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Identification of puffer fish species by native isoelectric focusing technique

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Abstract

This study was designed to validate the feasibility of using native isoelectric focusing electrophoresis (IEF) of water-soluble sarcoplasmic proteins in identifying puffer fish species. Each of the six tested species of puffer fish *Lagocephalus wheeleri*, *L. gloveri*, *L. lunaris*, *L. inermis*, *L. sceleratus*, and *Takifugu oblongus* showed species-specific protein band profiles after the focused gel was treated with Coomassie blue or silver stain. The majority of water-soluble puffer fish muscle proteins fell in the region with isoelectric point (pI) values of 5.85–8.65. Characteristic species-specific protein bands were present in all the three regions of pI 3.50–5.20, pI 5.85-6.55, and pI 7.35–8.15. Therefore, the species of puffer fish can be differentiated from the comparison of the characteristic native IEF protein band profiles.
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Keywords: Native IEF; Puffer fish; Species identification; Electrophoresis; Protein

1. Introduction

Species identification of seafood products is important for the implementation of the labeling regulations as set by many countries (Mermelstern, 1993). These regulations to prevent the substitution of some commercially important fish can be effectively achieved when species-specific data of all fish species are becoming available. Protein electrophoresis (Etienne et al., 1999, 2001; Pineiro et al., 1999) and molecular biological methods (Bossier, 1999; Mackie et al., 1999; Hold et al., 2001) are the two valuable methodologies that can be used for fish species identification.

Puffer fish, especially those of the Tetraodon order, are used in Taiwan to prepare for both the homemade food and the dried dressed fish fillet which is a favorite Taiwanese traditional snack food. *Lagocephalus gloveri* is the nontoxic puffer fish whose muscle contains no tetrodotoxin (TTX); it is therefore widely used for the preparation of the dried dressed fish fillets. However, the muscles of *L. lunaris* and *Takifugu oblongus* accumulate lethal levels of TTX (Hwang, Kao, Yang, Jeng, Noguchi, & Hashimoto, 1992). It is because of morphological similarities between *L. lunaris* and *L. gloveri* that both the manufacturers and consumers would identify the species ambiguously. Additionally, *T. oblongus*, one of the toxic puffer fish in Taiwan, is often abused as the material for the preparation of the dried dressed fish fillet. Therefore, serious food poisoning incidents due to ingestion of toxic puffer fish or toxic dried dressed fish fillets have occasionally occurred in Taiwan (Hwang, Cheng, Tsai, & Jeng, 1995; Hwang, Hsieh, Shiu, Chen, & Cheng, 2002). 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 an effort to prevent adulteration of fish species and to ensure consumers' rights, classic electrophoretic and immunological techniques, such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Chen & Hwang, 2002; Etienne et al., 2001), native or urea-isoelectric focusing electrophoresis (IEF) (Etienne et al., 1999, 2001; Mackie et al., 2000), two-dimensional electrophoresis (2-DE) (Pineiro, Barros-Velazquez,

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Sotelo, Perez-Martin, & Gallardo, 1998), and enzyme linked immunosorbent assay (ELISA) (Huang, Marshall, Kao, Otwell, & Wei, 1995), have been successfully applied in identifying fish species. High-performance liquid chromatography (HPLC) (Armstrong, Leach, & Wyllie, 1992), and capillary electrophoresis (CE) (Gallardo, Sotelo, Pineiro, & Perez-Martin, 1995) of muscle proteins to differentiate fish species have also been achieved. Pineiro et al. (1999) and Etienne et al. (1999) optimized the standard operation procedures (SOP) of SDS–PAGE and urea-IEF, respectively, for the identification of the species of raw and heat-processed fish. Recently, many researchers applied these SOPs to successfully characterize the processed fish species in their collaborative studies (Etienne et al., 2000, 2001; Mackie et al., 2000). The DNA-based techniques, such as the restriction fragment length polymorphism (RFLP) (Hold et al., 2001), the single strand conformation polymorphism (SSCP) (Mackie et al., 1999), and the sequence analysis of part of the species-specific gene (Chen, Hsieh, Tsai, Shiau, & Hwang, 2002; Cheng, Hsieh, Noguchi, Arakawa, & Hwang, 2001; Hsieh, Shiu, Cheng, Chen, & Hwang, 2002) provided a superior discriminatory power for the identification of processed fish products.

