A peptidomic approach to study the contribution of added casein proteins to the peptides profile in Spanish dry-fermented sausages

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Abstract

Peptidomics is a necessary alternative in the analysis of naturally generated peptides in dry-fermented processing. The intense proteolysis occurred during the processing of dry-fermented sausages is due to the action of endopeptidases and exopeptidases from both, endogenous muscle origin and lactic acid bacteria (LAB) added in the starter. Sodium caseinate is frequently used as an additive in this type of products because of its emulsifying properties, and consequently influences the protein profile available during the proteolysis. In this study, a mass spectrometry approach has been used to determine the impact of added sodium caseinate in the final peptide profile as well as to analyse its possible influence in the presence of certain previously described casein-derived bioactive peptides.

Keywords: peptidomics, sodium caseinate, mass spectrometry, dry-fermented sausages, bioactive peptides.

42 Introduction

43 A number of reactions responsible for the characteristic texture, flavour, and odour take
44 place during the processing of dry-fermented sausages. Key reactions are lipid
45 hydrolysis and autoxidation, proteolysis, and transformation of amino acids to aromatic
46 compounds. In this sense, although endogenous enzymes are main responsible for the
47 intense proteolysis that takes place during dry-fermented processing, the action of
48 microorganisms such as lactic acid bacteria has been proved to mainly influence the last
49 period of fermentation, also contributing to the generation of small peptides and free
50 amino acids (Toldrá and Flores, 2011). Frequently, additives such as sodium caseinate
51 are added into dry-fermented products as an ingredient of the formulation because of
52 their emulsifying properties. These added proteins also suffer similar transformations to
53 endogenous meat proteins, being also a source of small peptides and free amino acids.
54 These peptides, in addition to contribute to the development of the characteristic flavour
55 of dry-fermented products, have been described to exert important bioactive functions
56 such as antioxidant and antihypertensive activity in dry-cured meat products (Escudero
57 et al., 2013; and Mora et al., 2013).

58 During the last decade, the most common strategy used to study protein changes and to
59 identify, and quantitatively characterize, the proteomic profile of a complex mixture is
60 based on the isolation of proteins by using two dimensional SDS-PAGE electrophoresis
61 separations and the digestion with specific peptidases such as trypsin to be analyzed by
62 mass spectrometry (MS), what is commonly named *Peptide Mass Fingerprint* (PMF)
63 approach. However, when the aim of the study is to identify and sequence naturally
64 released peptides, this strategy commonly used in proteomics is not possible, and the
65 use of MS in tandem is necessary.

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Main difficulties in the study of naturally generated peptides arises from both, the small size of these fragments that cannot be trypsin digested and sometimes are in the limit of some mass spectrometry techniques, and the impossibility of controlling the hydrolysis, obtaining a complex mixture of peptides from different proteins with unspecific cleavage sites. For this reason, the use of advanced proteomic techniques such as mass spectrometry in tandem is essential to elucidate the sequence of these small peptides. Thus, recent advances in proteomics constitute an indispensable tool to develop fast, precise and sensitive analysis of released peptides in complex biological samples like dry-fermented products (López et al., 2015).

In this work, a peptidomic approach using a quadrupole/time-of-flight (Q/ToF) mass spectrometer has been used to study the influence of the addition of sodium caseinate in the final peptide profile of dry-fermented sausages.

Material and methods

Chemical and reagents

The chemical trifluoroacetic acid (TFA), was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile HPLC grade and formic acid were from Scharlab (Barcelona, Spain). For the MALDI-ToF/ToF analysis, α -Cyano-4-hydroxycinnamic acid from Sigma-Aldrich (St. Louis, MO, USA) was used as matrix, and Peptide Mass Standards kit for Calibration of AB SCIEX MALDI TOF Instruments (MA, USA) was also provided. All other reagents were of analytical grade.

Spanish dry-fermented sausages

Dry-fermented sausages were prepared by using a 75% of lean pork and a 25% of pork back fat. Additives such as NaCl at a concentration of 27 g/kg, lactose at 20 g/kg, dextrin at 20 g/kg and sodium caseinate at 20 g/kg were added, as well as glucose (7

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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 bactoform (Chr. Inc., Hansen, Denmark) containing *Lactobacillus pentosus* and *Staphylococcus carnosus*. Ripening process was developed in two steps at 75-85% of humidity that were 22 hours at 15-20°C, followed by 43 days at 9°C.

