecular masses became blurred and stayed together. The proteins of high molecular masses were difficult to be introduced into the IPG strips (*31*). Therefore, it brought about poor resolution of protein spots and reproducibility of the patterns in the higher molecular mass region. Recent proteomic studies on postmortem changes in salmon (*27*), sea bass (*28*), and cod (*29*) also demonstrated similar difficulties in separating proteins of higher molecular masses. Therefore, in this study, we used the partial proteome that showed optimal resolution of protein spots for protein pattern comparisons and protein spot matches from different puffer fish species. Most of the better resolved protein spots in the partial proteomes of the puffer fish had pI values of 3.5−7.0 and molecular masses of 7.4−45.0 kDa (**Figures 1** and **2**). Consequently, these puffer fish proteins found in the partial proteome are neutral/acidic in nature and had lower molecular masses. Similar observations were also noted with the 2DE patterns of sea bass (*28*) and cod (*29*).

The resolved protein spots of the five puffer fish species on the 2DE gels (**Table 1**) were compared for matches using Bio-Rad Discovery Series PDQuest System. *L. gloveri*, the more abundant and harmless puffer fish species in Taiwan, was chosen as the master species, and the other tested puffer fish served as member species. **Table 1** also shows the number of member species protein spots that were also present in the master species, *L. gloveri*. In addition, the ratios of the matched spots of a specific member species to its total protein spots or the total spots of the master species are shown. About 31.6−34.2% of the member species protein spots were also found in the 2DE gel of *L. gloveri*. Thus, the match rate of the protein spots for the four member species (*L. wheeleri*, *L. lunaris*, *L. inermis*, and *L. sceleratus*) to those of *L. gloveri* ranged from 20.8 to 29.6% (**Table 1**). When *L. wheeleri* was used as the master species for comparison with *L. inermis*, the match rate for the protein spots was determined to be above 40%, while the match rate between *L. wheeleri* and *L. lunaris* or *L. sceleratus* was lower than 30% (data not shown).

Examination of the partial proteomes of the five puffer fish showed that species specific proteins were acidic proteins of low molecular masses (**Figure 2**). Protein spots present in regions outside the pI range of 3.5−7.0 and molecular mass range of 7.4−45.0 kDa were not compared. The species specific protein spots with the estimated isoelectric points and molecular masses for each puffer fish are listed in **Table 2**. *L. gloveri* displayed five protein spots in a characteristic pattern unlike those of the other puffer fish species (**Figure 2A1**). Four (nos. 1−4) of these five protein spots were species specific for *L. gloveri*. Protein spot no. 5 (pI 5.2/22.4 kDa) was also detected in *L. wheeleri*, *L. lunaris*, and *L. sceleratus* (**Table 2**). Eight major protein spots were present in the partial proteome of *L. wheeleri* (**Figure 2B1**). Only two [no. 11 (pI 5.4/22.3 kDa) and no. 12 (pI 5.5/22.8 kDa)] of them were species specific for *L. wheeleri*. Some of the other six protein spots also existed in the partial proteomes of the other puffer fish species. For example, protein no. 10 (pI 5.0/22.4 kDa) was also detected in *L. lunaris*, *L. inermis*, and *L. sceleratus* (**Table 2**). Proteins nos. 6 and 7 (pI 3.8/19.4 kDa and pI 3.9/15.4 kDa) in *L. wheeleri* were also found in *L. lunaris* and *L. inermis* (**Table 2**). Similarly, *L. lunaris* had six unique protein spots in the partial proteome but with only protein spot no. 14 (pI 4.4/19.7 kDa) as its species specific protein (**Figure 2C1** and **Table 2**). Protein spot no. 13 (pI 4.3/22.4 kDa) was also present in *L. sceleratus*. Three species specific protein spots were each detected in *L. inermis* (nos. 15−17; **Figure 2D1** and **Table 2**) and *L. sceleratus* (nos. 18−20; **Figure 2E1** and **Table 2**). The existence of protein spots nos. 6−10 in both *L. wheeleri* and *L. inermis* may account for the greater than 40% match rate of proteins between these two species. Using the species specific proteins found here, monoclonal antibodies against these unique proteins of *L. lunaris* or other puffer fish species could be developed for incorporation in the rapid ELISA assays for practical use to protect the health of consumers.

These species specific proteins of puffer fish could belong to the group of proteins called parvalbumins. Parvalbumins are polymorphic, low molecular mass, calcium-binding acidic proteins. They have been considered as fish biomarkers because these proteins are particularly abundant in the white fast-contracting muscles of most fishes, where they may promote muscle relaxation (*32*). Isotypes of this group of proteins of trout and sea bass were found to link to their age of development (*33*). Because of the heat resistant nature of parvalbumins, they have been successively prepared as marker proteins for IEF (*34*) and in identifying fish products (*10*, *11*).

