



IDENTIFICATION OF CAUSATIVE FISH FOR A FOOD POISONING IN TAIWAN BY USING SDS-PAGE TECHNIQUE

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Key words: grouper, ciguatera, food poisoning, SDS-PAGE, species identification.

ABSTRACT

A food poisoning incident has occurred due to ingestion of red grouper meat on February 2006 in southern Taiwan. Two patients showed diarrhea, muscle weakness, vertigo and severe muscle pain. The 2 pieces of causative residues were investigated by toxicity assay. In order to identify the fish species of causative samples, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique was applied in this study. According to the low molecular weight region (<30.0 kD) of species-specific patterns extracted from both myofibrillar and sarcoplasmic proteins, two poisoning samples were identified as *Lutjanus bohar*. *L. bohar* is a notorious grouper species with ciguatera in the world.

I. INTRODUCTION

Ciguatera is a food poison from tropical or subtropical coral reef fish. In the world, more than 25,000 persons estimated were affected by ciguatera every year. The distribution of ciguatera is confined to discrete regions of Pacific Ocean, western Indian Ocean and Caribbean Sea [14]. The ciguateric toxin, ciguatoxin (CTX), is produced by the toxin dinoflagellate *Gambierbiscus toxicus*, and they will be accumulated first in herbivorous fish and subsequently in carnivorous fish through the food chain [20]. While some people eat the toxic fish, it will cause food poisoning [5]. The causative fish seem to be limited to several definite coral reef fish, including red grouper, moray eel, doctor fish and toothed jobfish. In

Taiwan, only one death was reported among all poisoning cases. However, the real fish species are almost not identified, except 2004's outbreak caused by moray eel *Gymnothorax javanicus* [24].

Groupers as genus *Lutjanus* are the commercially important fish species with high price in Taiwan. These fish distribute in the tropical and subtropical coral reef district in the world extensively and are carnivorous and edible important species [14]. Otherwise, the food poison incident occasionally occurred when people ate them. In Taiwan, there are more than 10 outbreaks happened. A food poisoning incident has occurred due to ingestion of red grouper meat on February 3, 2006 in Tainan, southern Taiwan. About 12 hr after eating, three patients showed the clinic symptoms of pricking of the lips, tongue and throat, vomiting, abdominal cramps, diarrhea, headache, dizziness, vertigo and paralysis. These symptoms are similar to those of ciguatera [9]. The toxicity and species of toxic fish samples implicated into ciguatera are less studied so far because of the causative residues lacked to retain for study and the morphological characteristics of retained residue have been removed.

Several official electrophoretic methods have been employed to differentiate species of seafood or seafood products [1]. Other feasible methods have been subsequently applied in identifying fish or shellfish species, including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), urea-isoelectric focusing (urea-IEF), high performance liquid chromatography (HPLC), capillary electrophoresis (CE), immunoassay, enzyme-linked immunosorbent assay (ELISA), and DNA techniques. Recent years, standardized methods of species identification for raw and heat-processed fish using SDS-PAGE and urea-IEF techniques have been established via collaborative studies [7, 19, 21, 23]. As previously reported [11], we have used DNA technique to identify the causative fish species as *Lutjanus bohar*. In this study, SDS-PAGE was used with Coomassie blue staining for visualizing protein bands. After comparing the species-specific protein band patterns, the species of causative grouper was identified as that of DNA technique.

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Table 1. Protein contents (mg/ml) of sarcoplasmic and myofibrillar extracts from different fish species muscle.

Fish species	Sarcoplasmic protein	Myofibrillar protein
Poisoning sample 1 (PS1) (Kaohsiung Chang-Gung Memorial Hospital)	3.4 ± 0.5*	5.1 ± 0.3
Poisoning sample 2 (PS2) (Tainan City Health Bureau)	3.1 ± 0.8	4.8 ± 0.4
<i>Lutjanus bohar</i> (LB)	7.5 ± 0.4	11.2 ± 1.4
<i>Variola albimarginata</i> (VA)	6.8 ± 0.3	8.3 ± 0.3
<i>Cephalopholis sonnerati</i> (CS)	5.3 ± 0.3	7.4 ± 0.5
<i>Lutjanus gibbus</i> (LG)	7.9 ± 0.5	10.3 ± 0.7
<i>Acanthurus dussumieri</i> (AD)	10.1 ± 1.1	14.8 ± 1.2

* Data are mean ± S.D. of triplicate assays.

