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Bio/sonochemical conversion of fish backbones into bioactive nanospheres

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ABSTRACT

Salmon backbones, co-streams of salmon processing industry, were transformed into stable, odour-free ingredients for cosmetics. First, the backbones were hydrolysed using commercial proteases (Bromelain + Papain, Trypsin, Corolase® 7089 and Protamex®) in order to accomplish the release of fish protein hydrolysates (FPH), which showed antioxidant activity and aptitude to inhibit skin-degrading and inflammatory enzymes. However, due to the FPH instability in aqueous solution and propensity for microbial contamination, their bioactive properties were entirely lost only after 24 h. To overcome the low stability and prevent the effect loss, a sonochemical technology was then employed to transform the FPH into stable tea tree oil-filled bioactive peptide-shell nanospheres (NS). Such transformation boosted the FPH antioxidant potential, which was further reflected in protection of fibroblasts from UV damage. In the form of NSs, the FPH resisted microbial contamination for more than 6 months and presented antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. In addition, the fish odour was eliminated after the NSs processing, thus addressing this important challenge for using fish raw materials in cosmetics. This work suggests an alternative high value use of the fishery co-streams and expands their application potential beyond their current use as fish or animal feed.

1. Introduction

Salmon processing industry creates large quantities of co-streams that include by-catch and parts of the fish that normally are not processed into food (heads, livers, guts, skins and bones). Despite the high content of valuable components such as proteins and lipids these co-streams contain, they are usually discarded or converted into low market-valued products, e.g., animal feed, fish meal and fertilizers [1]. Salmon backbones, in particular, represent a significant part of the cut offs from the fish filleting process that is currently processed into fish meal due to the high content of minerals that positively affect the growth and feed efficiency of aquaculture fishes [2]. Taking into consideration the proteinaceous nature of these still under-utilised rest materials, their alternative use as a resource for nutraceuticals and cosmoceuticals is a growing focus of interest [3,4]. New and valuable compounds can be extracted and purified from these co-streams and upgraded with technologies of varying complexity. One strategy that has been widely applied is the enzymatic hydrolysis of proteins. By cutting the protein into smaller fragments, peptides with enhanced biological activity may be obtained [4]. Fish protein hydrolysates (FPH) have been reported to present good amino acid balance [1] and nutritional properties [5–7] as well as antioxidative, antimicrobial, antihypertensive, anti-thrombotic and immunomodulatory activities [1]. However, the fish odour and low stability in aqueous medium, frequently associated with lipid oxidation and microbial contamination, pose a problem for their further exploitation [8].

Encapsulation techniques are commonly applied to address the stability challenge [9]. Multiple synthetic approaches are available to generate nanomaterials able to encapsulate bioactive compounds, but the use of methods such as the ultrasonic emulsification technique is gaining ground. This method involves mixing of a two-phase system at the oil/water interface with high intensity ultrasonic waves. An emulsion comprising oil-filled nanospheres (NSs) can be obtained in which the bioactive agent is found at the interface of the droplet, i.e., not encapsulated but the shell itself

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[10]. Besides maintaining the stability, nanospherisation assists the insertion of otherwise not penetrable bioactive agents in the dermis of the skin. In fact, the barrier function and lipophilic nature of the stratum corneum makes difficult the delivery of any large hydrophilic molecule into skin [11]. Thus, bioactive components processed into NSs may easily reach cellular proteins such as collagen and elastin as well as fibroblasts, all involved in the ageing process [12]. In addition, properties such as the small and uniform NSs size, large surface area to mass ratio and high reactivity aid to the desired application.

Skin is comprised of two main compartments: the avascular epidermis made up mostly by keranocytes and the vascularised matrix-rich dermis composed by sparsely distributed fibroblasts, which are responsible for the synthesis of dermal extracellular matrix (ECM) components [13]. With ageing, repetitive exposure to UV radiation or due to cutaneous inflammatory disease, major structural and functional changes occur in the ECM components, including the degradation of fibrillar collagens, elastic fibres (elastin and laminin) and loss of the oligosaccharide fraction [14]. Although known for their remarkable longevity, these biomolecules are affected by long-term and accumulated damage, which in turn results in the lowering of skin mechanical strength and elasticity [12,15]. The mechanisms that cause dermal damage ultimately lead to: (i) the up-regulation of the ECM-degrading enzymes such as collagenases and elastases, and (ii) cutaneous biomolecules damage that induces the overproduction of reactive oxidative species (ROS) and oxidative myeloperoxidase (MPO), which additionally damage proteins and DNA All these factors strongly contribute to skin ageing and/or development of many inflammatory skin disorders [14,16]. Therefore, an efficient strategy for skin care should comprise the control of collagenase, elastase and MPO activities, as well as the inhibition of free-radical formation and damage in fibroblasts.

