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(54) Title: USE OF A CASEIN-DERIVED PEPTIDE AND COMPOSITIONS THEREOF AS ANTIHYPERTENSIVE

(57) Abstract: Use of a casein-derived peptide with antioxidant and ACE-inhibitory activity *in vitro* and antihypertensive activity in mammals, and compositions thereof. This peptide may be use as active ingredient in pharmaceutical, cosmetic and/or dermatological compositions, dietary supplements or as food ingredient. Also relates with composition comprising this peptide in specific concentrations, and fermented compositions, including fermented dairy product, that have enhanced stability under various storage conditions, and their uses.



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USE OF A CASEIN-DERIVED PEPTIDE AND COMPOSITIONS THEREOF AS ANTIHYPERTENSIVE.

5 **FIELD OF THE INVENTION**

The present invention relates to the use of a casein-derived peptide with antioxidant and ACE-inhibitory activity *in vitro* and antihypertensive activity in mammals, and compositions thereof. Preferably, this peptide may be use as active ingredient in pharmaceutical, cosmetic and/or dermatological compositions, dietary supplements or as food ingredient. Also relates with composition comprising this peptide in specific concentrations, and fermented compositions, including fermented dairy product, that have enhanced stability under various storage conditions, and their uses.

BACKGROUND OF THE INVENTION

Uncontrolled oxidation processes have been recognized as one of the main causes of food quality decay. These processes are initiated by enzymes such as lipoxygenases (LOX), polyphenol oxidases (PPO) and peroxidases (POX). In order to inhibit such oxidation and peroxidation of unsaturated fatty acids, and to prevent deterioration of product quality, various antioxidants have been conventionally used. Antioxidants (AOX) act on peroxide radicals, which are generated in oxidation, to terminate chain oxidation, or as an alternative act on free radicals to terminate oxidative reaction. Synthetic antioxidants are commonly used antioxidants, such as butylhydroxyanisol (BHA) and butylhydroxytoluene (BHT). However, as their use expands, the safety of synthetic antioxidants has become questioned, since consumers are presenting growing rejections, which results in a decrease of their use. Furthermore, synthetic antioxidants are oil soluble, thus not suitable for their in aqueous solutions.

On the other hand, there are many well known high safety antioxidants such as natural vitamin E (α -tocopherol), vitamin C, and alike are known. However, these natural antioxidants are extremely fat- or water-soluble, so their applications are limited. The natural antioxidants have also disadvantages since
5 their activity cannot be maintained stable for a prolonged period of time.

Furthermore, the intake of peroxides produced from lipid oxidation exerts detrimental effects *in vivo*. Free radicals and active oxygen species generated in the course of oxidation reactions denature proteins *in vivo*, inactivate
10 enzymes (Szweda *et al.*, 1993 *Journal of Biological Chemistry* 268: 3342–3347), bring about mutations in DNA (Reiss *et al.*, 1972. *Biochemical and Biophysical Research Communication* 48:921-926), modify low density lipoproteins (Alaiz *et al.*, 1994. *Biological and Pharmaceutical Bulletin* 17:51-57), and contribute to aging and various diseases.

15 The reactive oxygen species (ROS), are implicated inter alia in the cause of carcinogenetic, inflammatory, infectious, cardiovascular and neurological diseases in man and animals (Nair *et al.*, 2003. *Mutagenesis* 18: 477-483). Antioxidants (AOX) are considered a promising therapeutic approach as they
20 may be playing neuroprotective (preventing apoptosis) and neuroregenerative roles (Moosmann and Behl, 2002. *Expert Opinion on Investigational Drugs* 11:1407-1435). AOX offer a promising approach in the control or slowing down progression of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and
25 ischaemic and haemorrhagic stroke (Maxwell, 1995; Floyd, 1999. *Proceedings of the Society for Experimental Biology and Medicine* 222:235-245; Mattson 2000. *Nature Rev. Mol. Cell Biol.* 1: 120-130; Moosmann and Behl, 2002. *Expert Opinion on Investigational Drugs* 11:1407-1435; Nair *et al.*, 2003. *Mutagenesis* 18: 477-483; Berger 2005. *Clinical Nutrition* 24:172-183). The
30 most important exogenous AOX are dietary phytochemicals (such as polyphenols, quinones, flavonoids, catechins, coumarins, terpenoids) and the smaller molecules like ascorbic acid (Vitamin C), alpha-tocopherol (Vitamin E)

and beta-carotene (pro-vitamin A). Because of the importance in preventing oxidation to biological processes and to improved stability of products subject to oxidation, there remains a need to identify new antioxidative compounds, such as antioxidant peptides.

