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

Leticia Mora, Elizabeth Escudero, M-Concepción Aristoy, and Fidel Toldrá✉

Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Avenue Agustín Escardino, 7, 46980, Paterna, Valencia, Spain

✉ Corresponding author: Tel: +34 390 00 22 ext. 2112; fax: +34 363 63 01.

E-mail address: ftoldra@iata.csic.es

Field Code Changed

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.

Introduction

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

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

Formatted: Font color: Red

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.

Formatted: Font color: Red

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

Formatted: Font color: Red

Formatted: Font color: Red

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 *Lactobacillus pentosus* and *Staphylococcus carnosus*. Ripening process was developed in two steps at 75-85% of humidity that were 22 hours at 15-20ºC, followed by 43 days at 9ºC.

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.

Formatted: Font color: Red

Formatted: Font color: Red

Formatted: Font color: Red

Formatted: Font color: Red

Peptide-mass mapping by MALDI-ToF MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectromety 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

fmol/µL) in 13 positions. Dry-fermented sausage extract was diluted ten times in H_2O-ACN (95:5) with 0.1% TFA, and 1 µL was spotted on 10 positions in the MALDI plate and allowed to air dry. Once dried, 0.5 µL of matrix solution (5 mg/mL of α-Cyano-4-hydroxycinnamic acid in H_2O-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 (3µ, 350µm x 0.5mm) (Eksigent of AB Sciex, CA, USA), at a flow rate of 3 µL/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 (3µm, 75µm 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 µL/min and running temperature of 30 ºC.

Formatted: Font color: Red

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, 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.

Formatted: Font color: Red

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**).

Formatted: Font color: Red

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,

Formatted: Font color: Red

Formatted: Font color: Red

Formatted: Font color: Red

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 α1-casein, Lys and Glu in α2-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 (Herreroset 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>α1-casein>κ-casein (Dalgleish, 1997) so a higher proportion of this molecule could also explain the higher amount of identified sequences in comparison with the other caseins.

Formatted: Font color: Red

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

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.

References

Aristoy, M. C., & Toldrá, F. (1995). Isolation of flavor peptides from raw pork meat and dry-cured ham. In G. Charalambous (Ed.), Food flavors: Generation, analysis and processes influence,1323–1344. Amsterdam: Elsevier Science.

Bintsis, T., Vafopoulou-Mastrojiannaki, A., Litopoulou-Tzanetaki, E. & Robinson, R.K. (2003). Protease, peptidase and esterase activities by lactobacilli and yeast isolates from Feta cheese brine. *Journal of Applied Microbiology*, 95, 68–77

Chaves-López, C., Serio, A., Paparella, A., Martuscelli, M., Corsetti, A., Tofalo, R., & Suzzi, G. (2014). Impact of microbial cultures on proteolysis and release of bioactive peptides in fermented milk. *Food Microbiology*, 42, 117-121.

Darewicz, M., Jerzy Dziuba, Marta Dziuba. 2006. Functional properties and biological activities of bovine casein proteins and peptides. *Pol. J. Food Nutr. Sci.* 15/56, 79–86.

Dalgleish D. G. (1997). Adsorption of protein and the stability of emulsions. *Trends Food Sci. Technol*., 8, 1–6

Escudero, E., Mora, L., Fraser, PD., Aristoy, MC., Arihara, K., & Toldrá, F. (2013). Purification and identification of antihypertensive peptides in Spanish dry-cured ham. *Journal of Proteomics*, 78, 499-507.

Fiat, A. M., & Jollès, P. (1989). Caseins of various origins and biologically active casein peptides and oligosaccharides: Structural and physiological aspects. *Molecular and Cellular Biochemistry*, 87(1), 5–30.

FitzGerald R.J., Murray B. A., & Walsh D.J. (2004). Hypotensive peptides from milk proteins. *J Nutr.*134(4), 980-988.