This work aimed to obtain skin care ingredients from defatted salmon co-streams implementing a two-step valorisation process that combines enzymatic hydrolysis to enrich the mixture with bioactive peptides and sonochemical stabilisation of the bioactives via their nanospherisation. Being mainly used for nutritional purposes, residues from fish bones have never been applied in skin care. The generation of bioactive agents from fish co-streams has the advantage of adding value to something that is currently either discarded or turned into comparatively low-value products.

2. Materials and methods

2.1. Materials

Fresh salmon (Salmo salar) backbones were obtained from local fish shop in Trondheim (Norway) where fish was hand filleted. The raw materials were kept on ice before processing. Commercial protease products such as Corolase® 7089 (AB Enzymes GmbH), Protamex® (Novozymes A/S), Papain FG (Enzybel), Bromelain 400 GDU/g (Enzybel) and Trypsin (Sigma–Aldrich) were received from the producer and used to produce FPHs. AlamarBlue® Cell Viability Reagent was purchased from Invitrogen, Life Technologies Corporation (Spain). Human foreskin fibroblasts cell line BJ-5ta (ATCC-CRL-4001) and bacteria strains of *E. coli* (*E. coli*, ATCC 25922) and *Staphylococcus aureus* (*S. aureus*, ATCC 35556) were purchased from American Type Culture Collection (LGC Standards S.L.U, Spain). All other chemicals were analytical/reagent or synthetic grade and were obtained from Sigma–Aldrich (Spain) and used without further purification.

2.2. Production of FPH

Fresh salmon backbones were first minced and the oil fraction was separated by centrifugation after mild thermal treatment. The obtained mixture of soluble protein fraction and sediments was used for enzymatic hydrolysis using five commercially available proteolytic enzyme preparations: (i) Corolase® 7089 (Source: *Bacillus subtilisis*, main activity: neutral metallloendopeptidase); (ii) Protamex® (Source: *Bacillus licheniformis, Bacillus amyloliquefaciens*, main activity: alkaline serine endopeptidase); (iii) Papain FG (Source: *Carica papaya*, main activity: cysteine endopeptidase); (iv) Bromelain 400 GDU/g (Source: *Ananas comosus*, main activity: cysteine endopeptidase); and (v) Trypsin (Source: Bovine pancreas, main activity: broad specificity endopeptidase). The enzyme treatments were carried out for 120 min at 50 °C in distilled water (1:1 of raw material mass), using an enzyme dosage of 0.1 % (w/w) of the raw material mixture. After heat inactivation of enzymes, the residual oil, emulsion, fish protein hydrolysate (FPH) and sludge fractions were separated by centrifugation. The FPH fractions were freeze-dried. Unhydrolyzed protein extract was used as a reference sample: it represents the profile obtained from the soluble protein fraction sample before the addition of commercial enzymes.

2.3. Characterization of FPH

The protein content of FPH was determined using DC Protein Assay Kit (Bio-Rad) while the amount of amino acid cysteine was determined spectrophotometrically using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) following a procedure adapted from Ellman [17]. Briefly, 100 μL of a serial dilution of FPH in 0.5 M PB, pH 8.0, were added to 100 μL of Ellman's reagent solution (0.3 mg/mL) in the same buffer in a 96-well plate. The samples were incubated for 1 h at room temperature in dark with gentle shaking. Absorbance was then measured at a wavelength of 412 nm, and the amount of free thiol groups were determined from a calibration curve obtained using L-cysteine standards. The degree of hydrolysis was evaluated as the proportion (%) of free α-amino nitrogen (i.e., peptides and free amino acids) with respect to the total N in the sample. Analyses were performed in duplicate. Protein was precipitated using sulfosalicylic acid and the supernatant was diluted using doubly distilled water. The samples were then analysed using reversed phase High Performance Liquid Chromatography (HPLC) (SIL-9A Auto Injector, LC-9A Liquid Chromatograph, RF-530 Fluorescence HPLC Monitor, Shimazdu). The results were used to calculate the content of the separate amino acids for each hydrolysate.

2.4. Sonochemical preparation of FPH NSs and characterization

FPH NSs were prepared by an adaptation of Wong and Suslick method [18]. Briefly, a two-phase solution containing 70% of a mixture of 2 g/L FPH aqueous solution and 0.05% (w/w) poloxamer and 30% of tea tree oil (organic phase) was prepared and placed into a thermostated (8 °C ± 1 °C) sonicator cell. The NSs were synthesized with a high-intensity ultrasonic probe (Sonic and Materials, VC-600, 20 kHz Ti horn at 40% amplitude). The bottom of the probe was positioned at the aqueous-organic interface, employing a density of ~0.5 W/cm^3 for 3 min using an ice-cooling bath to maintain the low temperature. The resulted emulsion was kept at 4 °C for 24 h and the non-reacted organic solvent was removed by three washing cycles with water and centrifugation. After the synthesis, the NSs were separated from the unreacted biopolymer and tea tree oil by leaving the reaction mixture at 4 °C for 24 h. The NSs were washed and separated with sufficient volumes of distilled water by centrifugation at 800 rpm for 15 min.