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Detection of tropomyosin and determination of proteins in crustacean oils

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ABSTRACT

Tropomyosin is known to be the main allergen in crustaceans and the objective of this study was to investigate if this protein could be detected in commercial crustacean oils from Antarctic krill (*Euphausia superba*) and the zooplankton *Calanus finmarchicus*. We also examined the possibility of determining the protein content in the oils by direct amino acid analysis. Western blotting showed that a commercial antibody against shrimp tropomyosin cross-reacted with a protein of similar size in Antarctic krill and *C. finmarchicus*. The protein tentatively identified as tropomyosin, was also detected in krill oil products, but not in oils from *C. finmarchicus*. The acetone–heptane method used for extracting proteins in the oils is however not optimal. Other extraction methods should therefore be considered when investigating the presence of allergenic proteins in oils. Direct amino acid analysis on oils should be further explored as a method for determining the total amount of proteins present.

1. Introduction

Crustaceans are one of eight foods or food groups that are thought to account for more than 90% of all immunoglobulin E food allergies world-wide (Hidalgo & Zamora, 2006). The myofibrillar protein tropomyosin (TM) is a cross-reacting allergen among crustaceans and has been shown to be the main allergen in species like shrimp, lobster, crab, and Antarctic krill (Nakano, Yoshinuma, & Yamada, 2008; Reese, Ayuso, & Lehrer, 1999). As reviewed by Hidalgo and Zamora (2006) proteins and peptides may be found in many crude and refined plant oils and several studies have shown that allergenic proteins may be found in oils extracted from peanuts (Ramazzotti et al., 2008), soybeans (Errahali et al., 2002), sunflower seeds (Zitouni et al., 2000), and gourmet nuts (Teuber, Brown, & Haapanen, 1997). Very few studies have however been carried out on the allergenicity of fish oils (Mark, Beaty, & Slavin, 2008) and to the best of our knowledge, the presence of allergens in oils from crustaceans has not been reported.

There has been a large increase in the demand for fish oils containing the unique long-chain polyunsaturated ω-3 fatty acids (*n*-3 LC-PUFA). The limited amount of fish oils available has led to extensive search for alternative sources of oils rich in *n*-3 LC-PUFA. These fatty acids in the fish oil originate from marine phytoplankton and one possibility is to harvest resources lower in the marine food web such as smaller crustaceans (Miller, Nichols, & Carter, 2008). Antarctic krill (*Euphausia superba*) and *Calanus finmarchicus* which are small shrimp-like crustaceans present in large amounts in the South and the North Atlantic Oceans, respectively (Greene et al., 2003; Nicol, Foster, & Kawaguchi, 2012), are currently harvested for the production of oils rich in *n*-3 LC-PUFA and astaxanthin. The oils are available on the nutraceutical markets and so far most studies on health effects have been carried out with krill oils (Eilertsen et al., 2012; Grimstad et al., 2012; Piscitelli et al., 2011; Tou, Jaczynski, & Chen, 2007).

When oil is extracted from a vegetable or animal source some proteins and peptides pass to the oil. The amount present can, however, be strongly reduced by refining the oil (Crevel, Kerkhoff, & Koning, 2000; Rigby et al., 2011). Oils extracted from crustacean species like Antarctic krill and *C. finmarchicus* are usually not refined to any extent since they contain desirable components like phospholipids and/or astaxanthin which are lost during traditional refining steps (Gunstone, 2004). Crustacean oils may therefore contain proteins that could cause reactions in people with crustacean allergies.

There is apparently no established method for extracting proteinous materials present in oils (Martín-Hernández, Bénet, & Obert, 2008). The proteins are commonly extracted with different aqueous buffers (Crevel et al., 2000; Ramazzotti et al., 2008) or precipitated using acetone or acetone together with hexane (Hidalgo, Alaiz, & Zamora, 2001; Martín-Hernández et al., 2008). When

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determining the protein content in oils, amino acid analysis of extracted proteins is often recommended instead of using colorimetric methods (Hidalgo et al., 2001; Martín-Hernández et al., 2008; Ramazzotti et al., 2008).

The main objective of this study was to investigate if TM could be detected in proteins extracted from commercial crustacean oil samples with the use of Western blotting. In addition, we also examined if the protein content in the oils could be estimated by direct amino acid analysis without prior extraction of proteins.

2. Materials and methods

2.1. Samples

Krill meal and Superba™ Krill Oil (KO) from Antarctic krill were provided by Aker Biomarine AS, Norway. Commercially available Superba™ Krill Oil capsules (KOc) were obtained from a local supermarket. Calanus® Oil (CO) from *C. finmarchicus*, Calanus® Oil capsules (COc) and whole frozen *C. finmarchicus* were supplied by Calanus AS, Norway. Fresh boiled shrimp (*Pandalus borealis)* were obtained locally and the muscle was recovered manually.

