Electrophoretic identification of muscle proteins in several puffer species with Coomassie blue/silver staining

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SUMMARY: Some commercially important fish species are often substituted for by lower price species. In order to detect fraud and to ensure that foods comply with regulations, species identification of puffer using SDS-PAGE with Coomassie blue/silver double staining was performed. A comparison was also made among extracts of sarcoplasmic, myofibrillar, SDS-soluble and urea-soluble proteins. The protein concentration of SDS and urea extracts was usually higher than that of the phosphate (sarcoplasmic and myofibrillar protein) extracts. Protein bands of low molecular weight showed the species-specific characteristics. Identification of all tested puffers could be achieved by judging from the SDS-PAGE patterns of the four protein extracts.

KEY WORDS: puffer, protein, electrophoresis, species identification, Coomassie blue/silver staining

INTRODUCTION

In Taiwan, a traditional food, dried seasoned fish fillet, is mostly made from non-toxic puffer (Lagocephalus gloveri and L. wheeleri). Although the muscle of these puffers does not contain the toxin tetrodotoxin (TTX), the muscle of other toxic puffers L. lunaris and Takifugu oblongus contain high amounts of toxin. Because L. lunaris and L. gloveri are morphologically so similar, manufacturers sometimes confuse them. T. oblongus is the most toxic species in Taiwan and often abused as the material of dried seasoned fish fillet. Hence, serious food poisoning incidents due to ingesting dried seasoned fish fillet or toxic puffers have occasionally occurred in Taiwan. Therefore, it is crucial to develop analytical methods for identifying fish species to prevent the use of toxic puffer species.

Several successful methods such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), urea-isoelectric focusing (urea-IEF), two-dimensional electrophoresis, high performance liquid chromatography (HPLC), capillary electrophoresis (CE), enzyme linked immunosorbent assay (ELISA), and DNA techniques have been recently applied in identifying fish or shellfish species.

The aims of this study are to establish basic data of SDS-PAGE patterns of sarcoplasmic and myofibrillar proteins (non-denaturing extraction), and 1% SDS and 8 M urea proteins (denaturing extraction) in puffer fish by using the Coomassie blue/silver double staining method.

MATERIALS AND METHODS

Authenticated samples of seven puffer species, Lagocephalus wheeleri (LW), L. gloveri (LG), L. lunaris (LL), L. inermis (LI), Takifugu oblongus (TO), T. xanthopterus (TX), and Sphoeroides pachygaster (SP) were purchased from seafood markets in Taiwan. Each species was represented by at least six fish. Among them, the muscles of LW, LG, LI, TX and SP are nontoxic, but those of LL and TO are toxic.

The extracts of fish sarcoplasmic and myofibrillar proteins were prepared by different phosphate buffers according to the procedure described by Hashimoto et al. All the operations were performed at 3-4°C. A 3 g of muscle was homogenized with 3 volumes of phosphate buffer, 15.6 mM Na2HPO4-3.5 mM KH2PO4 (ionic strength, I=0.05, pH 7.5), for 1 min using a Polytron (Kinematica Ag Littau, Switzerland). The homogenate was centrifuged at 10,000 g for 15 min. The precipitate was extracted by the same procedure again. These two supernatants were combined and used as the sarcoplasmic protein fraction. The residue from the above was homogenized with 3 volumes of KCl-phosphate buffer, 0.45 M KCl-15.6 mM Na2HPO4 -3.5 mM KH2PO4 (I=0.5, pH 7.5), in a Waring blender equipped with a baffle to prevent frothing and centrifuged. The precipitate was similarly homogenized and centrifuged again. Both supernatants were combined and used as myofibrillar protein fraction.

On the other hand, 3 g of muscle was homogenized with 3 volumes of either 1% SDS (w/v)
or 8 M urea solutions for 1 min using a Polytron. The 1% SDS mixture were boiled at 100°C for 2 min, homogenized again for 30 s and subsequently maintained at room temperature for 30 min. The 8 M urea mixture were kept at room temperature for 30 min before centrifuging. And then, all extracts centrifuged at 10,000 x g for 15 min at 20°C. The precipitate was similarly homogenized and centrifuged again. Both supernatants were separately combined and used as 1% SDS or 8 M urea extract. The protein concentration was determined by Lowry method. The protein extracts were adjusted with sample buffers to 0.3 mg/ml for Coomassie blue/silver staining.