II. MATERIALS AND METHODS

1. Materials

The remaining uneaten frozen fish fillets with small viscera and skin were weighed (154 and 182 g) and kept frozen below -20°C until use. Three specimens of each species from 11 ciguatoxin-suspected fish were collected from Pingtung and Kaohsiung in South Taiwan during April to October 2006. These species of collected samples included *Lutjanus bohar* (LB), *Variola albimarginata* (VA), *Cephalopholis sonnerati* (CS), *Lutjanus gibbus* (LG), *Acanthurus dussumieri* (AD), *Lutjanus sebae* (LS), *Lutjanus erythropterus* (LE), *Lethrinus olivaceus* (LO), *Lethrinus nebulosus* (LN), *Plectorhinchus vittatus* (PV) and *Plectorhinchus flavomaculatus* (PF). All samples were immediately transferred on ice to the laboratory.

2. Assay of Toxicity

Each of all viscera and muscles in the causative residues and fresh specimens was heated at 70°C water bath for 15 min, homogenized with 3 volume of acetone, and then centrifuged at 3,000 × g for 20 min. The residue was repeated to extract two more times. All supernatants were combined, evaporated to free from acetone. The aqueous solution was added with the same volume of ethyl ether to extract toxin twice. The upper phase (ether phase) were combined and evaporated to dry out. The residue was dissolved with 90% methanol and extracted with two volumes of n-hexane twice. The 90% methanol part was evaporated to free from methanol and the residue was dissolved with 0.1% Tween 20 solution for mouse assay. The toxicity was calculated by formula as follows: $\log(\text{MU}) = 2.3 \log(1 + 1/T)$. T (hr) is the survival time of 18-20 g ICR (Institute of Cancer Research) male mice after injection [15].

3. Protein Extraction

The extracts of fish sarcoplasmic and myofibrillar proteins were prepared according to the procedure described by Hashimoto *et al.* [10]. The 3 g sample was homogenized with 3 volumes of phosphate buffer (I = 0.05, pH 7.5) for 1 min using a Polytron (setting 4.0, Kinenatica Ag Littau, Switzerland). The homogenate was centrifuged at 10,000 × g for 15 min, using Himac CF15D2 with rotor RT15A6 (Hitachi, Japan).

Otherwise, 3 g sample was homogenized with 3 volumes of either 1% SDS (w/v) solution for 1 min, using a polytron. All extracts were centrifuged at 10,000 × 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 extract [19]. The protein concentration was determined by Lower method [16]. The protein extracts were adjusted with sample buffer to 2 mg/ml for Coomassie blue staining.

4. SDS-PAGE Analysis

SDS-PAGE was performed according to the modified procedure of Laemmli [12] and O'Farrell [18], using a Mini-Protean unit (Bio-Rad, Richmond, Calif.). Slab gels consisted of a separating gel (12.0%) and a stacking gel (4.0%). 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. Gels were stained with Coomassie blue reagent, 0.1% (w/v) Coomassie blue R-250 in 40% methanol and 10% acetic acid. After staining, gels were de-stained in 10% methanol and 10% acetic acid. Molecular weights were determined by comparing relative mobility of protein bands to standard proteins [25]. The gels were scanned and the acquired images were analyzed with the Image Master VDS (Pharmacia Biotech). The molecular weights were estimated by comparing R_f values on the gel with those of the protein standards. Statistical analysis for differences among the species was performed by the analysis of variance (ANOVA) procedure of Statistical Analysis System. The p value < 0.05 was considered statistically significant.

III. RESULTS AND DISCUSSION

After injecting with the extract of causative fish viscera, mice appeared diarrhea, breathing difficulty and then death. The toxicity of viscera was 2.45 MU/g (17.15 ng CTX/g) for sample 1 and 5.36 MU/g (37.52 ng CTX/g) for sample 2. The symptoms of mice injected with the extract from the muscle of causative samples and other species of ciguatoxin-suspected fish did not appear, so the toxicity of these tested samples was not detected. It means that the toxic ratio of related fish spe-