Peptide extraction

A total of 50 g of dry-fermented sausages made of pork were minced after the removal of fat, and homogenised with 200 mL of 0.01 N HCl for 8 min in cold. The homogenate was centrifuged (12,000 g for 20 min at 4 ° C), the supernatant was filtered through glass wool, and then deproteinized by adding three volumes of ethanol and maintaining the sample overnight at 4°C. Then, sample was centrifuged again (12,000g for 20 min at 4°C) and the supernatant was freeze-dried. Finally, the dried extract was dissolved in 25 mL of 0.01 N HCl and filtered through a 0.45 µm nylon membrane filter (Millipore, Bedford, MA, USA) until use.

Peptide-mass mapping by MALDI-ToF MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used to determine the molecular mass of the peptides contained in the extract. The analysis was performed using a 5800 MALDI-TOF/TOF (AB Sciex, MA, USA) in positive reflectron mode (3000 shots every position) in a range from 200 to 3500 Da; the laser intensity was manually adjusted to maximize the s/n ratio. Spectra were obtained from 3000 shots in every position with a final laser intensity of 3500. Plate model and acquisition method were calibrated by AB Sciex calibration mixture (des-Arg1-Bradykinin at 1 fmol/µL; Angiotensin I at 2 fmol/µL; Glu1-Fibrinopeptide B at 1.3 fmol/µL; ACTH (1–17 clip) at 2 fmol/µL; ACTH (18–39 clip) at 5 fmol/µL; and ACTH (7–38 clip) at 3

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fmol/ μ L) in 13 positions. Dry-fermented sausage extract was diluted ten times in H₂O-ACN (95:5) with 0.1% TFA, and 1 μ L was spotted on 10 positions in the MALDI plate and allowed to air dry. Once dried, 0.5 μ L of matrix solution (5 mg/mL of α -Cyano-4-hydroxycinnamic acid in H₂O-ACN (30:70) with 0.1% TFA was spotted.

The spectra obtained by MALDI-TOF MS were analysed using mMass - Open Source Mass Spectrometry Tool software v5.5 (Strohalm et al., 2010; Niedermeyer and Strohalm, 2012).

Nano-LC-MS/MS analysis

The nanoLC-MS/MS analysis was performed using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to a quadrupole-time-of-flight (Q-ToF) TripleTOF® 5600+ system from AB Sciex Instruments (Framingham, MA, USA) that is equipped with a nanoelectrospray ionization source.

A dilution of ten times of the dry-fermented sausages extract was done and five microlitres of the supernatant were injected into the LC-MS system through the autosampler. Samples were then preconcentrated on an Eksigent C18 trap column (3 μ , 350 μ m x 0.5mm) (Eksigent of AB Sciex, CA, USA), at a flow rate of 3 μ L/min and using 0.1% v/v TFA as mobile phase. After 5 min of preconcentration, the trap column was automatically switched in-line onto a nano-HPLC capillary column (3 μ m, 75 μ m x 12.3 cm, C18) (Nikkyo Technos Co, Ltd. Japan). The mobile phases consisted of solvent A, containing 0.1% v/v formic acid (FA) in water, and solvent B, containing 0.1% v/v FA in 100% acetonitrile. Chromatographic conditions were a linear gradient from 5% to 35% of solvent B over 90 min, and 10 min from 35% to 65% of solvent B, at a flow rate of 0.3 μ L/min and running temperature of 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

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information-dependent acquisition mode, in which a 0.25-s ToF MS scan from m/z of 100 to 1200 was performed, followed by 0.05-s product ion scans from m/z of 100 to 1500 on the 50 most intense 1 to 5 charged ions.

Results and discussion

In this study, MALDI-ToF mass spectrometry has been used to determine the amount and molecular masses of the peptides naturally generated during the dry-fermented processing. This information results very useful in the optimization of the conditions in the nLC-MS/MS analysis. Under controlled conditions of digestion, *in silico* strategy resulted in an economically accessible and fast way to know the theoretical size and sequence of the peptides generated from known proteins. However, when peptides have been generated by the action of endogenous enzymes and/or microorganisms, MALDI-ToF becomes a necessary alternative to determine the peptide profile. **Figure 1** shows the MALDI ToF spectra from 200 to 900 m/z (**A**) and from 850 to 3500 m/z (**B**), indicating that there is an extensive distribution of peptides in a wide range of molecular masses.