Field Code Changed

Field Code Changed

271 Gómez-Ruiz, J. A., Ramos, M., & Recio, I. (2002). Angiotensin converting enzyme-
272 inhibitory peptides in Manchego cheeses manufactured with different starter
273 cultures. *International Dairy Journal*, 12(8), 697–706.
274 González, L., Sacristán, N., Arenas, R., Fresno, J.M. & Tornadijo, E. (2010) Enzymatic
275 activity of lactic acid bacteria (with antimicrobial properties) isolated from a
276 traditional Spanish cheese. *Food Microbiology*, 27, 592-597.
277 Hayes M., Stanton C., Fitzgerald G.F., Ross R.P. (2007). Putting microbes to work:
278 Dairy fermentation, cell factories and bioactive peptides. Part II: Bioactive peptide
279 functions. *Biotechnology Journal*, 2, 435–449
280 Hafeez, Z., Cakir-Kiefer, C., Roux, E., Perrin, C., Miclo, L., Dary-Mourot, A. (2014).
281 Strategies of producing bioactive peptides from milk proteins to functionalize
282 fermented milk products, *Food Research International*, 63A, 71-80.
283 Hernández-Ledesma, B., Quirós, A., Amigo, L., & Recio, I. (2007). Identification of
284 bioactive peptides after digestion of human milk and infant formula with pepsin
285 and pancreatin. *International Dairy Journal*, 17, 42–49.
286 Herreros, M.A., Fresno, J.M., GonzálezPrieto, M.J., & Tornadijo, M.E. (2003).
287 Technological characterization of lactic acid bacteria isolated from Armada
288 cheese (a Spanish goats' milk cheese). *International Dairy Journal,* 13,469-479.
289 Kohmura, M., Nio, N., & Ariyoshi, Y. (1990). Inhibition of angiotensin-converting
290 enzyme by synthetic peptide fragments of human k-casein. *Agricultural and*
291 *Biological Chemistry*, 54(3), 835–836.
292 Lahov E., & Regelson W. (1996). Antibacterial and immunostimulating casein-derived
293 substances from milk: casecidin, isracidin peptides. *Fd Chem.Toxic*. 34(1), 131-
294 145.
295 Lametsch, R., Karlsson, A., Rosenvold, K., Andersen, H. J., Roepstorff, P., &
296 Bendixen, E. (2003). Postmortem proteome changes of porcine muscle related to
297 tenderness. *Journal of Agricultural and Food Chemistry*, 51(24), 6992–6997.
298 Liu, M., Bayjanov, J.R., Renckens, B., Nauta, A., & Siezen, R.J., (2010). The
299 proteolytic system of lactic acid bacteria revisited: a genomic comparison. *BMC*
300 *Genomics* 11, 36.
301 López, C.M., Bru, E., Vignolo, G. & Fadda, S.G. (2015). Identification of small peptides
302 arising from hydrolysis of meat proteins in dry fermented sausages. *Meat*
303 *Science,* 104, 20-29.
304 Macedo, A.C., Vieira, M., Poças, R. & Malcata, F.X. (2010) Peptide hydrolase system
305 of lactic acid bacteria isolated from Serra da Estrela cheese. *International Dairy*
306 *Journal,* 10, 769-774.

307 Maeno M., Yamamoto N., Takano T. (1996). Identification of an antihypertensive
308 peptide from casein hydrolysate produced by a proteinase from Lactobacillus
309 helveticus CP790. *Journal of Dairy Science*, 79, 1316-1321
310 Maruyama S., Miyoshi S., & Tanaka H. (1989). Angiotensin I-converting enzyme
311 inhibitors derived from ficus carica. *Agric.Biol.Chem*. 53(10), 2763-2767
312 Meisel, H., & Schlimme, E. (1994). Inhibitors of angiotensinconverting- enzyme derived
313 from bovine casein (casokinins). In V Brantl, & H. Teschemacher (Eds.), b-
314 Casomorphins and related peptides: Recent developments (pp. 27–33).
315 Weinheim: VCH, Germany.
316 Meisel H. (1998). Overview on milk protein-derived peptides. *International Dairy*
317 *Journal,* 8, 363-373.
Meisel H., Walsh D. J., Murray B., FitzGerald R. J. (2006). ACE inhibitory peptides. In: Nutraceutical proteins and peptides in health and disease. Mine Y., Shahidi F. (Eds.), CRC Taylor & Francis Group, Boca Raton, London, New York, 269-315.
Migliore-Samour D, Floc'h F, & Jollès P. (1989). Biologically active casein peptides implicated in immunomodulation. *Journal of Dairy Research* 56(3):357-62.
318 Mora, L., Escudero, E., Fraser, PD., Aristoy, MC. & Toldrá, F. (2013). Proteomic
319 identification of antioxidant peptides from 400 to 2500 Da generated in Spanish
320 dry-cured ham contained in a size-exclusion chromatography fraction. *Food*
321 *Research International*, 56, 68-76.
322 Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., & Takano, T.
323 (1995). Purification and characterization of angiotensin-I-converting enzyme
324 inhibitors from sour milk. *Journal of Dairy Science*, 78(4), 777–783.
325 Niedermeyer, T.H.J. & Strohalm, M. (2012). mMass as a Software Tool for the
326 Annotation of Cyclic Peptide Tandem Mass Spectra. *PLoS ONE,* 7 (9).
327 Ojeda M. J., Cereto-Massagué A., Valls C., Pujadas G. (2014). DPP-IV, An Important
328 Target for Antidiabetic Functional Food Design. *Foodinformatics* 177-212
329 Perpetuo, E. A., Juliano, L., & Lebrun, I. (2003). Biochemical and pharmacological
330 aspects of two bradykinin-potentiating peptides obtained from tryptic hydrolysis of
331 casein. *Journal of Protein Chemistry*, 22(7/8), 601–606.
332 Pihlanto-Leppala, A., Rokka, T., & Korhonen, H. (1998). Angiotensin I-converting
333 enzyme inhibitory peptides derived from bovine milk proteins. *International Dairy*
334 *Journal,* 8(4), 325–331.
335 Pihlanto, A., Virtanen, T., & Korhonen, H. (2010). Angiotensin I converting enzyme
336 (ACE) inhibitory activity and antihypertensive effect of fermented milk.
337 *International Dairy Journal*, 20, 3–10.