IEF electrophoresis, which separates proteins based on their differences in charges, is an excellent tool for the determination of protein isoelectric point (pI) and for identification of the species of raw and processed fish (Bossier & Cooreman, 2000; Etienne et al., 2001; Mackie et al., 2000). This protein electrophoresis technology is still cheaper and less sophisticated to operate than the DNA-based techniques (Pineiro et al., 1999). The aim of this study was to identify puffer fish species through the determination of the native isoelectric point characteristics of the muscle proteins.

2. Materials and methods

2.1. Fish material

Authentic samples of six puffer fish species, *Lagocephalus wheeleri* (LW), *L. gloveri* (LG), *L. lunaris* (LL), *L. inermis* (LI), *L. sceleratus* (LS), and *Takifugu oblongus* (TO), were purchased from the seafood markets in Ilan (eastern Taiwan) and Tainan (southern Taiwan). Each species was represented by at least six fish. All specimens were stored at −20 °C until use. Except for the species of LL and TO, the muscles of the other species are nontoxic (Hwang et al., 1992).

2.2. Protein extraction and determination

Extracts of fish sarcoplasmic proteins were prepared according to the previously published procedures except that double distilled water was used to replace phosphate buffer for protein extraction (Chen & Hwang, 2002). The protein concentration was determined by Coomassie protein assay following the instructions provided by the manufacturer (Pierce Co., Rockford, USA) and detected at 595 nm. The protein extracts were adjusted with double distilled water to 2 mg/ml for Coomassie blue staining and 0.3 mg/ml for silver staining.

2.3. Isoelectric focusing and protein staining

Native isoelectric focusing (IEF) of the puffer fish proteins was conducted by using Ampholine PAG plates (pH range 3.5–9.5, Pharmacia Biotech, Uppsala, Sweden) in connection with Multiphor electrophoresis equipment (Pharmacia Biotech) and a thermostatic circulator. The electrophoresis conditions of Bossier and Cooreman (2000) were modified. The gel support plate was set at 15 °C, and the gel was placed on the cooling plate. IEF electrode strips were soaked in 1 M H_3PO_4 for anode and 1 M NaOH for cathode, and the soaked electrode strips were then applied on the gel at the respective sides. Ten microliters of protein standard (Broad pI kit, pH 3–10, Pharmacia Biotech) was applied at 60 mm from the cathode using sample application pieces (Pharmacia Biotech). The standard is consisted of amyloglucosidase (pI 3.50), methyl red (pI 3.75), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin–acidic band (pI 6.85), horse myoglobin–basic band (pI 7.35), lentil lectin–acidic band (pI 8.15), lentil lectin–middle band (pI 8.45), lentil lectin–basic band (pI 8.65) and trypsinogen (pI 9.30). Fish protein samples, whose amounts were approximately 15 and 3 μg for Coomassie blue staining and silver staining, respectively, were placed at 10 mm from the cathode using sample application strip. Electrophoresis conditions were 1000 V, 50 mA, 30 W, and 150 min. Sixty minutes after the initiation of electrophoresis, the application pieces and strip were removed to avoid any smearing of proteins on the gel. Following electrophoresis, the proteins were fixed and stained using the PhastGel Blue R and Silver Staining Kit (Pharmacia Biotech), respectively. The gel was soaked in the preserving solution for 30 min, then covered with a cellophane preserving sheet, and dried at room temperature. The gels were scanned and the acquired images were analyzed by the Image Master system (Pharmacia Biotech).

3. Results and discussion

3.1. Protein concentration of the extracts

The concentrations of water-soluble protein from puffer fish muscles ranged from 5.9 to 10.4 mg/ml,

which are similar to the previously reported data (Chen & Hwang, 2002). Thus the protein extractability by distilled water and low ionic strength phosphate buffer was nearly identical. Although urea and SDS solutions seemed to extract more proteins than distilled water or phosphate buffer did (Chen & Hwang, 2002), some reports indicated that there appeared no apparent difference in protein contents between water and urea extracts (Etienne et al., 2001; Rehbein et al., 1995).