An extraction of the naturally generated peptides in dry-fermented sausages (at 43 days of processing) has been done and the resulting extract was analysed using nanoLC-MS/MS in order to identify the sequences of the peptides generated by the action of muscle enzymes and microorganisms as well as their proteins of origin. In this respect, a total of 347 proteins have been identified with a confidence higher than 95%. All these proteins were of muscle origin except casein, which was added as an ingredient during the preparation of dry-fermented sausages because of its emulsifying characteristics. From the total amount of peptides identified, the four types of casein proteins represent the 16% just beyond titin protein (31%), a giant cytoskeletal protein of vertebrate striated muscle with a molecular weight of 3MDa (see **Figure 2**).

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Thus, an intense proteolysis occurred during the dry-fermented processing releasing a large amount of peptides. The obtained results are similar to those observed in other type of dry-cured meat products of 10 and 14 months of curing (Escudero et al, 2013; and Mora et al, 2013). So a possible explanation for the very intense proteolysis occurred in the 43 days of processing of dry-fermented sausages described in this study could be due to the combined action of LAB and endogenous enzymes.

Table 1 shows a brief description of the results obtained by nanoLC-MS/MS. β -casein, α -S1- and α -S2-casein and κ -casein were identified with a sequence coverage of 94, 80, 69, and 71 %, respectively, indicating the high availability of this protein for the endogenous muscular enzymes and microorganisms action (see **Figure 3**). This fact is probably due to muscle proteins which are joined within the structure forming the muscle tissue which makes them less available for the proteolytic action, increasing the chance for added casein protein to be hydrolyzed.

In this study, parameters of the nLC-MS/MS analysis were adjusted for the identification of peptides comprised from 6 to 50 residues. The highest number of peptides have been identified from β -casein protein with a total of 603 peptides that represents a ratio (number of peptides identified vs total number of residues in the sequence) of 2.69, very high in comparison to ratios 1.27, 0.66, and 0.64 from α -S1- and α -S2-casein and κ -casein, respectively. Lactic acid bacteria such as *Lactobacillus pentosus* and *Staphylococcus carnosus* used in this study have a very complex proteolytic system that consisted on a proteinase attached to the cell wall that supports the extracellular casein degradation into oligopeptides (Darewicz et al., 2006). These oligopeptides are moved into the cytoplasm and finally degraded into smaller molecules and amino acids by intracellular peptidases (Chaves-López et al., 2014; Liu et al., 2010). On the other hand, endogenous enzymes can be divided into endopeptidases,

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responsible for the degradation of muscle into protein fragments and polypeptides, and endopeptidases, that acts on these fragments and are main responsible for the generation of small peptides and free amino acids.

Figure 4 shows the percentage distribution of amino acids in the protein sequences of the four types of caseins. Pro, Leu and Val are the most abundant amino acids of β -casein in comparison with the other types of caseins, whereas Leu and Glu are main amino acids of $\alpha 1$ -casein, Lys and Glu in $\alpha 2$ -casein, and Pro and Ala in κ -casein. The generation of this high level of peptides is due to aminopeptidase and carboxypeptidase activities from either endogenous enzymes (Aristoy and Toldrá, 1995; Toldrá and Flores, 1998; Lametsch et al., 2003) or acid lactic bacteria added in the starter (Herreraset al., 2003, Bintsis et al., 2003; Macedo et al., 2010). A possible reason for the higher number of peptides identified from β -casein in comparison with the other types of casein (see Table 2 and 3) could be the preference of aminopeptidases and carboxypeptidases for Pro, Leu and Val residues, although no studies regarding the enzymatic action of *Lactobacillus pentosus* and *Staphylococcus carnosus* in dry-fermented products have been reported. β -Casein is also considered the best emulsifying agent among caseins as casein ability to lower the surface tension decreases from β -casein> $\alpha 1$ -casein> κ -casein (Dalglish, 1997) so a higher proportion of this molecule could also explain the higher amount of identified sequences in comparison with the other caseins.

