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Effect of dry-cured ham maturation time on simulated gastrointestinal digestion: Characterization of the released peptide fraction

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ABSTRACT

This study deals with the identification of the peptides released after in vitro simulated gastrointestinal digestion of dry-cured Parma hams, using a physiological digestion model in terms of number of steps and composition of digestive juices. The obtained peptide mixture was analysed by ultra-high performance liquid chromatography with single quadrupole mass spectrometer detector and liquid chromatography hyphenated with triple quadrupole mass spectrometry and LTQ-orbitrap. This approach allowed for the identification of up to 81 different peptide sequences, mainly originating not only from myofibrillar proteins but also from sarcoplasmic proteins: the MW range spans between 200 and 1700 Da, with a high number of very short sequences (21 dipeptides and 12 tripeptides). Several identified released peptides are precursors of potentially bioactive sequences. The effect of the maturation time of the ham on the peptide profile obtained upon digestion was assessed: Principal Component Analysis allows for differentiating between 18 months and 24 months aged hams, underlying the importance of maturation on the digestibility of meat proteins and on the eventual release of bioactive sequences.

1. Introduction

Dry-cured hams are meat products mostly appreciated by consumers for their organoleptic properties; they also represent an important example of traditional methods to preserve, and eventually improve, the nutritional properties of meat. They are produced by pork thighs properly trimmed, salted and aged: their typical final characteristics are the results of proteolysis and lipolysis phenomena, produced by the action of endogenous muscle enzymes (Rodriguez, Aristoy, & Toldrà, 1995; Toldrà, 1998). In particular, proteolysis generates a considerable amount of small peptides and free amino acids, through the breaking of myofibrillar proteins (such as myosin, actin, troponin, tropomyosin) by endoproteases (calpains and cathepsins) and, subsequently, by exoprotease (peptidase and aminopeptidase). These processes acquire particular importance in long aged products such as Parma ham, one of the most known dry cured meat products, obtained by heavy pig thighs (12–14 kg), mildly salted and aged for a long period (at least 12 months, but usually commercialized later). Apart from its role in determining the final organoleptic and textural properties, the proteolysis process has also aroused great interest because of the potential functional properties of the released peptides. Indeed, several studies have reported health related activities of biopeptides derived from food protein sources of animal origin, in particular from dairy proteins (Haque, Chand, & Kapila, 2009; Meisel & FitzGerald, 2003). These bioactive peptides have been defined as "food components that may influence biological processes and thus have an impact on body functions or conditions and finally on health" (Roberts & Zaloga, 1994). Their activity depends on the composition of the amino acid sequence and on its length, and many peptides have shown multifunctional properties (on the cardiovascular, digestive, immune and nervous system) (Kitts & Weiler, 2003). In this contest, a number of research investigations are currently focusing on the identification of functional biopeptides from meat proteins (Udenigwe & Howard, 2013) as meat is widely used as a nutritional source of high quality proteins (Hoffmann, 1993).

Potential antioxidant (Samaranayaka & Li-Chan, 2011) and antihypertensive (Escudero, Sentandreu, Arihara, & Toldra, 2010) peptides have been identified in meat from various sources (Escudero, Mora, Fraser, Aristoy, & Toldrá, 2013; Escudero et al., 2013; Jang & Lee, 2005; Lafarga & Hayes, 2014): as far as pork meat, it has been demonstrated that biopeptides may be released by meat proteins by the action of endogenous or exogenous peptidases during processing (Lafarga & Hayes, 2014). Peptides exhibiting in vitro antioxidant activities have been characterized in water soluble fraction from dry cured Spanish hams

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(Escudero, Aristoy, Nishimura, Arihara, & Toldrá, 2012; Escudero, Mora, Fraser, Aristoy, Arihara, et al., 2013; Mora, Escudero, Fraser, Aristoy, & Toldrá, 2014). Antihypertensive peptides have also been identified and their activity has been proven both in vitro as well as on spontaneously hypertensive rats (Escudero, Mora, Fraser, Aristoy, & Toldrá, 2013; Escudero et al., 2012).

It is worth noting that biopeptides may exert their physiological actions both in the intestinal lumen as well as upon crossing intestinal epithelia and reaching blood circulation: thus, their modifications by the gastrointestinal digestion process, the transport mechanism across the intestinal epithelium and the enzymatic modifications taking place there, all have to be considered.

Indeed, some peptides found to be bioactive in in vitro tests failed to exert any effect in vivo, while, on the contrary, peptides that were not found active in in vitro tests have proven to be active in animal experiments (Iwaniak, Minkiewicz, & Darewicz, 2014).

These results can be related to degradation/modification of a peptide due to the action of enzymes existing in the digestive tract or to tractor of intracellular peptidases or to biotransformation in the blood serum or in the liver (Iwaniak et al., 2014; Yamada, Matoba, Usui, Onishi, & Yoshikawa, 2002).

Thus, the application of in vitro digestion models is a necessary step to study the release of potentially bioactive and functional peptides from food proteins and to identify the most interesting sequences (Bax et al., 2013; Bordoni et al., 2014; Storcksdieck, Bonsmann, & Hurrell, 2007; Weizheng, Feibai, Mouming, Bao, & Chun, 2011), taking also in consideration the eventual effect of processing or maturation time of food products on the general digestibility of proteins and on the release of these compounds.