Formatted: English (U.S.)

Formatted: English (U.S.)

Recio I., Gommans P. G. M., Slangen C. J., Visser S. (1998) Production of antibacterial peptides from biological fluids. Abstracts of Communications of the European Dairy Experts Symposium, Arnhem, 65 Conference proceedings.

Sandre, C., Gleizes, A., Forestier, F., Gorges-Kergot, R., Chilmonczyk, S., Leonil, J., Moreau, M. C., & Labarre, C. (2001). A peptide derived from bovine b-casein modulates functional properties of bone marrow-derived macrophages from germfree and human flora-associated mice. *The Journal of Nutrition*, 131(11), 2936–2942.

Schlimme, E., & Meisel, H. (1995). Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. *Die Nahrung*, 39(1), 1–20.

Shuang, Q., Harutoshi, S., & Taku, M. (2008). Angiotensin I-converting enzyme inhibitory peptides in skim milk fermented with Lactobacillus helveticus 130B4 from camel milk in inner Mongolia, China. *Journal of the Science of Food and Agriculture*, 88, 2688–2692.

Silva S.V., Pihlanto A., Malcata F.X. (2006). Bioactive peptdes in ovine and caprine cheeselike systems prepared with proteases from Cynara cardunculus. *Journal of Dairy Science*, 89 (2006), 336-3344.

Strohalm, M., Kavan, D., Novák, P., Volný, M., Havlíček, V. (2010). mMass 3: A Cross-Platform Software Environment for Precise Analysis of Mass Spectrometric Data. *Anal Chem,* 82 (11), 4648-4651.

Suetsuna, K., Ukeda, H., & Ochi, H. (2000). Isolation and characterisation of free radical scavenging activities peptides derived from casein. *Journal of Nutritional Biochemistry,* 11, 128–131.

Toldrá, F., & Flores, M. (2011). Microbial enzymatic activities for improved fermented meats (Review). *Trends in Food Science and Technology,* 22, 81-90.

Toldrá, F., & Flores, M. (1998). The role of muscle proteases and lipases in flavor development during the processing of dry-cured ham. *Critical Reviews in Food Science and Nutrition*, 38(4), 331–352.

Van der Ven C. (2002). Biochemical and functional characterisation of casein and whey protein hydrolysates. A study on correlations between biochemical and functional properties using multivariate data analysis. ISBN: 90-5808-653-4.

Field Code Changed

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.