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Dietary antioxidants may also help to prevent cardiovascular diseases (Krinksky 1993. *Annual review of nutrition* 13: 561-587); Parthasarathy, 1998. *Journal of Medicinal Food* 1:45-51). Cardiovascular diseases represent the first cause of morbidity and mortality in Western countries, with hypertension affecting about
10 20% of the world adult population (Miguel *et al.*, 2007. *Food Chemistry* 104: 163–168). High blood pressure is a common risk factor for cardiovascular diseases, and more accurately, this disease induces cerebrovascular incidents, heart failure and kidney disease, which all could lead to more complicated dysfunctions of the internal organs.

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Although most of the mechanisms associated with hypertension are unknown, is well studied that one regulator of hypertension, the angiotensin I-converting enzyme (ACE), which is a zinc-containing peptidyl-dipeptidase A (EC 3.4.15.1), plays an important physiological role in regulating blood pressure (Skeggs *et al.*,
20 1956. *The Journal of Experimental Medicine* 103: 295-299). The greater the ACE activity, the more angiotensin I is converted to angiotensin II, which induces high blood pressure. ACE converts an inactive form of the decapeptide, angiotensin I, to a potent vasoconstrictor, the octapeptide angiotensin II, and also inactivates bradykinin which reduces blood pressure. For these reasons,
25 specific inhibitors of ACE are useful for regulating physiological activities associated with ACE in the human body (Ondetti *et al.*, 1977. *Science* 196: 441–444).

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Potent synthetic ACE inhibitors such as captopril, enalapril, alacepril, lisinopril and ramipril are widely used in the clinical treatment of hypertension and heart disorders in humans. These synthetic drugs, however, have several side effects including coughing, taste disturbances and skin rashes. Food-derived ACE

inhibitors have safety advantages over the synthetic compounds (Atkinson & Robertson 1979. *Lancet* 2: 836–839). As a large number of individuals suffer from such disease, scientists believe that other methods rather than chemical and pharmacological medication should be identified in the effort to reduce
5 hypertensive diseases. Therefore, potential biological, functional and nutraceutical methods should be utilized as a treatment to minimize the number of individuals who fall into the hypertensive category and have been afflicted with diseases arising from this.

10 Some peptides have recently been reported to play an important role in controlling the development of hypertension by regulating the renin angiotensin system (Arihara *et al.*, 2001. *Meat Science*, 57: 319–324; Asihda *et al.*, 1996. *Clinical and Experimental Hypertension* 18: 851–871; Haws *et al.*, 1994. *Pediatric Research* 35: 671–676.; Katayama *et al.*, 2004. *Journal of Agricultural and Food Chemistry* 52: 771–775; Katayama *et al.*, 2007. *Journal of Food Science* 72: S702–S706; Katayama *et al.*, 2008. *Journal of Agricultural and Food Chemistry* 56: 355–360). There are of course other studies concerned with ACE inhibitory peptides with a view to enhance the value of food products. Many of these peptides have been sourced from animal products such as
20 bovine casein, porcine muscle, egg white, yak milk casein, sheep milk yoghurt and oyster (Miguel *et al.*, 2009, *Food Chemistry* 112: 211-214; Miguel and Fidel, 2007. *Food Chemistry* 102: 511–515; Miguel *et al.*, 2007. *Food Chemistry* 104: 163–168; Maoa *et al.*, 2007. *Food Chemistry* 103: 1282–1287; Papadimitriou *et al.*, 2007. *Food Chemistry* 105: 647–656; and Wang *et al.*, 2008. *Food Chemistry* 111: 302–308, respectively). Some have been found to be effective for antihypertensive agents *in vivo* without causing side effects in spontaneously hypertensive rats (SHR) and hypertensive humans (Li *et al.*, 2004. *Nutrition Research* 27:469–486).

30 Milk proteins (mainly α -lactalbumin, caseins, β -lactoglobulin, immunoglobulins, lactoferrin and serum albumin) are known for having a wide range of nutritional, functional and biological properties that make them important ingredients in

functional or health promoting foods. These properties are partly attributed to bioactive peptides coded in the different milk proteins. At present, numerous peptides exhibiting various activities, such as opiate, antithrombotic and antioxidant activities, cholesterol-lowering ability, antimicrobial, immunomodulatory, mineral carrier and antihypertensive activity have been reported. Moreover, some peptides are multifunctional and can exert more than one of the effects mentioned. Bioactive peptides are often inactive within the sequence of the parent protein and can be released by enzymatic hydrolysis.