### Table 1. Protein concentration of four extracts from puffer muscle (mg/ml)

<table>
<thead>
<tr>
<th>Fish</th>
<th>SP</th>
<th>MP</th>
<th>SDS</th>
<th>Urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>LW</td>
<td>6.6±1.6</td>
<td>10.3±3.2</td>
<td>12.8±3.5</td>
<td>13.2±4.8</td>
</tr>
<tr>
<td>LG</td>
<td>5.1±1.1</td>
<td>7.8±2.1</td>
<td>9.2±2.5</td>
<td>15.7±4.2</td>
</tr>
<tr>
<td>LL</td>
<td>8.3±2.2</td>
<td>14.2±3.7</td>
<td>8.5±1.5</td>
<td>14.6±3.1</td>
</tr>
<tr>
<td>LI</td>
<td>6.1±1.8</td>
<td>8.9±2.5</td>
<td>9.3±2.6</td>
<td>17.3±4.9</td>
</tr>
<tr>
<td>TO</td>
<td>9.2±2.4</td>
<td>15.3±3.1</td>
<td>14.6±4.8</td>
<td>20.6±5.1</td>
</tr>
<tr>
<td>TX</td>
<td>9.3±0.9</td>
<td>9.6±1.8</td>
<td>13.9±4.1</td>
<td>18.2±5.9</td>
</tr>
<tr>
<td>SP</td>
<td>4.8±0.8</td>
<td>7.5±2.1</td>
<td>13.4±3.3</td>
<td>23.1±3.7</td>
</tr>
</tbody>
</table>

SP: Sarcoplasmic protein; MP: Myofibrillar protein; SDS: SDS extracts; Urea: Urea extract. Data are mean±S.D. (n=6; specimens).

SDS-PAGE was performed according to the modified procedure using a Mini-Protean unit (Bio-Rad, Richmond, CA). Slab gels consisted of a separating gel (20%) that was polymerized for 1-2 h and a stacking gel (4.0%), which was poured 30 min before sample application. Five µl of protein standard and 10 µl of samples were applied in the wells of the gel. Electrophoresis was carried out at a constant voltage of 80 volts initially and increased to 120 volts when the tracking dye reached the separating gel. Electrophoresis was completed when the dye front reached the bottom of the gel. The gels were stained with silver reagent (Pharmacia Biotech, Piscataway, NJ) and followed by Coomassie blue reagent. After staining, gels were destained in 10% methanol and 10% acetic acid. Molecular weights were determined by comparing relative mobilities of protein bands to standard proteins. Protein standards were obtained from Bio-Rad (Broad range kit, myosin, 205 kD; β-galactosidase 120 kD; bovine serum albumin 84 kD; ovalbumin 52.2 kD; carbonic anhydrase 36.3 kD; soybean trypsin inhibitor 30.2 kD; lysozyme 21.9 kD; aprotinin 7.4 kD).

The gels were scanned and the acquired images were analyzed with the Image Master VDS (Pharmacia, Piscataway, NJ). Statistical analysis for differences was performed by the analysis of variance (ANOVA) procedure of the Statistical Analysis System. The p value <0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

Protein extractability of four extractants is shown in Table 1. Among these, 8 M urea was the most effective agent. Protein extractability of 1% SDS solutions was higher for five puffers (exceptions being L. lunaris, LL and T. oblongus, TO) than that of the two phosphate buffers (1=0.05, pH 7.5 and 1=0.5, pH 7.5). Thus, the protein concentration of 1% SDS and 8 M urea extracts was usually higher than that of the phosphate (sarcoplasmic and myofibrillar protein) extracts.

