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In vitro evidence for gut hormone stimulation release and dipeptidyl-peptidase IV inhibitory activity of protein hydrolysate obtained from cuttlefish (*Sepia officinalis*) viscera

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ABSTRACT

Two cuttlefish (*Sepia officinalis*) viscera protein hydrolysates were obtained with different enzymes extracted from cuttlefish and smooth hound (*Mustellus mustellus*). Their ability to stimulate the secretion of cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1), using the enteroendocrine STC-1 cell line, and to inhibit the DPP-IV activity during a simulated gastrointestinal digestion was assayed. The physico-chemical parameters of hydrolysates and their effects on intestinal cell viability were also determined. The hydrolysate obtained with cuttlefish enzymes (CVPH1) appeared to be the most promising for all assessed bioactivities. Thus CVPH1 was able to stimulate CCK and active GLP-1 releasing activities of enteroendocrine cells without any cytotoxicity and to inhibit DPP-IV activity. Moreover, these actions were enhanced after gastrointestinal digestion and CVPH1 was also able to inhibit the intestinal DPP-IV activity of Caco-2 cells. These very promising findings highlight, *via* two different mechanisms, the positive effect of CVPH1 on GLP-1 actions.

1. Introduction

In economically developed countries, lifestyles are characterized by excessive energy intake and consequent body weight increase leading to obesity that subjects to numerous diseases such as type 2 diabetes mellitus (T2DM), hypertension, cardiovascular diseases and respiratory problems. The development of new strategies to tackle this worldwide issue has lately become a major research field. Dietary proteins *via* their hydrolysis products have been identified to generate the highest satiety effect compared to lipids and carbohydrates (Bensaid et al., 2002; Foltz et al., 2008). During the digestion of food proteins, peptide sequences are generated under the action of digestive proteases in the different compartments of the gastrointestinal tract and can be involved in many physiological processes such as energy homeostasis through the regulation of food intake. Among the many effects of food-derived peptides on intestinal functions, numerous studies have shown that the biologically active peptides generated during the digestive process lead to the reduction of food intake by the stimulation of peptide hormone secretion such as cholecystokinin (CCK) and the glucagon-like peptide 1 (GLP-1), by the enteroendocrine cells (I and L) scattered along the intestine (Gardiner, Jayasena, & Bloom, 2008). CCKs are involved in a number of physiological functions, leading to food intake depletion and meal termination. Those functions include the stimulation of pancreatic secretion, the inhibition of gastric emptying and the stimulation of the gallbladder contraction (Moran & Kinzig, 2004). GLP-1 has been identified as an anorexigenic hormone and regulates food intake. As a glucose-dependent insulinotropic polypeptide (GIP), GLP-1 is also an incretin hormone that amplifies the secretion of insulin. It is likewise implicated in the glucose metabolism as a stimulator of pancreatic β-cell proliferation and as a glucagon secretion inhibitor (Brubaker & Anini, 2003; Kieffer & Habener, 1999; Turton et al., 1996). The physiological actions of intestinal hormones are then mediated via endocrine, paracrine and neurocrine ways (Moran & Dailey, 2011).

Among the protein sources, derived peptides from marine origin obtained by controlled hydrolysis are of a great interest due to the wide range of biological activities identified such as anti-hypertensive, antimicrobial, or opioid effects. Among these activities several recently

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reviewed studies (Cudennec & Ravallec, 2013) have shown the interesting potential of marine peptides to participate in energy metabolism *via* the regulation of food intake through different mechanisms e.g. acting as gut peptide receptors agonists or as CCK and GLP-1 secretion stimulating peptides.

GLP-1 and GIP activities on glucose homeostasis are regulated by the dipeptidyl peptidase IV (DPP-IV) that cleaves and *de facto* inactivates both hormones following their secretion (Drucker, 2006). The DPP-IV protease exists in a transmembrane protein active form, expressed in numerous tissues and cell types including the epithelial cells of the intestine, and in soluble active form in serum and several fluids such as saliva, seminal fluids and bile. DPP-IV is involved in several other regulatory processes such as chemokines and neuropeptides (Mulvihill & Drucker, 2014). The incretin effect is drastically reduced or lost in T2DM. Lately, DPP-IV inhibitors have indeed been considered as an innovative class of agents for the treatment of T2DM due to their actions on the enhancement of GLP-1 availability and on the recovery of the incretin effect. In this way, incretin-based therapies based on the oral administration of DPP-IV inhibitors (gliptins) are the most recent alternative treatments of T2DM as recently reviewed (Godinho et al., 2015). Nowadays, numerous works have focused on the strategy to identify "natural" peptide inhibitors of DPP-IV activity as an alternative for synthetic drugs to restore the incretin effect in T2DM. These peptides could be obtained by processing food (protein hydrolysis) or by proteolysis occurring during the digestive process of dietary proteins. In this way, numerous studies had first reported that milk derived products, like whey and dairy protein hydrolysates, could cause DPP-IV inhibition *in vitro* and could be a good source of DPP-IV inhibitors (Lacroix & Li-Chan, 2012; Nongonierma & FitzGerald, 2013a, 2013b; Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013; Tulipano, Sibilia, Caroli, & Cocchi, 2011). Other works found DPP-IV inhibitory peptides from different protein sources like defatted rice bran hydrolysates (Hatanaka et al., 2012). Regarding marine sources, the DPP-IV inhibitory activities of peptides obtained from fish, such as tuna cooking juice hydrolysate and barbell skin gelatin, and from macroalga like the protein extract hydrolysate of *Palmaria palmata* were identified (Harnedy, O'Keeffe, & FitzGerald, 2015; Huang, Jao, Ho, & Hsu, 2012; Sila et al., 2015).