On the other hand, the intense degradation of casein protein occurred when added as an additive in dry-fermented products will contribute not only to the characteristic flavor and aroma properties of these types of products but also to their bioactive activity. In this sense, casein-derived peptides have been described to exert antihypertensive, immunoregulating, antithrombotic, antimicrobial, and opioid activities in fermented

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milk products such as yogurt, sour milk, or kefir, being beneficial for the general health. Due to some studies using LAB have demonstrated their potential to generate bioactive peptides, some of the previously identified casein-derived bioactive sequences have been compared with the sequences identified in this study. **Tables 2 and 3** show some examples of the peptides identified in this study sharing sequences with previously described bioactive peptides. In this sense and as an example, peptides YQEPLV, YQEPVLGPVR and YQEPVLGPVRGPFPI have been identified in the studied extract, and were previously described as ACE inhibitory peptides obtained from fragments of β -casein protein. Similar sequences YQEPVLGPVRGPFPIIV and LLYQEPVLGPVRGPFPIIV have been also identified as antimicrobial (Sandre et al., 2001) and immunoregulatory (Hayes et al., 2007), respectively, as it is shown in **Table 2**. On the other hand, peptide YPVEPFTE identified in this study was described to display a selective potentiating activity on isolated guinea pig ileum for bradykinin, as well as showed in vitro ACE-inhibitory and opiate-like activity (Perpetuo et al., 2003). Finally, peptide KKYKVPQL identified in **Table 3** has also been described as responsible for ACE-inhibitory activity in Manchego cheeses (Gómez-Ruiz et al., 2002).

Conclusion

In this study, an intense proteolysis has been proved to occur during the dry-fermented processing. The obtained results are in agreement to those observed in dry-cured meat products due to the combined action of LAB and endogenous enzymes. The use of peptidomic analysis tools together with mass spectrometry in tandem allows the complex identification of those peptides released during the dry-fermented processing, helping in a better understanding of the proteolysis mechanisms as well as the influence of bacterial action in this type of meat products.

The action of LAB contributes to the generation of bioactive peptides, and the addition of sodium caseinate in dry-fermented sausages results on the formation of bioactive peptides that have been previously described as derived from milk products, influencing in the final peptides profile of this type of products. What is more, the optimised methodology might result very useful in the detection of fraudulent addition of casein protein by the identification of specific peptides generated during the dry-fermented processing.

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FIGURE CAPTIONS

Figure 1. MALDI-ToF spectra of the peptide extract of Spanish dry-fermented sausages. A) Values from 200 to 900 Da $[M-H^+]$ and B) Values from 850 to 3500 Da $[M-H^+]$.

Figure 2. Distribution of the peptides identified by nLC-MS/MS according to their protein of origin.

Figure 3. Sequences of the four types of caseins obtained from UniProt database: β -casein (CASB_BOVIN); α -S1-casein (CASA1_BOVIN); α -S2-casein (CASA2_BOVIN); and κ -casein (CASK_BOVIN).

Figure 4. Amino acids distribution for each type of casein protein calculated from their sequences.

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TABLES

Table 1. Caseins identified by nLC-MS/MS from the peptides naturally generated during dry-fermented processing of Spanish sausages.

Protein name	Accession No. ^a	No. Residues	Sequence Cov. (%) ^b	No. Peptides ^c	Ratio ^d
β-casein	CASB_BOVIN	224	94	603	2.69
α-S1-casein	CASA1_BOVIN	214	80	271	1.27
α-S2-casein	CASA2_BOVIN	222	69	147	0.66
κ-casein	CASK_BOVIN	190	71	122	0.64

a. Accession number in UniProt database (<http://www.uniprot.org/>). b. Percentage of sequence per protein identified by nLC-MS/MS. c. Total number of peptides identified in each protein with a confidence of 95%. d. Ratio number of peptides vs number of residues.

Table 2

β -Casein peptides identified by tandem mass spectrometry sharing sequences with previously described bioactive peptides.