10 Powerful ACE inhibitory hydrolysates were obtained from whey protein after hydrolysis with trypsin (Ferreira *et al.*, 2007. *International Dairy Journal* 17: 481–487; Mullally *et al.*, 1997. *International Dairy Journal* 7: 299–303) and α -chymotrypsin (Da Costaa *et al.*, 2007. *International Dairy Journal* 17: 632–640). Digestive enzymes have also been employed for the release of antihypertensive peptides from the milk casein fraction, for instance, this is the case of trypsin or pepsin (López-Expósito *et al.*, 2007. *Le Lait* 87: 241-249; Miguel *et al.*, 2009. *Food Chemistry* 112: 211-214). Spectrophotometric, fluorimetric, chromatographic and capillary electrophoresis techniques have been used to isolate the active peptides and to measure their ability to inhibit ACE in vitro.

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20 However, it is only through *in vivo* studies that the antihypertensive effects of a given hydrolysate or peptide can be reliably assessed. Several studies have been performed to determine the antihypertensive effects of ACE-inhibitory peptides using spontaneously hypertensive rats (SHR) (Miguel *et al.*, 2005. *British Journal of Nutrition* 94: 731–737; Mugerza *et al.*, 2006. *International Dairy Journal*, 16: 61–69; Nakamura *et al.*, 1995. *Journal of Dairy Science* 78: 1253–1257; for a review see, FitzGerald *et al.*, 2004. *Journal of Nutrition* 134: 980S–988S; López-Fandiño *et al.*, 2006. *International Dairy Journal* 16:1277–1293), but only few milk-derived peptides have been tested for their antihypertensive effect in humans (Hata *et al.*, 1996. *American Journal of Clinical Nutrition* 64: 767–771; Mizuno *et al.*, 2005. *British Journal of Nutrition* 94: 84–91; Jauhiainen *et al.*, 2005. *American Journal of Hypertension* 18: 1600–

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1605; Jauhiainen *et al.*, 2007. *International Dairy Journal* 17: 1209–1211; Seppo *et al.*, 2003. *American Journal of Clinical Nutrition* 77: 326–330).

The IC₅₀ value (inhibitor concentration leading to 50% inhibition) is used to estimate the effectiveness of different ACE inhibitory peptides. However, it is not always directly related to the *in vivo* hypotensive effect. Some peptides can be susceptible to degradation or modification in the gut, the vascular system and the liver. Being an inhibitor of ACE is not enough for a peptide to be able to reduce hypertension in a hypertensive subject; it should also be bioavailable in order to reach intact the renin-angiotensin mechanism of the smooth muscles of blood vessel walls. There are several examples of peptides, which showing a potent ACE-inhibitory activity, did not exert antihypertensive effect *in vivo*. For instance, peptide α s1-casein f(23-27) that showed a potent ACE-inhibitory activity but no hypotensive effect in SHR (Maruyama *et al.*, 1987. *Agricultural and Biological Chemistry* 51: 2557-2561), and peptide β -lactoglobulin f(142-148) with *in vitro* ACE-inhibitory activity but did not have effect in human volunteers (Walsh *et al.*, 2004. *Journal of Dairy Science* 87: 3845-3857).

For an industrial application of the antihypertensive peptides, a main consideration would be the optimization of the hydrolysis process, but also the evaluation of the resistance of the active peptides to processing conditions. It is now established that pressure-driven membrane-based processes such as ultrafiltration and nanofiltration can be used as strategy for enrichment and fractionation of peptide mixtures (López-Fandiño *et al.*, 2006. *International Dairy Journal*, 16, 1277-1293; Pouliot, 2008. *International Dairy Journal*, 18, 735-740). However, the transmission of bioactive peptides through membranes have to be exhaustively evaluated, since it involves complex mechanisms, such as size exclusion, electrostatic repulsion and ionic and hydrophobic interactions between peptides and membrane, but polarization concentration and fouling should be also taken into account (Butylina *et al.*, 2006. *Journal of Membrane Science*, 280, 418-426, Nau *et al.*, 1995. *Biotechnology and Bioengineering*, 46, 246-253; Wijers *et al.*, 1998. *Lait*, 78, 621-632). Dehydration preserves food

