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Key words: real-time PCR, vegetarian adulteration, surimi adulteration, Actinopterygii.

ABSTRACT

Vegetarian foods are abundant in the domestic market. It is found that animal ingredients have been added into vegetarian products to improve the texture and taste. Surimi adulteration in vegetarian food is an area of concern for vegetarians. The α-skeletal actin gene of aquatic animals as novel specific primers and probe of Actinopterygii in 36 fish species are tested using real-time PCR method. It was found that all of the 36 fish species of Actinopterygii showed positive results. The 10 surimi products, 10 surimi-based products containing fish components showed positive results. All of the 10 fresh foods containing no fish component showed negative results. It indicates that this novel specific primers and probe in Actinopterygii is feasible when applied in quantitative detection.

I. INTRODUCTION

In recent years, the population of strict vegetarians and vegetarians for health reasons has increased in nations around the world. The vegetarian food market scale is to expand rapidly within the near 3 years (2008-2010). According to the survey of 2005 Food Consumption Almanac, over 2.5 million people or 10% of the total population in Taiwan prefer vegetable diets. This percentage is considered to be rather high [14]. Due to moral values, health reasons, and doubts over food ingredients, Taiwan has a high percentage of vegetarians, most of whom are vegetarians for their religious faith. As a result, there have been higher demands for vegetarian foods in the market.

Because of fierce competitions, some manufacturers mixed animal ingredients into vegetarian foods to enhance the "texture" and "taste". As a result, 'the incident of animal ingredient adulteration' in June 2004 caused a great disturbance in Taiwan. The identification system of adulteration is largely based on physio-chemical properties of the food and is sometimes complicated and generally fails to give sufficient information. Even though surimi adulteration rarely presents a health hazard, however, such adulteration is an issue because it defrauds the consumer.

Adulteration of high quality and high priced food products is a commercial and economic problem. The widespread availability of surimi-based imitation makes it a morality adulterant with ethical implications for vegetarian products. Most of the initial research to detect adulteration has focused on detailed and expensive methodologies involving gas chromatography (GC) and mass spectrometry to identify unique chemicals that distinguish one ingredient from another [5]. In view of the problem caused by surimi adulterations of vegetarian products, VIS/NIR spectroscopy is a cheaper and more accurate method to detect 10% fish surimi adulteration in crabmeat surimi adulteration [6] as compared to GC, mass spectrometer, and agar-gel myosin light chains methods [12]. The pollock surimi in raw meat products is normally tested by means of agar-gel immunodiffusion [4].

Real-time PCR is a powerful advancement of the basic PCR technique. It has been successfully applied for detection of bacterial DNA in various environments, such as pathogenic bacteria in food [2], water [1] and feces [10]. In order to develop and correctly determine whether there is surimi adulteration in vegetarian foods, real-time PCR test method is adopted due to the many types of surimi used. Specific primers and probes are designed for the α-skeletal actin genes in fish. The α-skeletal actin is an important protein present in the eukaryote and participates in almost all forms of cells and cellular organelle movements. One of the most important actin is acting as a contractile protein in the skeletal muscle activities [3, 7, 11]. Therefore, the different fish species are detected in their homogeneity and real-time PCR method and quantitative test after adding surimi in vegetarian foods.

In this study, the α-skeletal actin gene of aquatic animals as specific primers and probes in fish species are designed and tested using real-time PCR method. A quantification performed for determining the proportion of golden threadfin surimi was adulterated in vegetarian foods.

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II. MATERIALS AND METHODS

1. Experimental Sample and Source

Raw fish samples: The 27 families and 36 species of raw fish samples below were purchased from grocery stores and traditional markets in Hsinchu: including Nemipteridae (*Nemipterus virgatus*), Gadidae (*Theragra chalcogramma*), Chanidae (*Chanos chanos*), Coryphaenidae (*Coryphaena hippurus*), Salmonidae (*Salmo salar* and *Oncorhynchus mykiss*, Scombridae (*Thunnus albacares*, *Scomberomorus commerson* and *Scomber austrlasicus*), Osmeridae (*Mallotus villosus* and *Plecoglossus altivelis*), Scomberesocidae (*Cololabis saira*), Channichthyidae (*Ereunias grallator*), Cichlidae (*Oreochromis hybrids* and *Oreochromis* hybrids *Tilapia* spp), Centrolophidae (*Psenopsis anomala*), Trichiuridae (*Trichiurus lepturus*), Serranidae (*Epinephelus malabaricus* and *Aethaloperca rogaa*), Teraponidae (*Bidyanus bidyanus*), Xiphiidae (*Xiphias gladius*), Sciaenidae (*Miichthys miiuy*) and *Larimichthys croceus*, Cyprinidae (*Carassius auratus*), Priacanthidae (*Priacanthus macracanthus*), Mugilidae (*Liza macrolepis*), Sparidae (*Dentex tumifrons*, *Acanthopagrus latus* and *Pagrus major*), Polynemidae (*Polydactylus sextarius*), Stromateidae (*Pampus argenteus*), Clupeidae (*Spratelloides gracilis*), Carangidae (*Trachinotus blochii*), Moronidae (*Lateolabrax japonicus*), Anguillidae (*Anguilla japonica*), and Siluridae (*Pangasius sutchi*). All those are belong to Actinopterygii.