Accession No.	Observed	Calculated	Mass			Previously identified bioactive peptides ^c				
UniProt	(m/z) ^a	(Da) ^b	Charge	P ₀	Identified sequence*	P _T	Sequence	IC ₅₀ (μM)	Activity	References
CASB_BOVIN	748.40	747.39	1	L	YQEPVL*	G	YQEPVL	280	ACE inhibitor	Pihlanto-Leppala et al (1998), Meisel et al (2006)
	717.45	1432.88	2	P	IQAFLLYQEPVL	G	YQEP	-	antioxidative	Silva, Pihlanto and Macata (2006)
	745.95	1489.89	2	P	IQAFLLYQEPVLG	P				
	579.32	1156.62	2	L	YQEPVLGPVR*	G	YQEPVLGPVR	300	ACE inhibitor	Meisel et al (2006)
	607.85	1213.68	2	L	YQEPVLGPVRG	P	YQEPVLGP	-	antioxidative	Silva, Pihlanto and Macata (2006)
	656.35	1310.69	2	L	YQEPVLGPVRGP	F				
	729.90	1457.79	2	L	YQEPVLGPVRGPF	P				
	769.44	1536.87	2	F	LLYQEPVLGPVRGP	F				
	778.43	1554.84	2	L	YQEPVLGPVRGPF	I	VRGPF	-	ACE inhibitor	Gómez-Ruiz et al (2002)
	834.93	1667.84	2	L	LYQEPVLGPVRGPF	I				
	834.97	1667.93	2	L	YQEPVLGPVRGPFPI*	I	YQEPVLGPVRGPFPI	-	ACE inhibitor	Meisel and Schlimme (1994)
	842.98	1683.94	2	F	LLYQEPVLGPVRGPF	P				
	891.48	1780.94	2	L	YQEPVLGPVRGPFPII	V				
	891.51	1781.00	2	F	LLYQEPVLGPVRGPF	I				
	891.51	1781.01	2	L	LYQEPVLGPVRGPFPI	I				
	941.05	1880.09	2	L	YQEPVLGPVRGPFPIIV*	-	YQEPVLGPVRGPFPIIV	-	antimicrobial	Sandre et al (2001)
	948.06	1894.10	2	F	LLYQEPVLGPVRGPFPI	I				
	948.06	1894.11	2	L	LYQEPVLGPVRGPFPII	V				
	997.58	1993.16	2	L	LYQEPVLGPVRGPFPIIV	-	GPVRGPFPIIV	-	ACE inhibitor	Nakamura et al (1995)
	1004.60	2007.19	2	F	LLYQEPVLGPVRGPFPII	V				
	1054.13	2106.25	2	F	LLYQEPVLGPVRGPFPIIV*	-	LLYQEPVLGPVRGPFPIIV	-	immunomodulating	Hayes et al (2007)
	1022.57	3064.70	3	Q	RDMPIQAFLLYQEPVLGPVRGPFPIIV	-				
	1064.60	3190.79	3	Y	PQRDMPIQAFLLYQEPVLGPVRGPFPII	V				
	776.41	1550.80	2	P	FAQTQSLVYFPGP	I	TQSLVYP	64	ACE inhibitor	Kohmura, Nio and Ariyoshi (1990)
	741.41	740.40	1	P	FPGPIP*	S	FPGPIP	260	DPP IV inhibitor	Ojeda, Cereto-Massagué, Valls and Pujadas (2014)
	593.85	1185.68	2	Q	SLVYFPGPIP	N	PGPIP	-	immunomodulating	Meisel (1998)
	650.87	1299.72	2	Q	SLVYFPGPIP	S	SLVYFPGPI	-	ACE inhibitor	Hafeez et al (2014)
	750.93	1499.85	2	Q	SLVYFPGPIPNSL	P				
	799.45	1596.89	2	Q	SLVYFPGPIPNSLP	Q				
	863.48	1724.95	2	Q	SLVYFPGPIPNSLPQ	N				
	920.50	1838.99	2	Q	SLVYFPGPIPNSLPQN	I				
	1074.10	2146.18	2	Q	SLVYFPGPIPNSLPQNIPP	L				
	754.10	2259.29	3	Q	SLVYFPGPIPNSLPQNIPPL	T				
	1181.17	2360.32	2	Q	SLVYFPGPIPNSLPQNIPPLT	Q				
	830.47	2488.39	3	Q	SLVYFPGPIPNSLPQNIPPLTQ	T				
	896.51	2686.51	3	Q	SLVYFPGPIPNSLPQNIPPLTQTP	V				