by reducing the water activity, thus inhibiting the activity of micro-organisms and enzymes, but it also reduces bulk which facilitates storage and transport. Dehydration by freeze-drying results in high quality products due to the most of deterioration and microbiological reactions are stopped for the absence of liquid water and the low temperatures (Ratti, 2001. *Journal of Food Engineering*, 49, 311-319). However, freeze-drying is an order-of-magnitude costlier than other drying methods, such as spray-drying, because of need of refrigeration, vacuum, and long running times. Spray-drying is a unit operation by which a liquid product is atomized in a hot gas current (generally air) to instantaneously obtain a powder. During this process a loss of a relatively large amount of dry powder with the exhaust air might occur, besides some negatively effects have been recently reported for food protein hydrolysates, such as changes in peptide composition, reduction of amino acid content and non enzymatic browning (Abdul-Hamid *et al.*, 2002. *Food Chemistry*, 78, 69-74 ; Bueno-Solano *et al.*, 2009. *Food Chemistry*, 112, 671-675; He *et al.*, 2008. *Bioresource Technology*, 99, 5956-5959). There are little data about the effects of dairy product technology on bioactive peptides, however process such as homogenization and pasteurization imply high pressures of the order of 14 MPa and temperature as high as 99°C. Incorporation of active hydrolysates to fermented milks implies that these peptides have to survive in the presence of the lactic acid bacteria (LAB) because of their cell-associated proteinases/peptidases systems that could further hydrolyse the bioactive sequences (Paul & Somkuti, 2009. *Letters in Applied Microbiology*, 49, 345-350; Paul & Somkuti, 2010. *Journal of Industrial Microbiology & Biotechnology*, 37, 173-178), even during refrigerated storage (WO 2006/042861).

Thus, the conditions of processing and storage may be detrimental to peptides. At this regard, changes in the molecular structure of an amino acid may lead to changes in the bioactivity and in the absorption of the peptide of interest in a bioavailable form. The most relevant degradation pathways of amino acids during processing were recently reviewed by López-Fandiño & co-workers (2006. *International Dairy Journal*, 16, 1277-1293). For example, thermal

processing favors racemization, amino acids decomposition (e.g. ornithine from arginine), glycation (non-enzymatic browning or Maillard reaction), and cross-linking. Furthermore, amino acids can be also oxidized during food processing (Elias *et al.*, 2008. *Critical Reviews in Food Science and Nutrition*, 48, 430-441),
5 and even D-amino acids can be synthesised out from L-amino acids by LAB.

As high blood pressure can lead to damaging effects on the brain, heart and blood vessels, it is essential to reduce the incidence of this condition among the population. Given the important role of diet in the prevention and treatment of
10 hypertension, and that is now demonstrated that the consumption of food products containing antihypertensive peptides produces a significantly reduction in blood pressure (Jauhiainen & Korpela, 2007. *Journal of Nutrition* 137: 825S-829S) it is necessary to identify peptides with antihypertensive activity *in vivo* that can be used as active ingredients in pharmaceutical and food
15 compositions, and develop pharmaceutical and food compositions with antihypertensive activity that can be processed and stored without change the bioactivity and/or the absorption of the bioactive peptide of interest.

20 DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a casein-derived peptide with antioxidant and ACE-inhibitory activity *in vitro* and antihypertensive activity in mammals, and compositions comprising this peptide, including fermented compositions with
25 antihypertensive activity, which can be processed and stored without change the bioactivity.

In one aspect the invention relates to an isolated peptide comprising the amino acid sequence of SEQ ID NO: 1, hereinafter peptide of the invention, for use as
30 antihypertensive. In a preferred embodiment of this aspect of the invention, the amino acid sequence of the peptide has no more than 10 amino acids. In another preferred embodiment the amino acid sequence of the peptide has no

more than 8 amino acids. In another preferred embodiment of this aspect of the invention, the amino acid sequence of the peptide consists of SEQ ID NO: 1. In a more preferred embodiment, the mammal is a human.