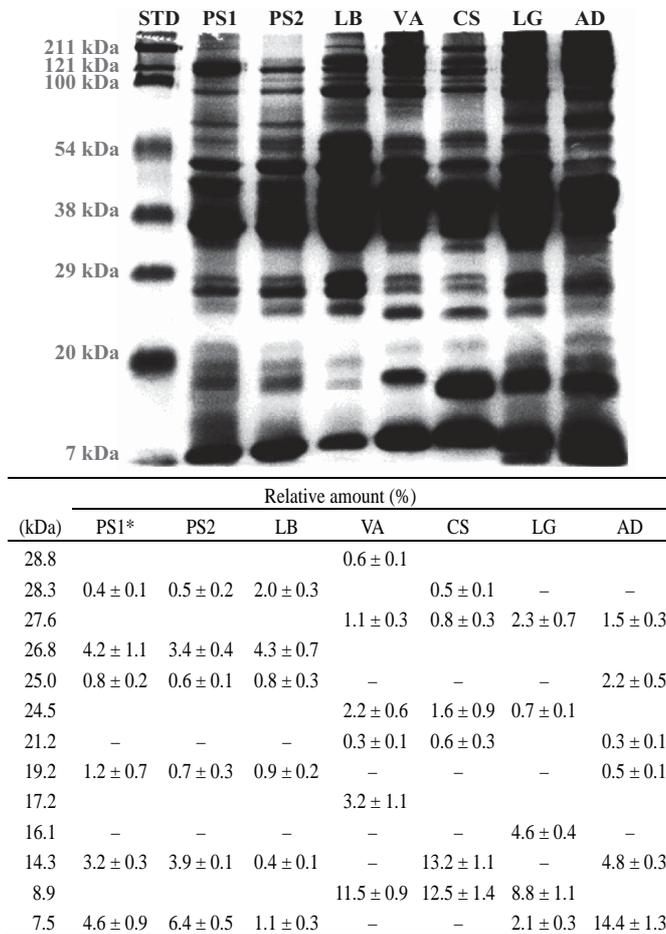


Fig. 1. SDS-PAGE patterns of sarcoplasmic proteins from different fish species with Coomassie blue staining and composition percentage of low molecular weight proteins ($n = 3$). STD: molecular standards, PS1: poisoning sample 1 (Kaohsiung Chang-Gung Memorial Hospital), PS2: poisoning sample 2 (Tainan Bureau of Health), LB: *Lutjanus bohar*, VA: *Variola albigarginata*, CS: *Cephalopholis sonnerati*, LG: *Lutjanus gibbus*, AD: *Acanthurus dussumieri*.

cies is not high in Taiwan and the toxicity may appear in the viscera when the fish become bigger. Previous researches pointed out that small size of coral reef fish usually do not contain toxicity [2, 8], and the content of ciguatoxin accumulated in viscera was more than in muscle [13].

After muscle extracting from 2 causative specimens and 5 ciguatoxin-suspected fish samples, protein extractability is shown in Table 1. The concentration of protein extracted from fish tissue represented the fresh degree and muscle quality in each sample. The protein concentration of the myofibrillar protein extracts was higher than that of the sarcoplasmic protein extracts in this study. Judging from the high molecular weight region (≥ 30.0 kDa) of both myofibrillar and sarcoplasmic protein extracts with Coomassie blue staining, there was no species-specific protein band among these investigated specimens (Figs. 1, 2). The bands of high molecular weight region (≥ 30.0 kDa) were apparently not useful in identifying fish species in this study.