3.2. Native IEF of water-soluble proteins following Coomassie blue staining

The native IEF patterns of the puffer fish sarcoplasmic proteins as shown in Fig. 1 exhibited many species-specific bands over a wide range of pI values of 3.50–9.50, with the pI 5.85–8.65 region containing the most abundant numbers of bands. These species-specific bands were clustered into three regions of pI 3.50–5.20, pI 5.85–6.55, and pI 7.35–8.15. Table 1 listed the characteristic bands in the three clustered regions for each of the six puffer species. The species-specific bands at the acidic region of pI 3.50–5.20 were pI 4.18 and 4.90 for *Lagocephalus wheeleri* (LW); pI 5.02 for *L. gloveri* (LG); pI 5.09 for *L. lunaris* (LL); pI 4.20 and 4.50 for *L. inermis* (LI); pI 4.13 for *Takifugu oblongus* (TO); and pI 4.59 for *L. sceleratus* (LS). At the region of pI 7.35–8.15, the species-specific protein bands were pI 7.47, 7.63, and 7.80 for LW; pI 7.38, 7.47, 7.63, and 7.82 for LG; pI 7.69, 7.86, and 7.97 for LL; pI 7.50, 7.77, and 7.99 for LI; pI 7.77 and 7.99 for TO; and pI 7.44, 7.51, and 7.68 for LS. Many of the numerous protein bands in the third region of pI 5.85–6.55 also provided good differential characteristics for species identification. Therefore, the Coomassie blue stained native IEF gels, which contain many sharp and species-specific protein bands, can be used as a valuable tool for puffer fish species identification. It is due to the reliability and precision nature of the technology that the US Food and Drug Administration makes the IEF patterns of many fish species available via the Internet (http://vm.cfscan.fda.gov) for species identification (Rehbein et al., 1995). Bossier and Cooreman (2000) successfully

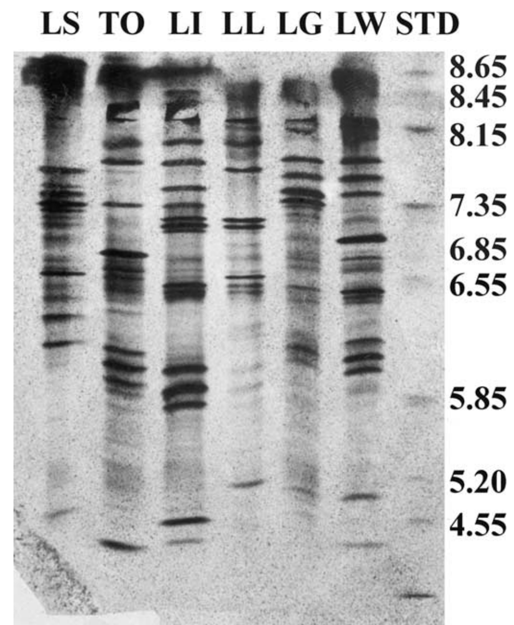

Fig. 1. Native IEF patterns of water-soluble puffer fish proteins with Coomassie blue staining.

applied another IEF databank to authenticate commercial flatfish.

3.3. Native IEF of water-soluble proteins following silver staining

Silver stained IEF gel also gave large numbers of protein bands, especially in the acidic region, for discriminatory application (Fig. 2). In the acidic region of pI 3.50–5.20, the major species-specific proteins were pI 4.19 and 4.85 for LW; pI 5.06 for LG; pI 5.20 for LL; pI 4.34 and 4.59 for LI; pI 4.23 for TO; and pI 4.59 for LS. The major characteristic protein bands in the pI 7.35–8.15 region were pI 7.42, 7.58, and 7.79 for LW; pI 7.37, 7.49, 7.61, and 7.81 for LG; pI 7.72 and 8.00 for LL; pI 7.47, 7.76, and 7.98 for LI; pI 7.76 and 7.92 for TO; and pI 7.37 and 7.67 for LS. Besides these unique bands, the resolving power of the protein bands at the regions of pI 5.85–6.55 was also noted (Table 2). According to our results, native IEF showed good reproducibility among at least six experiments for each fish species. A comparison

Table 1
Protein contents (mg/ml) and the species-specific bands of water-soluble puffer fish proteins with Coomassie blue staining