5 Hypertension, also referred to as high blood pressure, HTN or HPN, is a medical condition in which the blood pressure is chronically elevated. In current usage, the word "hypertension" without a qualifier normally refers to systemic, arterial hypertension. In individuals older than 50 years, hypertension is considered to be present when a person's blood pressure is consistently at
10 least 140 mm Hg systolic or 90 mm Hg diastolic. Persistent hypertension is one of the risk factors for strokes, heart attacks, heart failure and arterial aneurysm, and is a leading cause of chronic renal failure. Even moderate elevation of arterial blood pressure leads to shortened life expectancy. At severely high pressures, defined as mean arterial pressures 50% or more above average, a
15 person can expect to live no more than a few years unless appropriately treated. Beginning at a systolic pressure of 115 mm Hg and diastolic pressure of 75 mm Hg (commonly written as 115/75 mm Hg), cardiovascular disease (CVD) risk doubles for each increment of 20/10 mm Hg (Chobanian *et al.*, 2003. *Hypertension* 42 (6): 1206–1252).

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The term "isolated" herein, as used in "isolated peptide," refers to material, such as a nucleic acid, a peptide or a protein, which is (1) substantially or essentially free from components that normally accompany or interact with it as found in its naturally occurring environment. The isolated material optionally comprises
25 material not found which the material in its natural environment, or (2) if the material is in its natural environment, the material has been synthetically (non naturally) altered by deliberate human intervention.

Another aspect of the invention relates to the peptide of the invention,
30 comprising the amino acid sequence of SEQ ID NO: 1, for use as a medicament, or to the use of the peptide of the invention for the manufacture of a medicament.

In a preferred embodiment of this aspect of the invention, the amino acid sequence of the peptide has no more than 10 amino acids. In another preferred embodiment the amino acid sequence of the peptide has no more than 8 amino acids. In another preferred embodiment of this aspect of the invention, the amino acid sequence of the peptide consists of SEQ ID NO: 1.

Another aspect of the invention relates to the peptide of the invention, comprising the amino acid sequence of SEQ ID NO: 1, for use in the treatment or prevention of hypertension in a mammal including a human, or to the use of the peptide of the invention for the manufacture of a medicament for treatment of hypertension in a mammal including a human. In a preferred embodiment of this aspect of the invention, the amino acid sequence of the peptide has no more than 10 amino acids. In another preferred embodiment the amino acid sequence of the peptide has no more than 8 amino acids. In another preferred embodiment of this aspect of the invention, the amino acid sequence of the peptide consists of SEQ ID NO: 1. In a more preferred embodiment, the mammal is a human.

Another aspect of the invention relates to the peptide of the invention, comprising the amino acid sequence of SEQ ID NO: 1, for use in the treatment or prevention of a disease involving oxidation in a mammal including a human, or to the use of the peptide of the invention for the manufacture of a medicament for treatment of a disease involving oxidation in a mammal including a human. In a preferred embodiment of this aspect of the invention, the amino acid sequence of the peptide has no more than 10 amino acids. In another preferred embodiment the amino acid sequence of the peptide has no more than 8 amino acids. In another preferred embodiment of this aspect of the invention, the amino acid sequence of the peptide consists of SEQ ID NO: 1. In a more preferred embodiment, the mammal is a human. In another preferred embodiment, the disease involving oxidation is selected from the list comprising: osteoarthritis, rheumatoid arthritis, ischemia, cataract, corneal

pathology, glaucoma, retinal degeneration, vitreal degeneration, cancer, immune deficiency, hyperimmunity, autoimmunity, neurodegeneration, aging, Alzheimer's disease, Huntington's disease, Machado-Joseph disease, multiple sclerosis, muscular dystrophy, Parkinson's disease, senility, muscular atrophy, stroke, hepatopathies, systemic lupus erythematosus, mixed connective tissue disease, multiple sclerosis, and diabetes.

The peptide of the invention having an ability to reduce the hypertension in a mammal, could also show an ACE-inhibitory or antioxidative activity *per se*, or should be hydrolysed in order to release the bioactive peptide. As it is known by a person having ordinary skill in the art, the peptide of the invention could be like an acid or base addition salt, in particular pharmaceutically acceptable salts, esters, solvates and anhydrates. The term "pharmaceutically acceptable salts" or "solvates" refers to any pharmaceutically acceptable salt, ester, solvate, or any other compound which, upon administration to the recipient is capable of providing (directly or indirectly) a peptide as described herein. However, it will be appreciated that non-pharmaceutically acceptable salts also fall within the scope of the invention since those may be useful in the preparation of pharmaceutically acceptable salts. The preparation of salts and derivatives can be carried out by methods known in the art.