The major puffer fish protein spot on the 2DE gels appeared to be tropomyosin (pI 4.6/36.8−37.3 kDa) when compared to sea bass proteome (*28*). In this study, the puffer fish tropomyosin seemed to exist as a specific doublet spot, similar to the doublet tropomyosin band reported in squid (*35*). The tropomyosin of salmon (*27*), sea bass (*28*), and cod (*29*) appeared as one spot on the 2DE gels. However, sometimes, it is difficult to identify whether the salmon tropomyosin spot was a single spot or consisted of two very closely related polypeptides (*27*). Actin (pI 5.0/46.1 kDa) also appeared as another main spot on the puffer fish 2DE gels. The actin of salmon (pI 4.96−5.38/44.5 kDa) (*27*) and sea bass (pI 5.08−5.46/46 kDa) (*28*) existed as several spots on the 2DE gels, but the cod actin (*29*) only existed as one spot. Six of the nine puffer fish (*T. rubripes*) actin genes were muscle type genes, and two of them were expressed in skeletal muscle (*36*). The other primary protein spots detected in the 2DE gels of all five puffer fish species are an acidic protein (pI 4.7/28.2 kDa) and a neutral protein (pI 6.2/54.7 kDa).

Our previous study using native IEF (N-IEF) electrophoresis showed that the majority of puffer fish sarcoplasmic proteins had pI values of 5.85−8.65 with species specific protein bands that existed in regions of pI 3.50−5.20, pI 5.85−6.55, and pI 7.35−8.15 (*37*). The N-IEF seemed to separate the more alkaline proteins better than the acidic proteins. On the contrary, the IPG-2DE resolved the acidic proteins better than the N-IEF. Although proteins of higher molecular masses are better resolved by SDS−PAGE (*15*), this technique does not adequately improve species identification efficiency for puffer fish because the molecular masses of the species specific muscle proteins are less than 30 kDa (*6*). This limitation of application with the SDS−PAGE for species identification was overcome when using the IPG-2DE. As shown in this study, the species specific proteins of lower molecular masses were well-resolved on the IPG-2DE gels, making species differentiation of puffer fish possible. Furthermore, the IPG-2DE could be coupled with mass spectrometry for improved efficiency in species identification. Therefore, although the IPG-2DE technique is more complicated and expensive than one-dimensional electrophoresis, it is widely utilized for analysis of whole tissue proteins (*38*). We have also discovered cytochrome *b* gene as a valuable tool for identification of puffer fish species in the market (*19*). The combined