	1060.28	3177.82	3	Q	SLVYFPGPIPNSLPQNIPPLTQTPVVVPP	F				
	1109.31	3324.91	3	Q	SLVYFPGPIPNSLPQNIPPLTQTPVVVPPF	L				
	1147.01	3438.01	3	Q	SLVYFPGPIPNSLPQNIPPLTQTPVVVPPFL	Q				
	1189.69	3566.06	3	Q	SLVYFPGPIPNSLPQNIPPLTQTPVVVPPFLQ	P				
	916.79	3663.13	4	Q	SLVYFPGPIPNSLPQNIPPLTQTPVVVPPFLQP	E				
	949.05	3792.17	4	Q	SLVYFPGPIPNSLPQNIPPLTQTPVVVPPFLQPE	V				
	973.82	3891.24	4	Q	SLVYFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEV	M				
	1006.59	4022.32	4	Q	SLVYFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVM	G				
	1020.84	4079.32	4	Q	SLVYFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMG	V				
	876.9823	1751.95	2 S		LVYFPGPIPNSLPQN*	I	LVYFPGPIPNSLPQN	71	ACE inhibitor	Pihlanto, Virtanen and Korhonen, (2010)
	687.3968	2059.1684	3 S		LVYFPGPIPNSLPQNIPP*	L	LVYFPGPIPNSLPQNIPP	5	ACE inhibitor	Hernández-Ledesma, Quirós, Amigo, and Recio (2007)
	438.76	875.50	2	H	KEMPF	Y	EMPF	-	ACE inhibitor	Pihlanto-Leppala et al (1998);Perpetuo et al (2003)
	911.45	910.44	1	K	EMPF	P				
	338.53	1012.56	3	K	HKEMPF	Y				
	520.28	1038.55	2	H	KEMPF	P				
	588.81	1175.61	2	K	HKEMPF	P				
	637.34	1272.66	2	K	HKEMPF	V				
	652.86	1303.70	2	P	KHKEMPF	P				
	437.26	1308.76	3	M	APKHKEMPF	Y				
	467.93	1400.78	3	A	PKHKEMPF	P				
	480.93	1439.78	3	A	MAPKHKEMPF	Y				
	736.90	1471.79	2	M	APKHKEMPF	P				
	504.61	1510.82	3	E	AMAPKHKEMPF	Y				
	802.42	1602.83	2	A	MAPKHKEMPF	P				
	837.94	1673.87	2	E	AMAPKHKEMPF	P				
	601.98	1802.92	3	K	EAMAPKHKEMPF	P				
	499.79	1995.12	4	S	KVKEAMAPKHKEMPF	Y	VKEAMAPK		antioxidant	Suetsuna et al (2000); Korhonen and Pihlanto (2007)
	1016.05	2030.08	2	K	VKEAMAPKHKEMPF	P				
	1080.09	2158.17	2	S	KVKEAMAPKHKEMPF	P				
	801.44	2401.29	3	M	GVSKVKEAMAPKHKEMPF	P				
	565.53	2822.63	5	L	QPEVMGVSKVKEAMAPKHKEMPF	Y				
	960.80	3839.16	4	I	PPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEM	P				
	966.55	3862.17	4	V	PPFLQPEVMGVSKVKEAMAPKHKEMPFKYVPE	F				
	1006.59	4022.32	4	Q	SLVYFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVM	G				
	981.48	980.47	1	K	YPVEPFTE*	S	YPVEPFTE	-	ACE inhibitor	Perpetuo, Juliano and Lebrun (2003)
	603.82	1205.63	2	F	PKYPVEPFTE	S		-	opioid	Perpetuo et al (2003)
	647.34	1292.67	2	F	PKYPVEPFTE	Q				
	754.88	1507.75	2	F	PKYPVEPFTE	L				
	935.48	1868.95	2	P	FPKYVPEPFTE	L				
	860.77	3439.04	4	E	MPFPKYVPEPFTE	L				
	915.62	914.61	1	N	LHLPL	Q	LHLPL	5	ACE inhibitor	Hernández-Ledesma, Quirós, Amigo, and Recio (2007)
	458.80	915.59	2	E	NLHLPL	L	LHLPL		ACE inhibitor	