Fish species	Protein content	pI 3.50–5.20	pI 5.85–6.55	pI 7.35–8.15
Lagocephalus wheeleri (LW)	7.2	4.18, 4.90	5.90, 6.01, 6.08, 6.17, 6.28, 6.40, 6.43, 6.47	7.47, 7.63, 7.80
Lagocephalus gloveri (LG)	6.2	5.02	6.06, 6.12, 6.19, 6.41, 6.49	7.38, 7.47, 7.63, 7.82
Lagocephalus lunaris (LL)	6.3	5.09	5.90, 6.01, 6.45, 6.49, 6.54	7.69, 7.86, 7.97
Lagocephalus inermis (LI)	7.4	4.20, 4.50	5.89, 6.01, 6.45, 6.49	7.50, 7.77, 7.99
Takifugu oblongus (TO)	5.9	4.13	5.92, 6.01, 6.09, 6.33, 6.46, 6.53	7.77, 7.99
Lagocephalus sceleratus (LS)	10.4	4.59	6.14, 6.29, 6.41, 6.48, 6.55	7.44, 7.51, 7.68

Table 2
The species-specific bands of water-soluble puffer fish proteins with silver staining

Fish species	pI 3.50–5.20	pI 5.85–6.55	pI 7.35–8.15
Lagocephalus wheeleri (LW)	4.19, 4.85	5.91, 6.05, 6.12, 6.22, 6.27, 6.42, 6.47, 6.50	7.42, 7.58, 7.79
Lagocephalus gloveri (LG)	5.06	5.89, 5.95, 6.06, 6.13, 6.24, 6.43, 6.49	7.37, 7.49, 7.61, 7.81
Lagocephalus lunaris (LL)	5.20	6.08, 6.22, 6.31, 6.42, 6.47, 6.53	7.72, 8.00
Lagocephalus inermis (LI)	4.34, 4.59	6.01, 6.13, 6.26, 6.40, 6.45, 6.48, 6.52	7.47, 7.76, 7.98
Takifugu oblongus (TO)	4.23	5.92, 5.98, 6.12, 6.23, 6.36, 6.53	7.76, 7.92
Lagocephalus sceleratus (LS)	4.59	6.15, 6.22, 6.26, 6.41, 6.52	7.37, 7.67

of the Coomassie blue staining and the silver staining showed that IEF gels subjected to silver staining had high levels of background staining. In this study, the Coomassie blue staining method was adequate enough for the identification of puffer fish species. Hence, native IEF with either Coomassie blue stain or silver stain is a feasible tool for identifying puffer fish species. Recently, some researchers focused their attention on the small acidic and heat-stable calcium-binding proteins called parvalbumins for species identification of processed products (Etienne et al., 1999; Rehbein, Kundiger, Pineiro, & Perez-Martin, 2000). In the case of cold smoked products, native IEF was more useful than SDS–PAGE or urea IEF in identifying the fish species (Mackie et al., 2000). On the contrary, the SDS–PAGE and the urea-IEF seemed to be more suitable than native IEF for the identification of fish products subjected to heavy processing treatment (Etienne et al., 1999; Mackie et al., 2000). The characteristic species-specific acidic proteins, neutral proteins, and alkaline proteins can be used for the differentiation of puffer fish species.

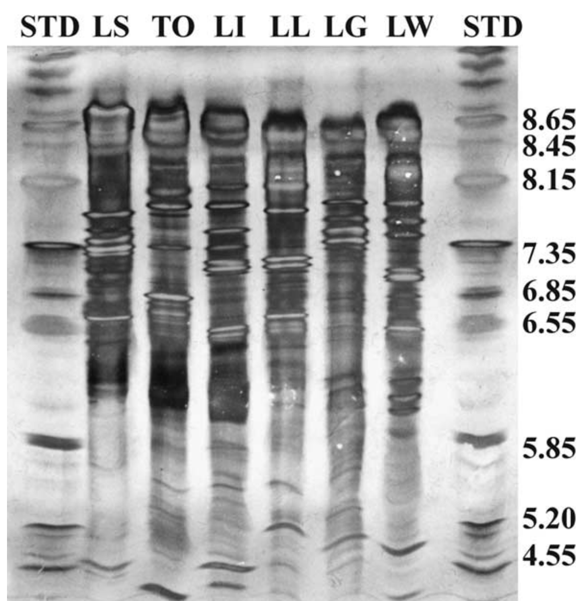

Fig. 2. Native IEF patterns of water-soluble puffer fish proteins with silver staining.

4. Conclusions

Species-specific protein bands were found among the six tested species of puffer fish in each of the three regions (pI 3.50–5.20, pI 5.85–6.55, and pI 7.35–8.15) of the native IEF gels as stained by Coomassie blue or silver staining. Therefore, native IEF is a feasible tool for the identification of puffer fish species.

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