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Establishment of immunological methods for the detection of soybean proteins in surimi products

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ABSTRACT

Recently, adulteration by adding soybean proteins to surimi products to replace fish muscle has been gaining momentum. In this study, soybean trypsin inhibitor (STI) was chosen as target protein for detection of soybean proteins in surimi products and both polyclonal antibody and monoclonal antibody (A11-6) against STI were prepared. A semi-quantitative means of analyzing soybean proteins in various commercial surimi products was performed by Western blot. A sandwich enzyme-linked immunosorbent assay (s-ELISA) was further developed to quantitatively detect soybean proteins in surimi products. The limit of detection (LOD) and the limit of quantification (LOQ) were 0.68 μg SPI/mL (13.6 mg SPI/kg food) and 3.09 μg SPI/mL (61.7 mg SPI/kg food), respectively. The rates of recovery of soybean proteins ranged from 100.1% to 122.2%, and the coefficient of variation (CV) was less than 4.1%. Our results indicated that the methods established can be applied to monitor soybean proteins in surimi products.

1. Introduction

Soybean proteins are widely used in both human food and animal feed industries owing to its high nutritional value and functional properties (Friedman & Brandon, 2001). Due to its high protein concentration and low fat content, soybean proteins are often used as meat extenders. Surimi, a paste made from fish that is used as an ingredient in foods such as fish balls, has recently become increasingly popular due to its convenience, unique taste, and high nutritional value. In China, the manufacturing of surimi products has seen an annual increase of approximately 10% (Anonymous, 2014). As the price of fish increases, the raw materials required for surimi preparation have become more and more expensive. Besides, soybean proteins would improve the gel-forming ability of surimi partly by protecting myofibrillar proteins from proteolysis by the endogenous proteinase (Jiang et al., 2006). Consequently, soybean proteins are being used increasingly in surimi products as a replacement for fish meat and to decrease the cost of the final product.

Since soybean is a major food allergen (L'Hocine & Boye, 2008), the presence of soybean proteins in surimi products can pose a serious problem for consumers with soy allergies. Furthermore, adding soybean proteins to surimi products without accurate labeling may constitute fraud. In order to guarantee the quality and safety of surimi products, it is necessary to establish a reliable and sensitive detection method to monitor the presence and quantity of soybean protein additives. However, surimi products are prepared through a series of steps, and added soybean proteins can be masked by other food matrixes, including proteins and starch, which complicate detection.

Many techniques have been developed to detect soybean proteins. Polyacrylamide gel electrophoresis is a simple method, but has low selectivity (Belloque, Garcia, Torre, & Marina, 2002). Chromatographic techniques, including high-performance liquid chromatography (HPLC) (Castro, García, Rodríguez, Rodríguez, & Marina, 2007; Castro-Rubio, García, Rodriguez, & Marina, 2005), reversed-phase HPLC (RP-HPLC) (García, Domínguez, García-Ruiz, & Marina, 2006), and perfusion RP-HPLC (Castro-Rubio, Castro-Rubio, García, & Marina, 2007) are also available. However, chromatographic methods require expensive equipment and suffer from matrix interference. Immunological methods, including ELISA, are powerful analytical tools for the detection of soybean proteins (Liu, Teng, Yang, Wang, & Wang, 2012; You et al., 2008). Previous

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publications have detailed the detection of soybean proteins in meat products by ELISA (Griffiths, Billington, Crimes, & Hitchock, 1984; Morishita et al., 2008). However, to our knowledge, there have been no reports to date on the detection of soybean proteins in surimi products. Therefore, the objective of our present study is to develop a protocol for the sensitive detection of soybean proteins in surimi products.

Soybean trypsin inhibitor (STI) is one of the main antinutritional factors in soybeans. It is a relatively thermally stable protein (Jiang et al., 2006) and is resistant to chemical denaturation and digestion (Roychaudhuri, Sarath, Zeece, & Markwell, 2003, 2004). Therefore, STI was chosen as the target protein to indicate the presence of soybean proteins, and the present investigation was aimed to produce antibodies against STI, and to develop specific immunological methods for sensitive and quantitative determination of soybean proteins in surimi products.

2. Materials and methods

2.1. Materials

Silver carp (*Hypophthalmichthys molitrix*) was purchased from a local market in Xiamen, China. Soybeans (*Glycine max*), SPI, fish balls, and surimi were purchased from commercial suppliers in Xiamen, China. Protein markers for SDS-PAGE and Western blot were from Fermentas (Vilnius, Lithuania). Bovine trypsin, streptavidin-HRP polymer and hypoxanthine-aminopterine-thymidine (HAT) were from Sigma (St. Louis, MO). Sulfosuccinimidyl-6-biotinamidohexanoate (Sulfo-NHS-LC-Biotin), enhanced chemiluminescent (ECL) substrate for Western blot, and 3,3′,5,5′-Tetramethylbenzidine (TMB) were from Pierce (Rockford, IL, USA). Culture media RPMI 1640 and fetal bovine serum (FBS) were from Invitrogen (California, USA). All chemicals and reagents were of analytical grade.

