International Journal of Food Microbiology 212 (2015) 9–15

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Small peptides hydrolysis in dry-cured meats

Leticia Mora [a], Marta Gallego [a], Elizabeth Escudero [a], Milagro Reig [b], M-Concepción Aristoy [a], Fidel Toldrá [a,*]

[a] Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Paterna, Valencia, Spain
[b] Instituto de Ingeniería de Alimentos para el Desarrollo, Universidad Politécnica de Valencia, Spain

ARTICLE INFO

Available online 17 April 2015

Keywords:
Proteolysis
Peptides
Proteomics
Enzymes
Peptidases

ABSTRACT

Large amounts of different peptides are naturally generated in dry-cured meats as a consequence of the intense proteolysis mechanisms which take place during their processing. In fact, meat proteins are extensively hydrolysed by muscle endo-peptidases (mainly calpains and cathepsins) followed by exo-peptidases (mainly, tri- and di-peptidyl peptidases, dipeptidases, aminopeptidases and carboxypeptidases). The result is a large amount of released free amino acids and a pool of numerous peptides with different sequences and lengths, some of them with interesting sequences for bioactivity. This manuscript is presenting the proteomic identification of small peptides resulting from the hydrolysis of four target proteins (glyceraldehyde-3-phosphate dehydrogenase, beta-enolase, myozenin-1 and troponin T) and discusses the enzymatic routes for their generation during the dry-curing process. The results indicate that the hydrolysis of peptides follows similar exo-peptidase mechanisms. In the case of dry-fermented sausages, most of the observed hydrolysis is the result of the combined action of muscle and microbial exo-peptidases except for the hydrolysis of di- and tri-peptides, mostly due to microbial di- and tri-peptidases, and the release of amino acids at the C-terminal that appears to be mostly due to muscle carboxypeptidases.

1. Introduction

Dry-cured meat products include a variety of processed meats where drying and ripening is involved. A first group is composed of dry-cured ham and dry-cured loin where main observed biochemical changes are due to the action of endogenous muscle enzymes whereas a second group includes a wide variety of semidry- and dry-fermented sausages where the observed changes are the result of a joint action of muscle and microbial enzymes (Toldrá and Reig, 2015). During processing, meat proteins are extensively hydrolysed by muscle endo-peptidases (mainly calpains and cathepsins) followed by exo-peptidases (mainly, tri- and di-peptidyl peptidases, dipeptidases, aminopeptidases and carboxypeptidases) (Toldrá, 2002). This extensive hydrolysis and the resulting identified peptides have been reported in dry-cured ham for several proteins such as creatin kinase (Mora et al., 2009), troponin T (Mora et al., 2010), glycolytic enzymes (Mora et al., 2011) and myoglobin (Mora and Toldrá, 2012). The result is a large amount of released free amino acids and a pool of numerous peptides with different sequences and lengths, some of them containing proline, phenylalanine, and tyrosine for antihypertensive activity or others rich in histidine and proline for antioxidant activity. Of course, the extent of proteolysis and the final quality will depend on a large number of variables related to the raw materials, enzyme activity, microbial population and type of microbial starter, and processing conditions.

Starter cultures have got an extended use worldwide for meat fermentation in recent decades (Leroy et al., 2015). The starter cultures usually consist of lactic acid bacteria alone or in combination with staphylococci and/or *Kocuria*, and the possible addition of yeasts or moulds. All these microorganisms contain relevant amounts of enzymes which are responsible of the enzymatic breakdown of carbohydrates, proteins and lipids (Cocconcelli and Fontana, 2015; Flores and Toldrá, 2011). Dry-cured meat products exhibit an intense proteolysis where most reported changes are based on following the protein breakdown or the generation of free amino acids as final outcome of proteolysis. For instance, several lactobacilli like *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum* and *Lactobacillus casei*, exhibited an intense action on sarcoplasmic and myofibrillar extracts using the whole cell, cell free extract and combinations of both (Fadda et al., 1999a,b, 2002; Sanz et al., 1999a,b), and an increase in free amino acids was reported for the yeast *Debaryomyces hansenii* acting on sarcoplasmic protein extracts (Santos et al., 2001). Proteolytic activity has been also detected in coagulase negative staphylococci and one isolate of *Kocuria* spp (Mauriello et al., 2004). The strains *Staphylococcus carnosus* and *Staphylococcus simulans* were reported to be able to hydrolyse sarcoplasmic but not myofibrillar proteins (Casaburi et al., 2005). However, in other staphylococci species no protease activity was detected but low aminopeptidase and high esterase activity (Casaburi et al., 2006). Further, several strains of *Penicillium* showed proteolytic activity in a culture media (Ockerman et al., 2001) and, more specifically, *Penicillium chrysogenum* showed proteolytic activity against sarcoplasmic and myofibrillar proteins (Benito et al., 2003).