Approaches using in vivo models were recently reported, which are particularly interesting but also difficult to replicate (Bauchart et al., 2007). Reliable in vitro digestion models, with a good level of approximation in simulating the real gastrointestinal conditions, may help in better understanding the phenomenon. Comparing in vitro and in vivo results often reveals important analogies in terms of released peptides, on account of the fact that the fundamental enzymes used in in vitro experiments (pepsin, chimotrypsin and trypsin) are the same operating in vivo (Escudero, Sentandreu, & Toldrà, 2010).

In any case, significant differences were observed between different in vitro studies, on account of the specific food component being analysed, the nature of the food matrix, and the sophistication of the in vitro digestion model used (Hur, Lim, Decker, & McClements, 2011), particularly as far as the composition of the digestive fluids used in each step, e.g., enzymes, salts, buffers, biological polymers, and surface-active components. Very often, digestive models applied to study peptide release in dry-cured hams are simplified models, based only on the use of gastric and duodenal enzymes at the correct pH typical of each environment (Escudero, Sentandreu, Arihara, et al., 2010; Escudero, Sentandreu, & Toldrà, 2010). This simplified approach is very useful in order to identify bioactive sequences to synthesize and test as future functional ingredients, but more physiological digestion model may allow to obtain better elucidation of the potential functionality of a complex food. Recently, a human simulated digestion model was applied to the in vitro digestion of Bresaola proteins (bovine meat) to characterize the peptide sequences released during the process and to identify bioactive released sequences (Ferranti et al., 2014). Also, a standardized model was very recently proposed by Minekus et al., based on the same principles and aiming at defining experimental conditions strictly mimicking the real human gastrointestinal environment (Minekus et al., 2014).

The aim of this work is to apply an in vitro physiological digestion model to characterize the peptides released upon digestion of dry-cured Parma hams of different ageing times, thus elucidating the effect of proteolysis during maturation on the peptide profile generated by digestion and to eventually identify potentially bioactive sequences.

2. Materials and methods

2.1. Samples

Dry-cured ham samples (n = 27) with 18 and 24 ripening months, were provided by the Experimental Station for Food Preserving Industry (Parma). Samples of biceps femoris muscle were minced by a common mill (Moulinex, Milano, Italia) and stored at freezing temperature (−22 °C) until analysis.

2.2. Reagents and solvents

Bidistilled water was produced in our laboratory by a Millipore Alpha Q purification system (Waters, Billerica, MA, USA). Pepsin from porcine gastric mucosa, trypsin from porcine pancreas, α-chymotrypsin from bovine pancreas, α-amylase from barley malt (type VIIIa), uric acid, mucin from porcine stomach (type III), glucose, glucuronic acid, glucosamine hydrochloride, bovine serum albumin, pancreatin from porcine pancreas, lipase from porcine pancreas (type II), bovine and ovine bile, sodium dihydrogen phosphate, potassium chloride, sodium hydroxide, sodium phosphate dibasic dodecahydrate, monobasic potassium phosphate, acetonitrile, methanol, urea, L-phenylalanyl-L-phenylalanine (Phe-Phe), acetic acid, and hydrochloric acid (0.1 N) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (37% v/v), sodium hydroxide, sodium hydrogen carbonate, calcium chloride, ammonium chloride, potassium thiocyanate, and potassium hydroxide were purchased from Carlo Erba (Milan, Italy). Formic acid was purchased from Acros Organics (Geel, Belgium). Sodium chloride was purchased from AnalaR Normapur (Milan, Italy). Sodium sulfate was purchased from Riedel de Haen (Seelze, Germany). Magnesium chloride hexahydrate was purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). SDS-PAGE standards Broad Range, XT Reducing Agent, XT sample buffer 4×, XT MES Running Buffer 20×, and Coomassie Brilliant Blue R-250 were purchased from BIO-RAD (Hercules, CA, USA).

2.3. Extraction of the peptide fraction (MW < 10 kDa)

To 5 g of minced dry-cured ham 45 ml of a HCl 0.1 N solution and 250 μl of a 1 mM Phe-Phe aqueous solution were added and the mixture was homogenized for 1 min with Ultra Turrax T50 Basic (IKAWerke, Staufen, Germany) at 322 g. After centrifugation at 4 °C (3200 g for 1 h), the supernatant was purified by a series of filtration steps using different filters (paper, 5 μm type SMWP, 0.45 μm type HPLV). 4 ml of the obtained extract was evaporated to dryness under vacuum by rotary evaporator and the residue was dissolved with 2 ml of a 0.1% formic acid aqueous solution. This solution was then filtered on a Vivaspin 2 (Sartorius, Goettingen, Germany) filtration system equipped with 10 kDa cut off filters, at room temperature and 5423 g for 45 min. The filter was washed 3 times with 2 ml of a 0.1% formic acid aqueous solution. The filtered sample was evaporated to dryness under vacuum by rotary evaporator and the residue dissolved in 150 μl of a 0.1% formic acid aqueous solution and centrifuged at 4 °C, at 16060 *g*, for 10 min before the UPLC–ESI-MS analysis.

2.4. SDS-PAGE analyses

Extracted samples: 5 μl of extract filtered at 0,45 μm was evaporated to dryness under nitrogen flux and dissolved in 25 μl of reducing sample buffer 1×. Digested samples (after clean-up): 500 μl of digested sample was evaporated under nitrogen flux and dissolved in 25 μl of reducing sample buffer 1×.

Reducing sample buffer was composed by 5% of XT Reduce Agent in sample buffer 1×. The running buffer (1×) was obtained by dilution of XT MES Running Buffer 20× with bidistilled water. Coomassie staining solution: 1 litre of solution was prepared by dissolving 1 g of Coomassie