395

396

397

398

399

400

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 numer 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_f	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	IQAFLL**YQEPVL**	G	YQEP	-	antioxidative	Silva, Pihlanto and Malcata (2006)
	745.95	1489.89	2	P	IQAFLL**YQEPVL**G	P				
	579.32	1156.62	2	L	**YQEPVLGPVR***	G	YQEPVLGPVR	300	ACE inhibitor	Meisel et al (2006)
	607.85	1213.68	2	L	**YQEPVLGP**VRG	P	YQEPVLGP	-	antioxidative	Silva, Pihlanto and Malcata (2006)
	656.35	1310.69	2	L	**YQEPVLGP**VRGP	F				
	729.90	1457.79	2	L	**YQEPVLGP**VRGPF	P				
	769.44	1536.87	2	F	LL**YQEPVLGP**VRGP	F				
	778.43	1554.84	2	L	**YQEPVLGP**VRGPFP	I	VRGPFP	-	ACE inhibitor	Gómez-Ruiz et al (2002)
	834.93	1667.84	2	L	L**YQEPVLGP**VRGPFP	I				
	834.97	1667.93	2	L	**YQEPVLGPVRGPFPI***	I	YQEPVLGPVRGPFPI	-	ACE inhibitor	Meisel and Schlimme (1994)
	842.98	1683.94	2	F	LL**YQEPVLGP**VRGPF	P				
	891.48	1780.94	2	L	**YQEPVLGP**VRGPFPII	V				
	891.51	1781.00	2	F	LL**YQEPVLGP**VRGPFP	I				
	891.51	1781.01	2	L	L**YQEPVLGP**VRGPFPI	I				
	941.05	1880.09	2	L	**YQEPVLGPVRGPFPIIV***	-	YQEPVLGPVRGPFPIIV	-	antimicrobial	Sandre et al (2001)
	948.06	1894.10	2	F	LL**YQEP**VLGPVRGPFPI	I				
	948.06	1894.11	2	L	L**YQEP**VLGPVRGPFPII	V				
	997.58	1993.16	2	L	L**YQEP**VL**GPVRGPFPIIV**	-	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	RDMPIQAFLL**YQEPVLGPVRGPFPIIV**	-				
	1064.60	3190.79	3	Y	PQRDMPIQAFLL**YQEPVLGP**VRGPFPII	V				
	776.41	1550.80	2	P	FAQ**TQSLVYP**FPGP	I	TQSLVYP	64	ACE inhibitor	Kohmura, Nio and Ariyoshi (1990)
	741.41	740.40	1	P	**FPGPIPN***	S	FPGPIPN	260	DPP IV inhibitor	Ojeda, Cereto-Massagué, Valls and Pujadas (2014)
	593.85	1185.68	2	Q	**SLVYPFPGPI**P	N	PGPIPN	-	immunomodulating	Meisel (1998)
	650.87	1299.72	2	Q	**SLVYPFPGPI**PN	S	SLVYPFPGPI	-	ACE inhibitor	Hafeez et al (2014)
	750.93	1499.85	2	Q	**SLVYPFPGPI**PNSL	P				
	799.45	1596.89	2	Q	**SLVYPFPGPI**PNSLP	Q				
	863.48	1724.95	2	Q	**SLVYPFPGPI**PNSLPQ	N				
	920.50	1838.99	2	Q	**SLVYPFPGPI**PNSLPQN	I				
	1074.10	2146.18	2	Q	**SLVYPFPGPI**PNSLPQNIPP	L				
	754.10	2259.29	3	Q	**SLVYPFPGPI**PNSLPQNIPPL	T				
	1181.17	2360.32	2	Q	**SLVYPFPGPI**PNSLPQNIPPLT	Q				
	830.47	2488.39	3	Q	**SLVYPFPGPI**PNSLPQNIPPLTQ	T				
	896.51	2686.51	3	Q	**SLVYPFPGPI**PNSLPQNIPPLTQTP	V				
	1060.28	3177.82	3	Q	**SLVYPFPGPI**PNSLPQNIPPLTQTPVVVPP	F				
	1109.31	3324.91	3	Q	**SLVYPFPGPI**PNSLPQNIPPLTQTPVVVPPF	L				
	1147.01	3438.01	3	Q	**SLVYPFPGPI**PNSLPQNIPPLTQTPVVVPPFL	Q				