* Corresponding author. Tel.: +34 963900022; fax: +34 963636301.
E-mail address: ftoldra@iata.csic.es (F. Toldrá).

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.04.018
0168-1605/

Peptide transporters and peptidases were found generally ubiquitous in *L. sakei* (Freiding et al., 2011). In fact, several di-peptidyl peptidases, dipeptidases and aminopeptidases have been characterised in lactic acid bacteria essentially from *L. sakei* (Montel et al., 1995; Sanz and Toldrá, 1997, 2001, 2002; Sanz et al., 1998), *L. plantarum*, *Lactobacillus brevis* and *L. casei* subesp *casei* (Bintsis et al., 2003; González et al., 2010; Herreros et al., 2003; Macedo et al., 2010) or from the yeast *D. hansenii* (Bolumar et al., 2003a,b, 2008). *Leuconostoc mesenteroides* and *L. curvatus* strains have been reported to show high aminopeptidase and X-prolyl dipeptidyl aminopeptidase activity although the enzymatic activity may vary between strains of *L. plantarum*, *Lactobacillus pentosus* and *Weissella cibaria* (Zotta et al., 2007). In general, these exo-peptidases are involved in the release of small peptides and generation of free amino acids that affect flavour development but also could contribute to the generation of some bioactive peptides.

Endogenous muscle proteinases were reported to be the responsible for the released polypeptides after comparing the RP-HPLC chromatographic profiles of the starter inoculated sausages and the controls (Hughes et al., 2002) corroborating previous findings (Molly et al., 1997). However, very little is known about the small peptides generated as finishing intermediate products. A recent manuscript provided a first insight on some of the small peptides generated in Argentinian fermented sausages and gave some hypothetical potential routes for their generation (López et al., 2015).

This manuscript is presenting the proteomic identification of small peptides resulting from the hydrolysis of four key proteins (glyceraldehyde-3-phosphate dehydrogenase, beta-enolase, myozenin-1 and troponin T) and discusses the enzymatic routes for their generation and hydrolysis in dry-fermented sausage and dry-cured ham at initial stages.

2. Materials and methods

2.1. Dry-cured meats preparation

Dry-fermented sausages were prepared by triplicate using a mixture of 75% of lean pork and 25% of pork back fat. The raw mixture also contained sodium chloride (27 g/kg), lactose, dextrin and sodium caseinate at 20 g/kg each, glucose (7 g/kg), sodium ascorbate (0.5 g/kg), sodium nitrite (0.15 g/kg), and potassium nitrate (0.15 g/kg). Dry-fermented sausages were inoculated with a starter culture C-P-77S bactoferm (Chr. Inc., Hansen, Denmark) containing *L. pentosus* and *S. carnosus*. Fermentation took 22 h at 15–20 °C, followed by 43 days of ripening at 9 °C and 75–85% of humidity. Samples were taken at the end of the process.

Dry-cured hams were prepared by triplicate from 6 months old pigs (Landrace × Large White) and followed the traditional procedures consisting on pre-salting for 30 min, salting for 10 days at 2–4 °C and 90–95% relative humidity and post-salting for 60 days at 4–5 °C and 75–85% relative humidity. Hams contained sodium chloride (40 g/kg) and potassium nitrate (0.15 g/kg). Samples were taken just at the end of this stage with a total of 70 days dry-curing process in order to compare with those of fermented sausages.