a. Relation of mass/charge observed in the nLC-MS/MS spectrophotometer expressed in m/z .

b. Calculated relative molecular mass of the matched peptide in Daltons.

c. Sequences and data of previously described bioactive peptides sharing same or part of the sequence with the casein-derived peptides identified in this study from dry-fermented sausages.

* Sequences identified in this study showing an asterisc indicates that the sequence has been previously described as bioactive. Bold residues are sequences showing bioactive properties.

α -S1-, α -S2- and κ -Casein peptides identified by tandem mass spectrometry sharing sequences with previously described bioactive peptides.

Accession No.	Observed	Calculated	Mass		P _r	Sequence	IC ₅₀ (μM)	Activity	References
UniProt	(m/z) ^a	(Da) ^b	Charge	P ₀	Identified sequence*				
CASAA1_BOVIN	599.37	1196.72	2	A	RPKHPKHHQG	L RPKHPKHHQ	13	ACE inhibitor	FitzGerald, Murray and Walsh (2004)
	499.96	1406.87	3	A	RPKHPIKHOGGLP	Q RPKHPKHHQGLPQEVLENLLRF	0	immunomodulating	Lahov and Regelson (1996)
	512.65	1534.92	3	A	RPKHPIKHOGGLPQ	E TTMPLW	-	opioid	Migliore-Samouri et al. (1989)
	555.66	1663.97	3	A	RPKHPIKHOGGLPQE	V	16	ACE inhibitor	Mariyama S., Miyoshi S., Tanaka (1989)
	939.04	1876.07	2	A	RPKHPIKHOGGLPQEV	N	-	immunomodulating	Hayes et al (2007)
	996.09	1990.16	2	A	RPKHPIKHOGGLPQEVLN	E KKYKVPQ	-	ACE inhibitor	Gómez-Ruiz et al (2002)
	707.39	2119.16	3	A	RPKHPIKHOGGLPQEVLENE	N KKYKVPQL	-	ACE inhibitor	Gómez-Ruiz et al (2002)
	745.42	2233.24	3	A	RPKHPIKHOGGLPQEVLENN	L	-		
	1007.51	2013.00	2	I	IPNPGENSEKTMTPLW	-			
	1108.55	2215.08	2	F	SDPNPVGENSEKTMTPLW	-			
	873.44	2617.31	3	D	APSFSDPNPVGENSEKTMTPLW	-			
	999.82	2996.45	3	Q	YTDAPISFSDPNPVGENSEKTMTPLW	-			
	1003.64	1002.63	1	L	KKYKVPQL*	E L KKYKVPQL			
	558.87	1115.73	2	R	LKKYKVPQL	E L KKYKVPQL			
	378.23	1131.67	3	L	KKYKVPQLE	I L KKYKVPQLE			
	636.92	1271.83	2	L	RLKKYKVPOL	E L RLKKYKVPOL			
	701.45	1400.88	2	L	RLKKYKVPQLE	I L RLKKYKVPQLE			
	807.39	1612.76	2	L	RLKKYKVPQLEIV	P L RLKKYKVPQLEIV			
	837.49	2509.44	3	L	RLKKYKVPQLEIVPSAEERL	H L RLKKYKVPQLEIVPSAEERL			
CASAA2_BOVIN	738.40	737.39	1	K	FALPOV*	L FALPOV	4.3	ACE inhibitor	FitzGerald R.J., Murray B. A., Walsh (2004)
	1157.60	1156.59	1	R	YQKFALPOV	L AMKPWPQPK	600	ACE inhibitor	Van der Ven C.(2002); Maeno, Yamamoto and Takano (1996)
	635.86	1269.70	2	R	YQKFALPQYL	K VYQHOKAMKPWPQPKTKVIPYVRY	-	antibacterial	Recio, Gommaris, Slangen, and Visser (1998)
	546.34	1635.99	3	K	AMKPWPQPKTKVIP	Y VYQHOKAMKPWPQPKTKVIPYVRYL	-	antibacterial	Recio, Gommaris, Slangen, and Visser (1998)
	628.68	1883.03	3	V	YQHOKAMKPWPQPKTK	K			
	633.72	1898.13	3	K	AMKPWPQPKTKVIPYV	R			
	671.38	2011.12	3	V	YQHOKAMKPWPQPKTK	V			
	685.74	2054.21	3	K	AMKPWPQPKTKVIPYVR	Y			
	728.45	2182.34	3	Q	KAMKPWPQPKTKVIPYVR	Y			
	771.13	2217.26	3	K	AMKPWPQPKTKVIPYRV	L			
	771.13	2310.38	3	H	QKAMKPWPQPKTKVIPYVR	Y			
	774.44	2320.30	3	V	YQHOKAMKPWPQPKTKVIP	Y			
	913.86	2738.56	3	V	YQHOKAMKPWPQPKTKVIPYVR	Y			
	710.42	2837.65	4	T	YVQHOKAMKPWPQPKTKVIPYVR	Y			
	735.68	2938.68	4	K	TYVQHOKAMKPWPQPKTKVIPYR	Y			
CASK_BOVIN	1043.09	2084.17	2	H	PHPHLSFMAPPKNQDK	T LSFMAPPKK MAIPPCKNQDK MAIPPCK NQDK	- - - -	antithrombotic antithrombotic antithrombotic antithrombotic	Fiat et al (1993) Fiat and Jollès (1989); Schimme and Meisel (1995) Fiat and Jollès (1989); Schimme and Meisel (1995) Fiat and Jollès (1989); Schimme and Meisel (1995)
	505.7982	1009.5818	2	M	AIPPCKNQD*	K AIPPCKNQD	19.9	ACE inhibitory	Shuang, Harutoshi and Taku (2008)