The 10 non-fish samples purchased from grocery stores and traditional markets in Hsinchu area were beef, pork, chicken, shrimp, egg, refined rice, brown rice, water celery, burdock hamburger and vegetable hamburger meat. Raw surimi and refined products: Raw surimi and cooked refined products were obtained from 6 fishery product factories. They included 20 food items: Raw surimi obtained from Factory A included pollack surimi and golden threadfin surimi, the cooked refined products were pollack ball (containing pollack surimi) and squid ball (containing golden threadfin surimi). Raw surimi obtained from Factory B included golden threadfin surimi, milkfish surimi, and dolphinfines surimi, and the cooked refined products were Olen (containing dolphinfines surimi). Raw surimi obtained from Factory C included golden threadfin surimi and pollack surimi, and the cooked refined products were lobster-flavored ball (containing pollack surimi and golden threadfin surimi). Raw surimi obtained from Factory D included golden threadfin surimi, and the cooked refined products were pollack ball (containing golden threadfin surimi), larval fish ball (containing golden threadfin surimi), and squid ball (containing golden threadfin surimi). Raw surimi obtained from Factory E included golden threadfin surimi. Raw surimi obtained from Factory F included golden threadfin surimi, and the cooked refined foods were squid ball (containing golden threadfin surimi), one fragile (containing golden threadfin surimi), lobster's ball (containing pollack surimi and golden threadfin surimi).

The professional surimi manufacture process: fish meat is crushed, blended into surimi, and stored in chilling. The cooked refined product manufacture process: surimi is defrosted and added with little amounts of cornstarch, salt, and pepper. The ingredients are mixed evenly and the mixture extruded into different shapes by use of a machine, then cooked in 80-85°C hot water for 25-30 min and refrigerated after cooling.

2. DNA Extraction

A sample of 25 g was placed in a 225 mL 0.85% NaCl homogenated bottle (blender) and homogenized for 2 min. An extract of 1mL homogenated was obtained and placed in 1.5 mL centrifuge tube, and centrifuged for 5 min using 15700 × g to remove the supernatant. Obtained 20 mg was extracted DNA using Genomic DNA Mini Kit (Geneaid, Taipei, Taiwan). 200 μL LGT solution and 20 μL proteinase K (final concentration: 10 mg/mL) were added and shaken. It was left for 30 min at 60°C to dissolve, 200 μL GB solvent was added, shaken evenly for 5 sec, and left for 20 min to further dissolve. Then, 200 μL alcohol was added and shaken for 10 sec. The mixture was moved to GD Column and centrifuged at 15700 × g for 2 min to remove the centrifuged substances. Added 400 μL W1 solvent and centrifuged for 30 sec at 15700 × g to complete the first cleaning. Then, added 600 μL cleaning solution and centrifuged for 30 sec at 15700 × g to complete the second cleaning, then, removed the remaining organic solvent by centrifuging for 3 min at 15700 × g. Next, 100 μL elution at 70°C was added and left for 3-5 min and centrifuged for 30 sec at 15700 × g to extract GD Column from the DNA. Finally, the DNA was collected using a 1.5 mL centrifuge tube.