	1189.69	3566.06	3	Q	**SLVYPFPGPI**PNSLPQNIPPLTQTPVVVPPFLQ	P				
	916.79	3663.13	4	Q	**SLVYPFPGPI**PNSLPQNIPPLTQTPVVVPPFLQP	E				
	949.05	3792.17	4	Q	**SLVYPFPGPI**PNSLPQNIPPLTQTPVVVPPFLQPE	V				
	973.82	3891.24	4	Q	**SLVYPFPGPI**PNSLPQNIPPLTQTPVVVPPFLQPEV	M				
	1006.59	4022.32	4	Q	**SLVYPFPGPI**PNSLPQNIPPLTQTPVVVPPFLQPEVM	G				
	1020.84	4079.32	4	Q	**SLVYPFPGPI**PNSLPQNIPPLTQTPVVVPPFLQPEVMG	V				
	876.9823	1751.95	2	S	**LVYPFPGPIPNSLPQN***	I	LVYPFPGPIPNSLPQN	71	ACE inhibitor	Pihlanto, Virtanen and Korhonen, (2010)
	687.3968	2059.1684	3	S	**LVYPFPGPIPNSLPQNIPP***	L	LVYPFPGPIPNSLPQNIPP	5	ACE inhibitor	Hernández-Ledesma, Quirós, Amigo, and Recio (2007)
	438.76	875.50	2	H	K**EMPFPK**	Y	EMPFPK	-	ACE inhibitor	Pihlanto-Leppala et al (1998);Perpetuo et al (2003)
	911.45	910.44	1	K	**EMPFPK**Y	P				
	338.53	1012.56	3	K	HK**EMPFPK**	Y				
	520.28	1038.55	2	H	K**EMPFPK**Y	P				
	588.81	1175.61	2	K	HK**EMPFPK**Y	P				
	637.34	1272.66	2	K	HK**EMPFPK**YP	V				
	652.86	1303.70	2	P	KHK**EMPFPK**Y	P				
	437.26	1308.76	3	M	APKHK**EMPFPK**	Y				
	467.93	1400.78	3	A	PKHK**EMPFPK**Y	P				
	480.93	1439.78	3	A	MAPKHK**EMPFPK**	Y				
	736.90	1471.79	2	M	APKHK**EMPFPK**Y	P				
	504.61	1510.82	3	E	AMAPKHK**EMPFPK**	Y				
	802.42	1602.83	2	A	MAPKHK**EMPFPK**Y	P				
	837.94	1673.87	2	E	AMAPKHK**EMPFPK**Y	P				
	601.98	1802.92	3	K	EAMAPKHK**EMPFPK**Y	P				
	499.79	1995.12	4	S	K**VKEAMAPK**HK**EMPFPK**	Y	VKEAMAPK		antioxidant	Suetsuna et al (2000); Korhonen and Pihlanto (2007)
	1016.05	2030.08	2	K	**VKEAMAPK**HK**EMPFPK**Y	P				
	1080.09	2158.17	2	S	K**VKEAMAPK**HK**EMPFPK**Y	P				
	801.44	2401.29	3	M	GVSK**VKEAMAPK**HK**EMPFPK**Y	P				
	565.53	2822.63	5	L	QPEVMGVSK**VKEAMAPK**HKEMPFPK	Y				
	960.80	3839.16	4	I	PPLTQTPVVVPPFLQPEVMGVSK**VKEAMAPK**HKEM	P				
	966.55	3862.17	4	V	PPFLQPEVMGVSK**VKEAMAPK**HKEMPFPKYPVEP	F				
	1006.59	4022.32	4	Q	SLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVM	G				
	981.48	980.47	1	K	**YPVEPFTE***	S	YPVEPFTE	-	ACE inhibitor	Perpetuo, Juliano and Lebrun (2003)
	603.82	1205.63	2	F	PK**YPVEPFTE**	S		-	opioid	Perpetuo et al (2003)
	647.34	1292.67	2	F	PK**YPVEPFTE**S	Q				
	754.88	1507.75	2	F	PK**YPVEPFTE**SQS	L				
	935.48	1868.95	2	P	FPK**YPVEPFTE**SQSLT	L				
	860.77	3439.04	4	E	MPFPK**YPVEPFTE**SQSLTLTDVENLHLPLP	L				
	915.62	914.61	1	N	**LHLPLP**LL	Q	LHLPLP	5	ACE inhibitor	Hernández-Ledesma, Quirós, Amigo, and Recio (2007)
	458.80	915.59	2	E	N**LHLPLP**L	L	HLPLP		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.