2.2. Extraction of proteins from oils

Proteins in KO and CO samples were extracted using a modified version of the acetone-hexane method described by Martín-Hernández et al. (2008). To 25 g of oil 62.5 ml cold acetone–heptane (AH, 1:1 w/w) was added. The mixture was shaken vigorously every 10 min for 1 h at 0 °C. It was then left on ice overnight in a refrigerator and subsequently centrifuged at 4000*g* and 4 °C for 20 min. The supernatant was discarded and the precipitate washed 6 times with vigorous shaking before centrifugation. Calanus oil samples were first washed with 30 ml AH followed by washing with 10 ml acetone-methanol (AM, 1:1 w/w) and subsequently 5 ml AH. The mixtures were centrifuged at 4000*g* and 4 °C for 20 min and the supernatant discarded between each wash. The final three washings steps were performed with 5 ml of AH, AM, and AH, respectively. The mixtures were centrifuged at 15000*g* and 4 °C for 20 min and the supernatant discarded after each wash. Krill oil samples followed the same procedure, however due to large amounts of polar lipids (phospholipids) the precipitate was washed with AM, AH, and AM in the first three washing steps followed by AM, AH, and AM as the final 3 washes. The pellets were left to air dry and samples intended for SDS-PAGE were dissolved in 500 µl sample buffer (25% glycerol, 2% SDS, 0.1% bromphenol blue, 14.4 mmol 2-mercaptoethanol, 60 mM Tris/HCl, pH 6.8). Separate protein samples intended for amino acid analysis were extracted in the same manner.

Oils in the commercial capsules were obtained by using a syringe. Each oil sample (10 g) was submitted to the same protein extraction procedure as described above using the same oil to solvent ratios. The final pellets were air dried and dissolved in 200 µl electrophoresis sample buffer.

As positive controls, 25 g of CO were spiked with 50, 200, 500, and 1000 µg bovine serum albumin (BSA, Standard, Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL, USA). The proteins in the oils were extracted as described and dissolved in 500 µl sample buffer. As a second control, BSA was dissolved directly in sample buffer to a concentration of 1 mg/ml.

2.3. Extraction of proteins from krill meal, C. finmarchicus and shrimp muscle

The proteins present in krill meal, frozen *C. finmarchicus* and shrimp muscle were extracted using the trichloroacetic acid (TCA)-acetone method of Méchin, Damerval, and Michel (2007) with minor modifications. A TCA-acetone extraction solution (10% TCA, 0.07% 2-mercaptoethanol dissolved in cold acetone) was made fresh and kept at −20 °C before each extraction. The acetone washing solution (0.07% 2-mercaptoethanol in cold acetone) was also stored at −20 °C prior to use.

Raw frozen *C. finmarchicus* (0.4 g) was added 3.6 ml of ice-cold extraction solution and homogenised with a T25 Ultra-Turrax (IKA Laboratory and Analytical Equipment, Staufen, Germany) for 30 s while kept on ice. The sample was stored at −20 °C for 2 h before centrifugation at 20000*g* and 4 °C for 15 min. The supernatant was removed and the pellet suspended in 1.8 ml cold acetone washing solution. The sample was kept at −20 °C overnight before centrifugation and removal of supernatant. The washing step was repeated twice with an incubation time of 1 h at −20 °C before centrifugation. After the final washing step the pellet was dried under nitrogen. The sample was added 1 ml of 10% SDS and heated at 90 °C for 10 min before being diluted 1 + 1 in SDS electrophoresis sample buffer. Samples of krill meal and shrimp muscle were extracted in the same manner. The krill meal sample was added 3 ml of 10% SDS due to the high protein concentration and heated at 90 °C for 10 min before being diluted 1 + 1 in SDS electrophoresis sample buffer.

2.4. Determination of protein by amino acid analysis

The protein content in KO and CO and in the protein pellets extracted from the oils by the AH method were determined by amino acids analysis. KO (200 mg) and CO (600 mg) were diluted to 1.1 ml with distilled water and 0.1 ml of 20 mM norleucine (internal standard) was added. The protein pellets extracted from 25 g of oils were added 1.0 ml distilled water and 0.2 ml of 20 mM norleucine. Finally, in all samples, 1.2 ml of 12 M hydrochloric acid (HCl) was added and the mixture was flushed with N_2-gas for 15 s before hydrolysis at 110 °C for 24 h (Moore & Stein, 1963). Aliquots of the supernatants were dried under N_2-gas and dissolved in a 0.2 M lithium citrate buffer (pH 2.2) prior to analysis. The analysis of amino acids was performed using a Biochrom 30 amino acid analyser (Biochrom Co, Cambridge, UK) and the UV signals were analysed by Chromeleon software (Dionex, Sunnyvale, CA, USA) and compared with A9906 physiological amino acids (Sigma Chemicals Co, St. Louis, MO, USA). Protein content in the samples was calculated as the sums of the individual molecular weight of the amino acid residues after the deduction of the molecular weight of water according to recommendations by FAO (2003).

2.5. SDS-PAGE and Western blotting

The extracted proteins dissolved in sample buffer were heated to 90 °C for 10 min and analysed by SDS-PAGE performed basically according to Laemmli (1970) using 4–12% NuPAGE® Novex® Bis-Tris polyacrylamide gels in an XCell *SureLock*™ Mini-Cell (Life Technologies Corporation, Carlsbad, CA, USA) with MES-buffer, 200 V for 45 min at room temperature. SeeBlue® Plus2 Pre-Stained Standard (Life Technologies) was used as a molecular weight marker. The gel was stained with Coomassie blue (SimplyBlue™ SafeStain, Life Technologies Corporation, Carlsbad, CA, USA).

The protein content in the SDS-PAGE and Western blotting samples originating from the oils, including oils from the capsules, were based on the amino acid analysis of the protein pellets extracted from the KO and CO as described above. The protein content in the electrophoresis samples extracted from krill meal and *C. finmarchicus* was estimated on basis of amino acid analysis of krill meal and *C. finmarchicus*. In the shrimp muscle sample, the protein content was based on dry weight of the extracted pellet.