2.2. Sample extraction and deproteinisation

A total of 50 g of sample were minced and homogenised with 200 mL of 0.01 N HCl for 8 min in a stomacher (IUL Instrument, Barcelona, Spain). The homogenate was centrifuged at 4 °C and 12,000 *g* for 20 min, filtered through glass wool and then the solution was deproteinised by adding 3 volumes of ethanol and maintaining the sample at 4 °C for 20 h. Afterwards, the sample was centrifuged again at 4 °C and 12,000 *g* for 10 min and the supernatant was dried in a rotatory evaporator. Finally, the dried deproteinised extract was dissolved in 25 mL of 0.01 N HCl, filtered through a 0.45 μm nylon membrane filter (Millipore, Bedford, MA, USA) and stored at −20 °C until use.

2.3. Size-exclusion chromatography

To fractionate deproteinised meat product extracts according to molecular mass, a 5 mL aliquot of each extract was subjected to size-exclusion chromatography. A Sephadex G25 Fine column (2.5 × 65 cm, GFE Healthcare Bio-Science AB, Uppsala, Sweden), previously equilibrated with 0.01 N HCl which had been vacuum-filtered through a 0.45 μm nylon membrane filter (Millipore, Bedford, MA, USA), was employed for this purpose. The molecular mass range is 700–5000 Da. The separation was performed using the same 0.01 N HCl as mobile phase, at a flow rate of 15 mL/h and 4 °C. Fractions of 5 mL were collected using an automatic fraction collector and were further monitored by ultraviolet absorption at 214 nm (Ultrospec 3000 UV/Visible spectrophotometer, Pharmacia Biotech, Cambridge, England). Fractions corresponding to elution volumes from 125 to 160 mL were pooled together and aliquots of 100 μL were lyophilised.

2.4. Peptide identification by nanoliquid chromatography and mass spectrometry in tandem (nLC-MS/MS)

The identification of the peptides was done by nLC-MS/MS using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to the quadrupole/time-of-flight (Q-ToF) TripleTOF® 5600+ system (AB Sciex Instruments, MA, USA) with a nanoelectrospray ionisation source (ESI), according to the methodology described by Gallego et al. (2015).

Briefly, lyophilised samples were resuspended in 100 μL of H_2O with 0.1% of trifluoroacetic acid (TFA) and after concentrating, 5 μL were injected into the nESI-LC-MS/MS system. Samples were then preconcentrated on an Eksigent C18 trap column (3 μ, 350 μm × 0.5 mm; Eksigent of AB Sciex, CA, USA), using 0.1% TFA as mobile phase A at a flow rate of 3 μL/min. The trap column was automatically switched in-line onto a nano-HPLC capillary column (3 μm, 75 μm × 12.3 cm, C18) (Nikkyo Technos Co, Ltd. Japan), with a linear gradient from 5% to 35% over 90 min of solvent B, containing 0.1% formic acid in 100% acetonitrile, and 10 min from 35% to 65% of solvent B at a flow rate of 0.30 μL/min and 30 °C. The outlet of the capillary column was directly coupled to a nano-electrospray ionisation system (nano-ESI). The Q/ToF was operated in positive polarity and information-dependent acquisition mode. A 0.25-s ToF MS scan from m/z values of 300 to 1250 was performed, followed by 0.05-s product ions scans from m/z values of 100 to 1500 on the most intense 1–5 charged ions.

Automated spectral processing, peak list generation, and database search were performed using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA, USA) (hppt://www.matrixscience.com). The identification of protein origin of peptides was done using UniProt and NCBInr protein databases, with a significance threshold $p < 0.05$. The tolerance on the mass measurement was 0.3 Da in MS mode and 0.3 Da for MS/MS ions.

3. Results and discussion

This work has presented the proteomic identification of small peptides resulting from the hydrolysis of four key proteins (glyceraldehyde-3-phosphate dehydrogenase, beta-enolase, myozenin-1 and troponin T) extracted at 70 days in dry-cured ham (see Tables 1 to 4) and 43 days in dry-fermented sausage (see Tables 5 to 8). It can be observed that peptides are extensively hydrolysed step-wise from initially higher than 30 amino acids length down to sequences with just a few amino acids. The comparison of peptide profiles can help to elucidate the role of muscle and microbial exo-peptidases in such extensive peptide hydrolysis.

When observing Tables 1 to 4 corresponding to hydrolysed peptides in 3 months dry-cured ham, there is an evidence for the release of many amino acids from the N-terminal and, consequently, the progressive reduction in size of the remaining peptides. This is observed for the 4