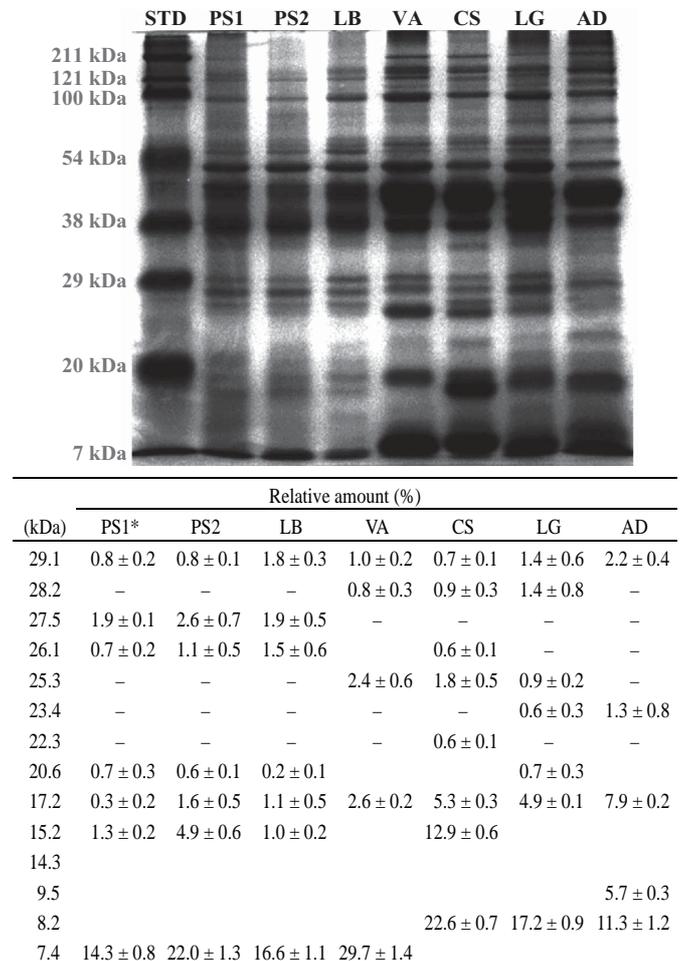


Fig. 2. SDS-PAGE patterns of myofibrillar proteins from different fish species with Coomassie blue staining and composition percentage of low molecular weight proteins ($n = 3$). The Symbols refer to authentic species and the footnote detailed in Fig. 1.

SDS-PAGE pattern of sarcoplasmic proteins extracted with phosphate buffer following Coomassie blue staining and the densities of the low molecular weight protein bands are shown in Fig. 1. In the low molecular weight region (< 30.0 kDa) of sarcoplasmic proteins, specific bands were observed in each species. Therefore, species of 5 tested fish could be identified by using 7.5 kDa to 28.8 kDa protein bands. The characteristic protein bands of these 5 species were respectively as follows: 28.3, 26.8, 25.0, 19.2, 14.3 and 7.5 kDa for LB; 28.8, 27.6, 24.5, 21.2, 17.2 and 8.9 kDa for VA; 28.3, 27.6, 24.5, 21.2, 14.3 and 8.9 kDa for CS; 27.6, 24.5, 16.1, 8.9 and 7.5 kDa for LG; 27.6, 25.0, 21.2, 19.2, 14.3 and 7.5 kDa for AD. Otherwise, the specific bands in both PS1 and PS2 were 28.3, 26.8, 25.0, 19.2, 14.3 and 7.5 kDa. The patterns of low molecular weight region (< 30.0 kDa) of sarcoplasmic proteins extracted from 2 causative specimens were totally the same as the pattern in LB.

In the low molecular weight region (< 30.0 kDa) of myofibrillar proteins, a number of bands were presented by composition of myosin light chains, troponins and parvalbumins.

The pattern of low molecular weight region (<30.0 kD) of myofibrillar proteins extracted by phosphate buffers also shows the specific bands from each species (Fig. 2). Inspection of the 7.4 kD to 29.1 kD protein band region displays the characteristic bands for these 5 species as follows: 29.1, 27.5, 26.1, 20.6, 17.2, 15.2 and 7.4 kD for LB; 29.1, 28.2, 25.3, 17.2 and 8.2 kD for VA; 29.1, 28.2, 26.1, 25.3, 22.3, 17.2, 15.2 and 8.2 kD for CS; 29.1, 28.2, 25.3, 23.4, 20.6, 17.2 and 8.2 kD for LG; 29.1, 23.4, 17.2, 9.5 and 8.2 kD for AD. The specific bands in both PS1 and PS2, including 29.1, 27.5, 26.1, 20.6, 17.2, 15.2 and 7.4 kD, were also the same with that in LB.

According to the low molecular weight region (<30.0 kD) of both myofibrillar and sarcoplasmic pattern, two poisoning samples (PS1 and PS2) were identified as *Lutjanus bohar*. The result reveals that species identification could be achieved by using SDS-PAGE analysis. In many reports also indicated that the characteristic protein bands could be extracted by SDS-containing solution and were usable for species identification [3, 4, 6, 22].

Up to the present, there was still no reliable prevention against ciguatera poisoning. It was suggested that the weight of coral reef fish should be less than 1.0 kg for diet. Furthermore, the viscera from coral reef fish are not allowed for eating [17]. In this study, the species of causative grouper is successfully identified as *L. bohar*. This grouper is a notorious species of ciguatera in the world because of causing food poisoning usually. For guaranteeing edible safety of grouper, the toxicity of *L. bohar* needs to be investigated in Taiwan.

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