3. Design of the Oligonucleotide Primers and Probe

According to National Center for Biotechnology Information (NCBI) gene pool search results, specific primers and probe of α-skeletal actin were selected. The oligonucleotides of primers and probes were synthesized by Tib Molbiol Company (Syntheselabor GmbH, Berlin, Germany). The sequences of primers are as follows:

Primer F: 5'-GCTCTGGACTTCGAGAACGAGAT-3';
Primer R: 5'-TGTTGTAGGCGGTCTCATGGATA-3';

the amplified DNA length is 103 bp. The probe was a fluorogenic TaqMan probe (Tib Molbiol, Berlin, Germany) labeled with a reporter dye (FAM^{TM}) (Tib Molbiol) at the 5' end and a quencher dye (TAMRA^{TM}) (Tib Molbiol) at the 3' end. The sequences of probe are as follows: 5'-FAM-CTGGAGAAGA GCTACGAGCTTCCCGA-TEMRA-3'. This system used the nuclease of Taq DNA polymerase to dissolve the internal fluorogenic probe in order to test the target gene amplification. The real-time PCR system exploited Taq DNA polymerase (5'-nuclease) to cleave internal fluorogenic probe and detect the increasing amount of target gene.

4. Real-Time PCR Optimization and Conditions

LightCycler TaqMan Master Kit (Roche Molecular Bio-

Table 1. Detecting the fresh no-fish foods using the α-skeletal actin gene as specific primers by real-time PCR.

Samples	*No. of samples	Real-time PCR detected (+/–)
Animal-made	5	–
Plant-made	3	–
Processed-made	2	–

*: See materials and methods.

chemicals) was first used to produce Master Mix: Taq DNA polymerase, reaction buffer, $MgCl_2$, and dNTP mix. 5 μL DNA extract was added in 15 μL master mix. By use of real-time PCR detect system (Light Cycler™ instrument; Roche Diagnostic, Mannheim, Germany), real-time PCR amplification was conducted. The conditions were as follows: (1) Preheating: it was conducted for 10 min at 95°C. (2) Amplification: each cycle contains at 95°C, 5 sec; 55°C, 20 sec; 72°C, 1 sec; total for 45 cycles. (3) Cooling is conducted for 30 sec at 40°C. The PCR product result was determined directly by LightCycler software version 3.5.3 (Roche Molecular Biochemicals). The fluorogenic value of the PCR product had reached the threshold of 0.1, its cycle number was then set as the Ct (cycle threshold) value. A value higher than the threshold value 0.1 is determined as a positive reaction, and a value lower than the 0.1 value is determined as a negative reaction.

The components for PCR were supplied in master mix provided by a real-time PCR Kit (LightCycler FastStart DNA Master Hybridization Probes Kit; Roche Applied Science). The standard master mixed contained 1 mM $MgCl_2$, 0.5 μM primers, 0.2 μM TaqMan probe and 2 μL of "HotStart" PCR reaction mix (FastStart Taq polymerase, reaction buffer, dNTP, and 10 mM $MgCl_2$) in a volume of 18 μL. The amplification reactions were carried out by mixing 2 μL of DNA template with master mix to a volume of 20 μL per reaction.

III. RESULTS

1. Analytical Specificity

All the positive results of 36 fish species of 27 genus in Actinopterygii, and specific set primers F and R and the probe designed from the α-skeletal actin tested by real-time PCR showed at Ct value 23-36 (not tabulated). Therefore, universal primer F and R possess specificity for Actinopterygii.

In addition, Table 1 shows the 10 non-fish animal and plant samples that were tested using these two sets of primers by real-time PCR tests. The foods of animal-made (beef, pork, chicken, shrimp and egg) and plant-made (refined rice, brown rice and water celery) as well as processed foods (burdock hamburger meat and vegetable hamburger meat) were shown to be negative. Therefore, it showed that the sets of primers are only able to detect the specificity of fishes.

Table 2. Using the α-skeletal actin gene as universal primers and probes for detecting the surimi and surimi-based products by real-time PCR.

Factories	*No. raw or cooked products	Real-time PCR detected (+/–)
A factory	2 raw	+
	2 cooked	+
B factory	3 raw	+
	1 cooked	+
C factory	2 raw	+
	1 cooked	+
D factory	1 raw	+
	3 cooked	+
E factory	1 raw	+
F factory	1 raw	+
	3 cooked	+

*: See materials and methods.

2. Qualitative Test on Surimi and Refined Products of Surimi

The source of surimi-based products sold in the market is versatile. The surimi types frequently used in domestic market are golden threadfin surimi, sailfish surimi, pollack surimi and milkfish surimi. Golden threadfin surimi is sold at the lowest prices in the market. In order to confirm the surimi adulteration added soybean-protein to vegetarian foods, Table 2 shows 20 products out of 6 surimi factories that raw surimi and the heated refined products can be detected using universal primers and probes by real-time PCR test. It showed that the constituents of the 10 raw frozen surimi products that can be detected, the constituents of the other 10 cooked refined products could be tested by real-time PCR test. The extracted DNA of these products was not all destroyed after freezing and heating.