Table 3

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							Previously identified bioactive peptides[c]			
UniProt	(*m/z*)[a]	(Da)[b]	Charge	P_0	Identified sequence*		P_f	Sequence	IC_{50} (μM)	Activity	References
CASA1_BOVIN	599.37	1196.72	2	A	**RPKHPIKHQ**G		L	RPKHPIKHQ	13	ACE inhibitor	FitzGerald, Murray and Walsh (2004)
	469.96	1406.87	3	A	**RPKHPIKHQ**GLP		Q	RPKHPIKHQGLPQEVLNENLLRF	0	immunomodulating	Lahov and Regelson (1996)
	512.65	1534.92	3	A	**RPKHPIKHQGLPQ**		E	TTMPLW	-	opioid	Migliore-Samour et al. (1989)
	555.66	1663.97	3	A	**RPKHPIKHQGLPQE**		V		16	ACE inhibitor	Maruyama S., Miyoshi S., Tanaka (1989)
	939.04	1876.07	2	A	**RPKHPIKHQGLPQEVL**		N		-	immunomodulating	Hayes et al (2007)
	996.09	1990.16	2	A	**RPKHPIKHQGLPQEVLN**		E	KKYKVPQ	-	ACE inhibitor	Gómez-Ruiz et al (2002)
	707.39	2119.16	3	A	**RPKHPIKHQGLPQEVLNE**		N	KKYKVPQL	-	ACE inhibitor	Gómez-Ruiz et al (2002)
	745.42	2233.24	3	A	**RPKHPIKHQGLPQEVLNEN**		L				
	1007.51	2013.00	2	D	IPNPIGSENSEK**TTMPLW**		-				
	1108.55	2215.08	2	F	SDIPNPIGSENSEK**TTMPLW**		-				
	873.44	2617.31	3	D	APSFSDIPNPIGSENSEK**TTMPLW**		-				
	999.82	2996.45	3	Q	YTDAPSFSDIPNPIGSENSEK**TTMPLW**		-				
	1003.64	1002.63	1	L	**KKYKVPQL***		E				
	558.87	1115.73	2	R	L**KKYKVPQL**		E				
	378.23	1131.67	3	L	**KKYKVPQ**LE		I				
	636.92	1271.83	2	L	RL**KKYKVPQL**		E				
	701.45	1400.88	2	L	RL**KKYKVPQL**E		I				
	807.39	1612.76	2	L	RL**KKYKVPQL**EIV		P				
	837.49	2509.44	3	L	RL**KKYKVPQL**EIVPNSAEERL		H				
CASA2_BOVIN	738.40	737.39	1	K	**FALPQY***		L	FALPQY	4.3	ACE inhibitor	FitzGerald R.J., Murray B. A., Walsh (2004)
	1157.60	1156.59	1	R	YQK**FALPQY**		L	AMKPWIQPK	600	ACE inhibitor	Van der Ven C.(2002); Maeno, Yamamoto and Takano (1996)
	635.86	1269.70	2	R	YQK**FALPQYL**		K	YYQHQKAMKPWIQPKTKVIPYVRY	-	antibacterial	Recio, Gommans, Slangen, and Visser (1998)
	546.34	1635.99	3	K	**AMKPWIQPK**TKVIP		Y	YYQHQKAMKPWIQPKTKVIPYVRYL	-	antibacterial	Recio, Gommans, Slangen, and Visser (1998)
	628.68	1883.03	3	V	YQHQK**AMKPWIQPKT**		K				
	633.72	1898.13	3	K	**AMKPWIQPK**TKVIPYV		R				
	671.38	2011.12	3	V	YQHQK**AMKPWIQPK**TK		V				
	685.74	2054.21	3	K	**AMKPWIQPK**TKVIPYVR		Y				
	728.45	2182.34	3	Q	K**AMKPWIQPK**TKVIPYVR		Y				
	740.09	2217.26	3	K	**AMKPWIQPK**TKVIPYVRY		L				
	771.13	2310.38	3	H	QK**AMKPWIQPK**TKVIPYVR		Y				
	774.44	2320.30	3	V	YQHQK**AMKPWIQPK**TKVIP		Y				
	913.86	2738.56	3	V	YQHQK**AMKPWIQPK**TKVIPYVR		Y				
	710.42	2837.65	4	T	**VYQHQKAMKPWIQPKTKVIPYVR**		Y				
	735.68	2938.68	4	K	**TVYQHQKAMKPWIQPKTKVIPYVR**		Y				
CASK_BOVIN	1043.09	2084.17	2	H	PHPH**LSFMAIPPKKNQDK**		T	LSFMAIPPKK	-	antithrombotic	Fiat et al (1993)
								MAIPPKKNQDK	-	antithrombotic	Fiat and Jollès (1989); Schimme and Meisel (1995)
								MAIPPKK	-	antithrombotic	Fiat and Jollès (1989); Schimme and Meisel (1995)
								NQDK	-	antithrombotic	Fiat and Jollès (1989); Schimme and Meisel (1995)
	505.7982	1009.5818	2	M	**AIPPKKNQD***		K	AIPPKKNQD	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*.

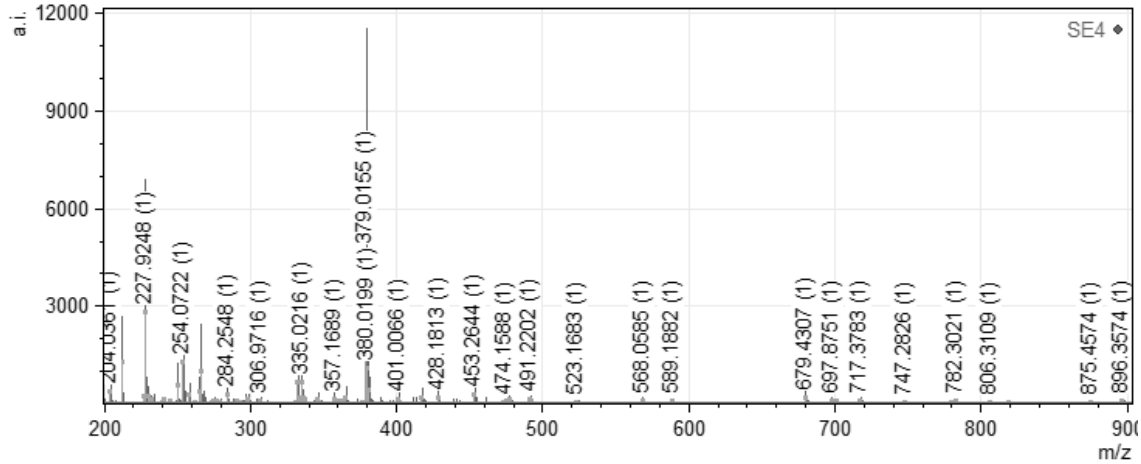
b. Calculated relative molecular mass of the matched peptide in Daltons.