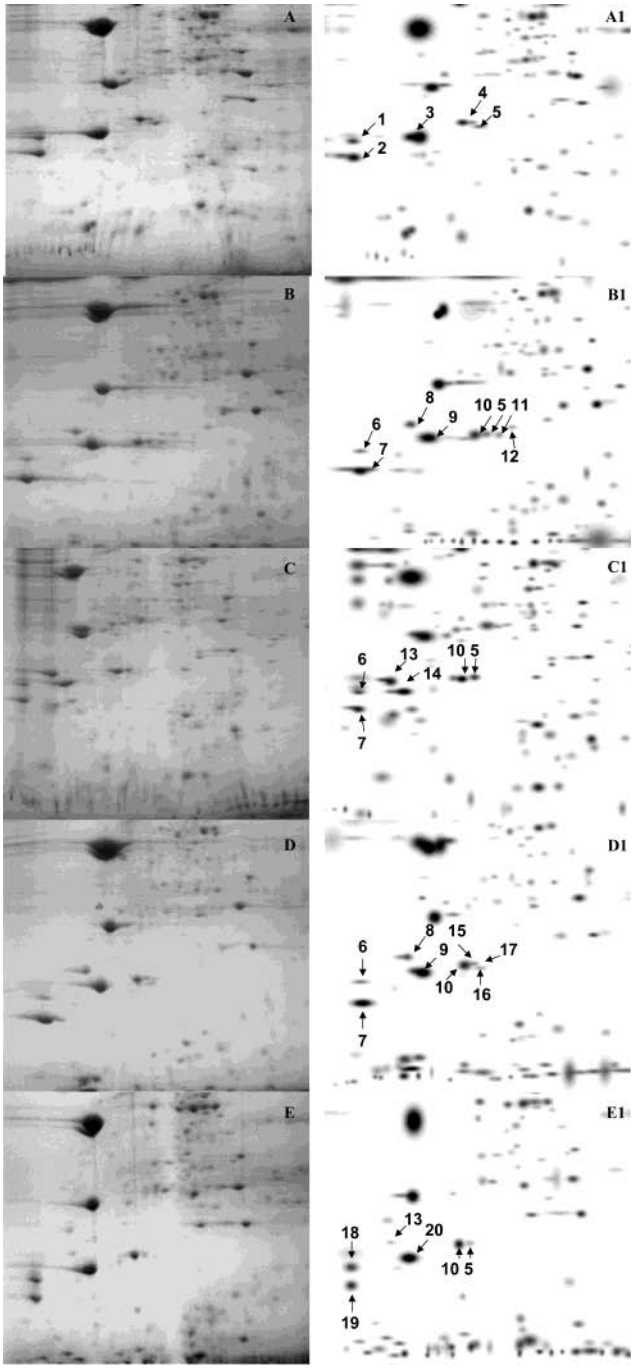

Figure 2. Partial original 2DE protein patterns (**A**–**E**) and their relevant computer-synthesized patterns (**A1**–**E1**) of all five puffer fish species: (**A**) *L. gloveri*, (**B**) *L. wheeleri*, (**C**) *L. lunaris*, (**D**) *L. inermis*, and (**E**) *L. sceleratus*.

use of gene sequence with RFLP has successfully identified puffer fish egg product (*39*).

The Fugu genome has been characterized as an invaluable compact model for vertebrate genomes (*40*). The whole genome of *Fugu rubripes* was identified as 365 megabase (*41*). The authors also highlighted that three-quarters of the predicted human proteins have strong matches to fugu proteins (*41*), implying that there exists an interesting linkage between human and fugu proteomes. Proteome analysis, defined as the investigation of the protein content expressed by a genome, focuses on elucidation of the expressions and turnovers of cellular proteins (*38*). However, because fish genomes are still poorly characterized, this renders difficulty for protein identification.

Table 2. Species Specific Proteins Found in the Partial Proteomes of the Five Puffer Fish Species

fish species	protein spots[a] (pI/Mw)			
L. gloveri	1 (3.7/18.5)[b]	2 (3.7/14.9)[b]	3 (4.4/19.3)[b]	4 (4.9/22.9)[b]
	5 (5.2/22.4)			
L. wheeleri	5 (5.2/22.4)	6 (3.8/19.4)	7 (3.9/15.4)	8 (4.4/23.5)
	9 (4.6/22.2)	10 (5.0/22.4)	11 (5.4/22.3)[b]	12 (5.5/22.8)[b]
L. lunaris	5 (5.2/22.4)	6 (3.8/19.4)	7 (3.9/15.4)	10 (5.0/22.4)
	13 (4.3/22.4)	14 (4.4/19.7)[b]		
L. inermis	6 (3.8/19.4)	7 (3.9/15.4)	8 (4.4/23.5)	9 (4.6/22.2)
	10 (5.0/22.4)	15 (5.1/22.4)[b]	16 (5.2/22.3)[b]	17 (5.3/22.4)[b]
L. sceleratus	5 (5.2/22.4)	10 (5.0/22.4)	13 (4.3/22.4)	18 (3.8/16.2)[b]
	19 (3.8/14.2)[b]	20 (4.6/19.6)[b]		

[a] The protein spots with the isoelectric points (pI) and molecular masses (kDa) for each puffer fish species match those shown in **Figure 2**. [b] Species specific protein spots for each puffer fish species.

A total of 132 675 sequences are characterized in the Swissport database, and 935 of these are fish sequences, of which 74 are puffer fish sequences (http://us.expasy.org/). In the 940 641 TrEMBL sequences, 6574 sequences belong to fish and 570 of these are puffer fish sequences (http://us.expasy.org/). The puffer fish genome sequences together with related information are available at http://fugu.hgmp.mrc.ac.uk, http://www.fugu-sg.org, and http://www.jgi.doe.gov/fugu. On the contrary, no marine species could be found as the target organism from the WORLD-2DPAGE database (http://us.expasy.org/ch2d/2d-index.html). In the Swiss-2DPAGE database, there are 406 protein data for human, 233 proteins for mouse, 157 proteins for *Escherichia coli*, but none for fish (http://us.expasy.org/ch2d/).

This study shows that IPG-2DE is a practical and powerful tool for creating two-dimensional protein profiles for puffer fish identification and to minimize foodborne intoxication by TTX. As with hake (*11*) and mussel (*24*) species, the use of IPG-2DE can lead to development of amino acid sequences and mass spectrometric profiles of species specific proteins for more efficient identification of puffer fish. The results of this study will also contribute to the establishment of fugu proteome database for linking the functional proteins to fugu genome.

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