The SDS-PAGE patterns of sarcoplasmic proteins are shown in Fig. 1. Judging from 11.8 kD to 6.7 kD, the characteristic protein bands for each puffer were as follows: 11.8, 7.5, 5.9 kD for LW; 7.5, 5.9 kD for LG; 9.7, 6.7 kD for LL; 9.7, 7.0 kD for LI; 9.7, 8.0 kD for TO; 11.8, 8.0 kD for TX and 9.1 kD for SP (Fig. 1). The sarcoplasmic proteins pattern could be used for discriminating different species of puffer.

The SDS-PAGE patterns of myofibrillar proteins are shown in Fig. 1. The species-specific characteristic protein bands of myofibrillar proteins were those below 18.9 kD, and were minor components. Specific characteristic protein bands for each puffer were largely in the region ranging from 14.4 kD to 8.5 kD, and were as follows: 11.8, 7.5, 5.9 kD for LW; 7.5, 5.9 kD for LG; 9.7, 6.7 kD for LL; 9.7, 7.0 kD for LI; 9.7, 8.0 kD for TO; 11.8, 8.0 kD for TX and 9.1 kD for SP (Fig. 1). The recognition of myofibrillar proteins could be conducted for species identification by using SDS-PAGE with both Coomassie blue and silver staining.
LW; 13.9, 10.3, 7.7 kD for LG; 17.0, 13.9, 10.3 kD for LL; 17.0, 13.9, 11.5, 7.4 kD for LI; 13.9, 10.3, 8.7 kD for TO and TX, and 16.6, 15.0, 10.3, 8.7 kD for SP. TO and TX could be differentiated by the presence of 18.9 kD and 19.5 kD, respectively. Hence, species identification of puffer could be achieved by comparing the profiles of SDS extracts. The characteristic protein bands could be extracted by SDS-containing solution and were usable for species identification.4,16,17)

Fig. 1. SDS-PAGE patterns of sarcoplasmic (upper) and myofibrillar (bottom) proteins extracted with phosphate buffers from seven puffer species with Coomassie blue/silver staining.

The lanes from left to right are protein standards, Lagocephalus wheeleri, L. gloveri, L. lunaris, L. inermis, Takifugu oblongus, T. xanthopterus, Sphoeroides pachygastrus.

The SDS-PAGE patterns of 8 M urea extracts are shown in Fig. 2. In the 28.6-24.0 kD region, all species except for TO and TX could be identified as follows: 28.0, 24.9 kD for LW; 28.6, 24.9 kD for LG; 28.6, 24.4 kD for LL; 28.6, 25.6, 24.0 kD for LI; 28.6, 26.8, 24.0 kD for TO and TX; and 28.6, 26.8 kD for SP. All species could be differentiated by the molecular weight region (≤ 13.9 kD). Species-specific protein bands were as follows: 7.6 kD for LW; 13.9, 7.6, 5.9 kD for LG; 13.9, 10.0, 7.6 kD for LL; 13.9, 10.9, 7.6, 6.3 kD for LI; 13.9, 10.9, 8.5 kD for TO; 13.5, 10.9, 8.5 kD for TX and 13.9, 8.5, 7.6, 6.3 kD for SP. Therefore, the SDS-PAGE pattern of 8 M urea extract could be used for identifying puffer species. This result was similar to other reports.4,16,17) In this study, the individual mobility variation between fish of the same species was not found. There were only small difference when comparing the patterns of 8 M urea and 1% SDS extracts for TO and TX. However, 1% SDS seems to be a more efficient agent than 8 M urea when inspecting of the low molecular weight region.

Fig. 2. SDS-PAGE patterns of SDS-soluble (upper) and urea-soluble (bottom) proteins from seven puffer species with Coomassie blue/silver staining.

The representations of lanes see Fig. 1.
In conclusion, species identification of all tested puffers could be achieved by examining the low molecular weight protein bands following SDS-PAGE of any of the above four extracts using double staining. Double staining from SDS-soluble proteins seems to be the best combined procedure for identifying the different puffer species.

ACKNOWLEDGMENTS

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