2.2. Preparation and labeling of an anti-STI mAb (A11-6)

Preparation of anti-STI monoclonal antibody (mAb) was performed as previously described (Cai et al., 2013; Jiang, Shen, et al., 2013). Purified STI was heated at 110 °C for 30 min and used as the antigen. Five female BALB/c mice on a soybean-free diet were injected subcutaneously with 150 μg of the antigen emulsified in Freund's complete adjuvant (Sigma, USA) at six weeks of age. Three subsequent injections were given at two week intervals and the antigen was emulsified in Freund's incomplete adjuvant (Sigma, USA). Ten days after the final immunization, the serum of each mouse was tested, and the mouse with the highest titer and sensitivity toward STI received a booster injection. Four days later, the mouse spleen was removed for hybridoma production.

Hybridoma production was performed as previously reported with some modifications (Liu et al., 2012). Mouse splenocytes were fused with myeloma cells (SP2/0) in the presence of polyethylene glycol (PEG). Following fusion, cells were cultured in RPMI 1640 medium containing 20 mL/100 mL FBS and 1 g/100 g HAT at 37 °C, and antibody-producing cells were screened. Positive subclones were cloned by the limiting-dilution method. The hybridoma cell line that produced immunoglobulin G (IgG) with the highest binding capacity to STI was labeled as A11-6. The mAb obtained from the culture supernatant was further purified with a Protein G affinity column.

Biotinylation of the mAb was carried out according to the manufacturer's instructions. Briefly, purified A11-6 was exchanged into phosphate-buffered saline and a 100-fold molar excess of biotin reagent was prepared immediately before use and was mixed with A11-6 on ice for 2 h. After labeling, excess non-reacted biotin reagent was removed using a desalting column.

2.3. Preparation of polyclonal anti-STI antibody

Preparation of anti-STI polyclonal antibody (pAb) was performed as previously described with modification (Jiang, Cai, et al., 2013). Two New Zealand white rabbits fed a soybean-free diet were subcutaneously immunized with 100 μg STI (previously heated at 110 °C for 30 min) in Freund's complete adjuvant (Sigma, USA). Immunizations were repeated three times at two week intervals using Freund's incomplete adjuvant. Three days after the last injection, the whole blood was collected and centrifuged at 4000 *g* for 15 min. The animal experiments were all carried out in accordance with the guidelines issued by the Ethical Committee of Jimei University. Polyclonal IgG was purified from the antiserum using a Protein A affinity column, and biotinylation of the antibody was performed as described above.

2.4. Preparation of surimi and fish balls

Fresh silver carp muscle was deboned, and 300 g of minced meat was stirred in 1500 mL ice cold water for 5 min and allowed to stand for 10 min, followed with dewatering by centrifugation at 3000 *g* for 5 min. This process was repeated 3 times. The minced muscle was ground for 10 min in a meat grinder and ground a second time in the presence of 3 g/100 g NaCl for 20 min. The resulting product was used as surimi sample. The fish balls were made by mixing starch and surimi at a ratio of 1:5 (g:g). In order to evaluate the sensitivity and the recovery of the established methods, SPI was added to surimi and fish balls respectively, resulting in eight groups of surimi with different contents of SPI (0, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, and 2.0 g/100 g) and four groups of fish balls with 0, 0.5, 1.0, and 2.0 g/100 g of SPI.

2.5. SDS-PAGE and Western blot

SDS-PAGE was performed using the Laemmli method (Laemmli, 1970). Samples were mixed with sample buffer and heated at 95 °C for 10 min in the presence of reducing agent DTT. Samples were separated in 12 g/100 mL polyacrylamide gels, and the gels were stained with Coomassie Brilliant Blue R-250 (CBB).

Western blot was carried out using the method as described (Towbin, Staehelin, & Gordon, 1979). Briefly, following SDS-PAGE, the separated proteins were transferred to a nitrocellulose membrane and the membrane was blocked with 5 g/100 g skim milk at room temperature for 1.5 h. After washing with 20 mmol/L Tris–HCl (pH 8.0) containing Tween-20 (TBST), the membrane was incubated with biotinylated A11-6 mAb for 1.5 h, and the unbound antibody was removed. The membrane was then incubated with Streptavidin-HRP for 1 h, followed by extensive washes with TBST. Results were developed by ECL, and a FluorChemQ instrument (Alpha Innotech, France) was used to record the results.

2.6. Sandwich ELISA

A sandwich ELISA was developed to quantify soybean proteins in surimi products. The mAb A11-6 was coated on the 96-well microtiter plate at 37 °C for 2 h. The plate was washed with TBST to remove unbound A11-6 and was then blocked with 5 g/100 g skim milk at 37 °C for 1.5 h. After washes, the extracted samples or SPI samples with known contents were added to each well and incubated at 37 °C for 1.5 h. After washes with TBST, the biotin-labeled polyclonal antibody was added and incubated at 37 °C for 1.5 h. After extensive washing, a streptavidin-HRP conjugate was