Cuttlefish (*Sepia officinalis*) is one of the most exploited marine species in the Mediterranean and Atlantic waters. In the Mediterranean, the Gulf of Gabes is the main site for cuttlefish resources and landings occur essentially in the fishing port of Sfax (southeast of Tunisia). In Tunisia, cephalopod processing is one of the important areas in the marine industry and frozen cuttlefish is mainly exported to Asian countries, especially, Japan. During the processing of cuttlefish, large quantities of waste, including viscera, are generated and discarded. These by-products are approximately 30% of the raw original material that is not utilized, causing serious environmental pollution and an important commercial loss (Balti, Barkia, Bougatef, Ktari, & Nasri, 2009). Cuttlefish viscera contain high levels of digestive proteolytic enzymes and proteins, making them a suitable source from which proteases for food application can be recovered. In previous works, we purified and characterized trypsin, chymotrypsin and cathepsin D from the hepatopancreas of cuttlefish (*S. officinalis*) (Balti et al., 2009, 2012; Balti, Hmidet, et al., 2010).

In this work, we assess the potential of two by-product hydrolysates obtained with different enzymes extracted from cuttlefish and smooth hound to regulate the secretion of CCK and GLP-1, using the enteroendocrine STC-1 cell line and to inhibit the DPP-IV activity during a simulated gastrointestinal digestion. We compare this with a non-hydrolyzed by-product as control. The mode of DPP-IV activity inhibition of the most promising hydrolysate has been assayed and its ability to inhibit DPP-IV activity of intestinal human Caco-2 cells has been evaluated. At the same time, the physico-chemical parameters of the hydrolysates and their effect on intestinal cell viability have been determined.

2. Materials and methods

2.1. Material and chemicals

Cuttlefish by-products were obtained from the seafood processing company "Calembo" (Sfax, Tunisia). The collected by-products were washed twice with water to eliminate the dark ink and then packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (*w*/*w*), and transported to the research laboratory within 30 min. Upon arrival, the cuttlefish by-products were separated, and the viscera only were collected and stored in sealed plastic bags at −80 °C for later use.

Dulbecco's modified Eagle's Medium (DMEM), fetal calf serum, and all other cell culture reagents were purchased from PAN-Biotech GmbH (Aidenbach, Germany). All other chemicals were of reagent grade.

2.2. Obtaining cuttlefish by-products hydrolysates

2.2.1. Fish digestive proteases

Crude protease extracts from smooth hound (*M. mustelus*) intestines (Balti et al., 2009; Bougatef, Balti, Jellouli, Triki-Ellouz, & Nasri, 2008; Kembhavi, Kulkarni, & Pant, 1993) and cuttlefish (*S. officinalis*) hepatopancreas (Balti et al., 2009; Bougatef et al., 2008; Kembhavi et al., 1993), prepared in our laboratory were used for the production of protein hydrolysates. Protease activity was determined according to the method of Kembhavi et al. (Balti et al., 2009; Bougatef et al., 2008; Kembhavi et al., 1993), using casein as a substrate. One unit of protease activity was defined as the amount of enzyme required to release 1 μg of tyrosine per minute under the experimental conditions used.

2.2.2. Preparation of cuttlefish viscera protein hydrolysates

Cuttlefish viscera (500 g) in 1000 mL distilled water were first minced using a grinder (Moulinex Charlotte HV3, France), and then cooked at 90 °C for 20 min to inactivate endogenous enzymes. The cooked by-product sample was then homogenized in a blender for about 2 min and hydrolyzed with enzymes in optimal conditions (pH 8.0 and 50 °C). The enzyme was added to the reaction at the same enzyme/protein ratio (E/S = 15 $U \cdot mg^{-1}$) to compare hydrolytic efficiencies. During the reaction, the pH of the mixture was maintained constant by the continuous addition of a 4 M NaOH solution. The hydrolysis was carried out for 4 h and the reaction was stopped by heating the solution at 80 °C for 20 min to inactivate the enzyme. After cooling at room temperature, the cuttlefish viscera protein hydrolysates were then centrifuged (500 g) for 20 min to separate insoluble and soluble fractions. Finally, the soluble phase was freeze-dried using a freeze-dryer (Bioblock Scientific Christ ALPHA 1-2, IllKrich, France) and stored at −20 °C for further use.

2.2.3. Determination of the degree of hydrolysis

The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds broken (h) to the total number of peptide bonds per unit weight (h_{tot}) was in each case, calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis as shown below (Adler-Nissen, 1986).

$$DH(\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times Nb}{MP} \times \frac{1}{\alpha} \times \frac{1}{h_{tot}} \times 100$$

where B is the amount of NaOH consumed (mL) to keep the pH constant during the reaction. *Nb* is the normality of the base, *MP* is the mass (g) of protein (N × 6.25), and α is the average degree of dissociation of the α-NH_2 groups released during hydrolysis expressed as:

$$\alpha = \frac{10^{pH-pK}}{1 + 10^{pH-pK}}$$