a. Relation of mass/charge observed in the nLC-MS/MS spectrophotometer expressed in m/z.
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c. Sequences and data of previously described bioactive peptides sharing same or part of the sequence with the casein-derived peptides identified in this study from dry-fermented sausages.
d. Sequences identified in this study showing an asterisk indicates that the sequence has been previously described as bioactive. Bold residues are sequences showing bioactive properties.

Figure 1

A) From 200 to 900 m/z

B) From 850 to 3500 m/z

Figure 1.

Figure 2

Figure 2.

Figure 3

CASB_BOVIN				
1	MKVLILACLV	ALALARELEE	LNVPGEIVES	LSSSEESITR
51	EEQQQTEDEL	QDKIHPFAQT	QSLVYPFPGP	IPNSLPQNIP
101	PFLQPEVMGV	SKVKEAMAPK	HKEMPFPKYP	VEPFTESQSL
151	PLPLLQSWMH	QPHQPLPPTV	MFPPQSVLSL	SQSKVLPVPQ
201	PIQAFLLYQE	PVLGPVRGPF	PIIV	
CASA1_BOVIN				
1	MKLLILTCLV	AVALARPKHP	IKHQGLPQEV	LNENLLRFFV
51	KVNELSKDIG	SESTEDQAME	DIQOMEAESI	SSSEEIVPNS
101	VPSERYLGYL	EQLRLKKYK	VPQLEIVPNS	AEERLHSMKE
151	IGVNQELAYF	YPELFRQFYQ	LDAYPSGAWY	YVPLGTQYTD
201	IGSENSEKTT	MPLW		
CASA2_BOVIN				
1	MKFFIFTCLL	AVALAKNTME	HVSSSEESII	SQETYKQEK
51	CSTFCKEVVR	NANEEYSIG	SSSEESAIVA	TEEVKITVDD
101	NQFYQKFPQY	LQYLYQGPIV	LNPWDQVKRN	AVPITPTLNR
151	KKTVDMESTE	VFTKKTKLTE	EEKNRLNFLK	KISQRYQKFA
201	HQKAMKPWIQ	PKTKVIPYVR	YL	
CASK_BOVIN				
1	MMKSFFLVVT	ILALTLPFLG	AQEQNQEQPI	RCEKDERFFS
51	YVLSRYPSYG	LNYYQQKPVA	LINNQFLPYP	YYAKPAAVRS
101	SNTVPAKSCQ	AQPTTMAPHP	HPHLSFMAIP	PKKNQDKTEI
151	PTSTPTTEAV	ESTVATLEDS	PEVIESPPEI	NTVQVTSTAV

Figure 3.

Figure 4

Figure 4.