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.

Figure 1

A) From 200 to 900 *m/z*



B) From 850 to 3500 *m/z*

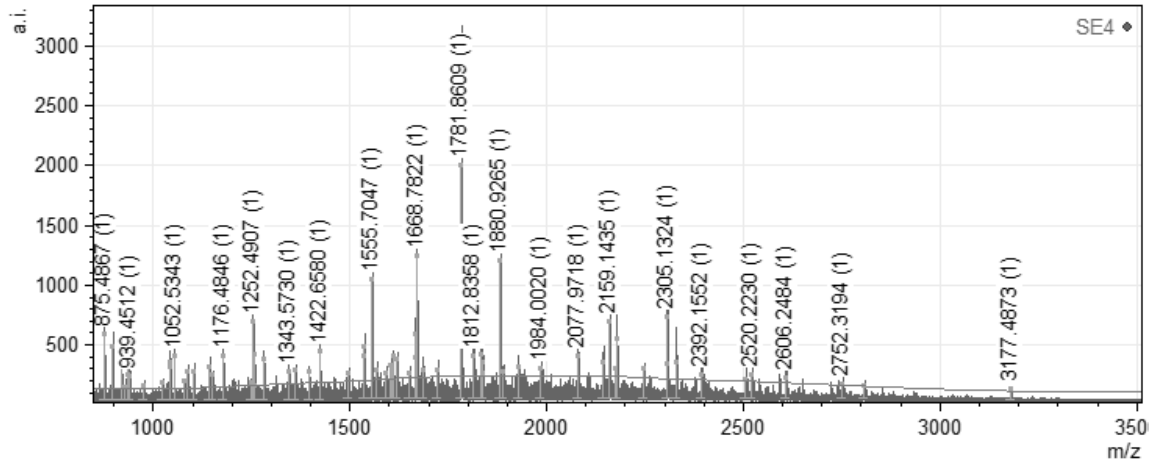


Figure 1.

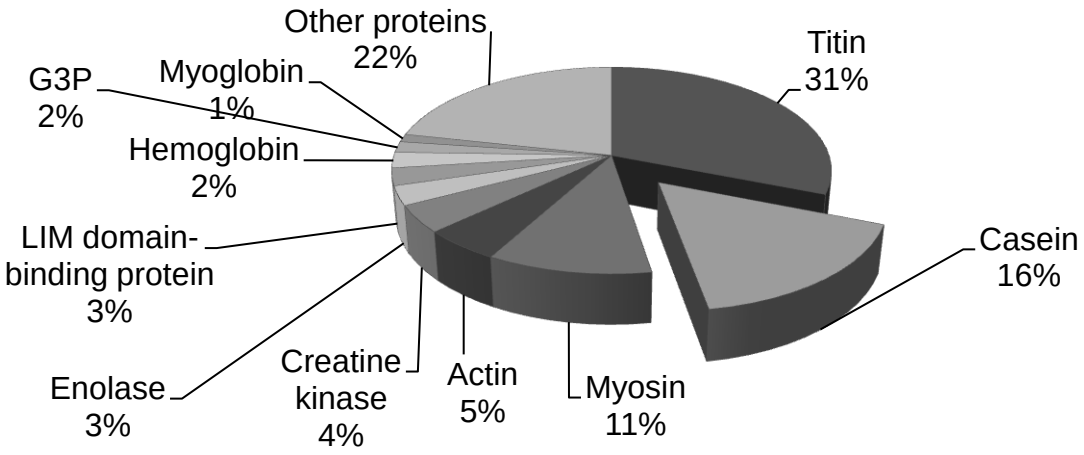

Other proteins
22%
Titin
31%
G3P
2%
Myoglobin
1%
Hemoglobin
2%
LIM domain-
binding protein
3%
Casein
16%
Enolase
3%
Creatine
kinase
4%
Actin
5%
Myosin
11%

Figure 2.