3. Quantitative Test after Adding Surimi in Vegetarian Foods

Fig. 1 mimics the condition of golden threadfin surimi (*Nemipterus virgatus*) at 1%, 4%, 16% and 64% (w/w) added in soybean-protein of vegetarian foods. The standard curve prepared using real-time PCR is adopted. The straight line of the equation is $y = -3.2x + 34.5$, and the linear correlation coefficient R^2 was 0.98. The Ct value range was 28.5-34.5. When golden threadfin surimi of weight percentage 64%, 16%, 4% and 1% was added, the mean Ct value were 28.5, 30.5, 32.5, and 34.5. The absolute value of the slope is 3.2. The real-time quantitative PCR method proposed in this study allowed us to detect surimi adulteration over a very wide range. Hence, the percentage of golden surimi threadfin in an unknown sample can be measured by interpolation from a standard curve of Ct value generated from known starting DNA concentration. In the case of determination of surimi content (w/w, %) in foods, it is necessary to convert from the

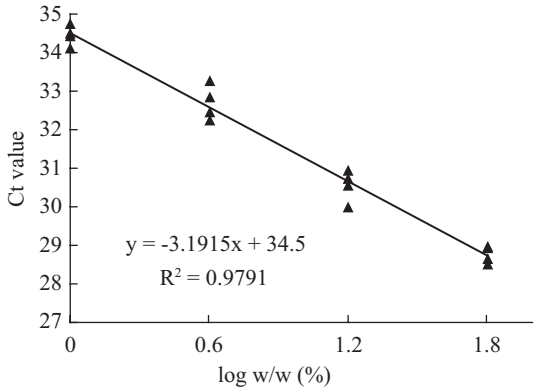

Fig. 1. Standard curve for the golden threadfin surimi content in vegetarian foods and PCR cycle threshold (n = 4) log w/w (1%, 4%, 16%, 64%) contained in the vegetarian protein by real-time PCR.

determination of the copy numbers of fish target DNA sequences and from that of the copy numbers of the fish universal specific gene. Therefore, further study is need to determine a way to convert the ratio of measured copy numbers to units of weight/weight. It showed that the surimi adulteration content was detected and the sensitivity was about 1%.

IV. DISCUSSION

Real-time PCR method is a fast detection method. It not only has the advantage of specificity and better sensitivity that is not possible in chemical method, it also has the quantitative tests. Actinopterygii is a radiated fish type and the most predominant and abundant fish among all vertebrates. There are over 27,000 species (96% of all the present fish types) that are distributed throughout fresh water and seawater. The morphological variety and specialized functions of skeletal muscles provide vertebrates with highly organized body movements [8]. Therefore, one set of specific primers and a probe were designed by α-skeletal actin in this experiment to detect whether surimi had been added in vegetarian foods. The 36 fish species of Actinpterygii can all be detected. The test results of the 10 non-fish samples are not false-positives. The results from this study indicate that it is possible to detect adulteration using real-time PCR in surimi adulterated with surimi-based imitation vegetarian foods.

The adulteration of golden threadfin surimi is added in soybean-protein of vegetarian foods. Real-time PCR quantifies the linear correlation. The results can be applied in vegetarian food surimi adulteration quantitative tests. The detection limit is about 1%.

As shown in Fig. 1, the real-time quantitative PCR method proposed in this study used to detect golden threadfin surimi. The amplification was clearly observed in a range between 1~64% in the vegetarian protein. The quantification in unknown samples are accomplished by measuring Ct and using the standard curve to determine the starting copy number. Even though, different fishes have various standard curves, it may increase the detection labor. The detection the limits is 1%, we had not test lower, because it is enough to the surimi adulteration, the lower 1% of surimi is not to enhanced the texture and taste. In previous study, Laube *et al*. [9] and Tanabe *et al*. [13] detected hidden meat mince in processed foods, the limit were lower 0.1%.

As for different factors might influencing the amount of fish species determined. Including the number of cells per unit of mass, the degree of ploidy and the genome size have an influence on the result of quantification [9]. The correction factors for the determination of the proportion of species are a possibility to overcome the stated uncertainty in the future.

This is the first report use of the α-skeletal actin gene utilizing real-time PCR to detect surimi adulteration in vegetarian foods. Since the method is rapid, specific, sensitive, and highly quantitative, it would be particularly useful in the detection of hidden surimi in vegetarian foods.

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