CASB_BOVIN

```
  1 MKVLILACLV ALALARELEE LNVPGEIVES LSSSEESITR INKKIEKFQS
 51 EEQQQTEDEL QDKIHPFAQT QSLVYPFPGP IPNSLPQNIP PLTQTPVVVP
101 PFLQPEVMGV SKVKEAMAPK HKEMPFPKYP VEPFTESQSL TLTDVENLHL
151 PLPLLQSWMH QPHQPLPPTV MFPPQSVLSL SQSKVLPVPQ KAVPYPQRDM
201 PIQAFLLYQE PVLGPVRGPF PIIV
```

CASA1_BOVIN

```
  1 MKLLILTCLV AVALARPKHP IKHQGLPQEV LNENLLRFFV APFPEVFGKE
 51 KVNELSKDIG SESTEDQAME DIKQMEAESI SSSEEIVPNS VEQKHIQKED
101 VPSERYLGYL EQLLRLKKYK VPQLEIVPNS AEERLHSMKE GIHAQQKEPM
151 IGVNQELAYF YPELFRQFYQ LDAYPSGAWY YVPLGTQYTD APSFSDIPNP
201 IGSENSEKTT MPLW
```

CASA2_BOVIN

```
  1 MKFFIFTCLL AVALAKNTME HVSSSEESII SQETYKQEKN MAINPSKENL
 51 CSTFCKEVVR NANEEEYSIG SSSEESAEVA TEEVKITVDD KHYQKALNEI
101 NQFYQKFPQY LQYLYQGPIV LNPWDQVKRN AVPITPTLNR EQLSTSEENS
151 KKTVDMESTE VFTKKTKLTE EEKNRLNFLK KISQRYQKFA LPQYLKTVYQ
201 HQKAMKPWIQ PKTKVIPYVR YL
```

CASK_BOVIN

```
  1 MMKSFFLVVT ILALTLPFLG AQEQNQEQPI RCEKDERFFS DKIAKYIPIQ
 51 YVLSRYPSYG LNYYQQKPVA LINNQFLPYP YYAKPAAVRS PAQILQWQVL
101 SNTVPAKSCQ AQPTTMARHP HPHLSFMAIP PKKNQDKTEI PTINTIASGE
151 PTSTPTTEAV ESTVATLEDS PEVIESPPEI NTVQVTSTAV
```

Figure 3.

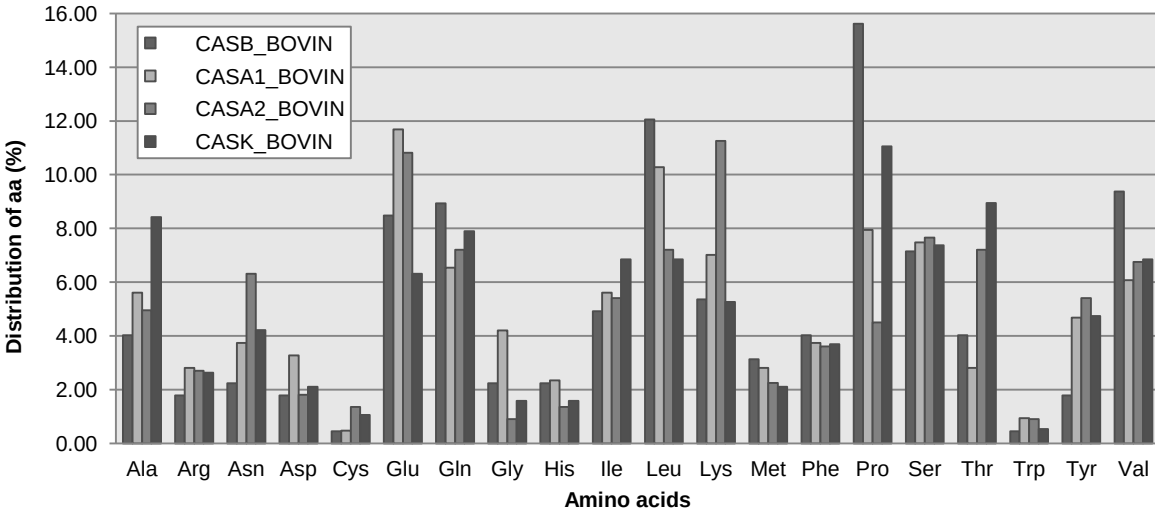

16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Distribution of aa (%)
CASB_BOVIN
CASA1_BOVIN
CASA2_BOVIN
CASK_BOVIN
Ala Arg Asn Asp Cys Glu Gln Gly His Ile Leu Lys Met Phe Pro Ser Thr Trp Tyr Val
Amino acids

Figure 4.