The peptide of the invention can be administered in a substantial pure form to a person or can be given as part of a more complex composition. When the peptide is given to a person in need thereof as part of a more complex mixture, the amount of this mixture given to this person should be such that the peptide mentioned in this invention reaches the effective amount. Examples of mixtures that contain this peptide or to which the peptide might be added are dried protein extracts, hydrolyzed protein extracts, dried plant extracts, cacao powder, a dried food composition, etc. A person in need of the mentioned peptide, can consume the peptide of this invention in a pure form as a pharmaceutical composition, as part of a composition containing this peptide, for instance as a dietary supplement, or as part of a food matrix whereto this peptide has been

added in an effective amount. Examples of food matrixes to which an anti-hypertensive and/or antioxidative composition containing the peptide of this invention can be added include, but are not limited to: beverages, infused foods, milk, yogurts, cheese, fermented milk, flavoured milk drinks, soybean milk, 5 precooked cereals, bread, cake, butter, margarine, sauces, frying oils, vegetable oils, corn oil, olive oil, soybean oil, palm oil, sunflower oil, cottonseed oil, condiments, salad dressings, fruit juices, syrups, desserts, icings and fillings, soft frozen products, confections, chewing gum and intermediate food.

10 Then, another preferred aspect of the invention relates to a composition, hereinafter first composition of the invention, comprising the peptide of the invention. In a preferred embodiment of this aspect of the invention, the amino acid sequence of the peptide has no more than 10 amino acids. In a preferred embodiment of this aspect of the invention, the amino acid sequence of the 15 peptide has up to 8 amino acids. In another preferred embodiment of this aspect of the invention, the amino acid sequence of the peptide consists of SEQ ID NO: 1.

In another preferred embodiment of this aspect of the invention, the amount of 20 the peptide of the invention is between 0.06 to 0.08 mg for kg of body weight of the mammal.

In a further preferred embodiment, the amount of the peptide of the invention in the composition is between 0.5 mg and 10 mg. In a more preferred 25 embodiment, the amount of the peptide of the invention is between 1,0 mg and 5,0 mg. In an even more preferred embodiment, the amount of the peptide of the invention is between 1.5 mg and 2.0 mg.

In a further preferred embodiment, the first composition of the invention also 30 comprises the peptide, hereinafter second peptide of the invention, comprising the amino acid sequence of SEQ ID NO: 9. In a more preferred embodiment, the amino acid sequence of the peptide consists of SEQ ID NO: 9. In a further

preferred embodiment, the amount of the peptide comprising the amino acid sequence of SEQ ID NO: 9 is between 1.5 mg and 15 mg. In a more preferred embodiment, the amount of the peptide comprising the amino acid sequence of SEQ ID NO: 9 is between 2.0 mg and 10 mg. In an even more preferred embodiment, the amount of the peptide comprising the amino acid sequence of SEQ ID NO: 9 is between 2.5 mg and 3.5 mg.

In a further preferred embodiment, the first composition of the invention consists in a food composition. The food composition comprises the peptide of the invention in an effective amount to reduce hyperthension in mammals and/or to reduce the excess reactive oxygen species (ROS) in a mammal, including a human. Preferred food compositions are selected from: a beverage, infused food, milk, yogurt, cheese, fermented milk, flavoured milk drink, soybean milk, precooked cereals, bread, cake, butter, margarine, sauces, frying oils, vegetable oils, corn oil, olive oil, soybean oil, palm oil, sunflower oil, cottonseed oil, condiments, salad dressings, fruit juices, syrups, desserts, icings and fillings, soft frozen products, confections, chewing gum and intermediate food.. Also within the food compositions, the present invention contemplates a protein hydrolysate, obtained from mammal milk, enriched in the peptide of the invention or derivatives thereof. In a preferred embodiment the protein hydrolysate is obtained from bovine milk. In a preferred protein hydrolysate according to the invention, the amount of the peptide of the invention or salts thereof is between 0.005% and 3%, preferably between 0,01% and 1% of the protein hydrolysate. It is clear that the use of the peptide and derivatives thereof, as well as the protein hydrolysates of the invention can be employed in the preparation of food compositions, which include dietary supplements and food ingredients. In another preferred embodiment the nutritional or dietary supplement comprises a sterile composition containing the peptide of invention, preferably provided with a gastric juice resistant release delaying coating. In another preferred embodiment the food composition, including the protein hydrolysate and the nutritional or dietary supplement comprises appropriate "carriers" such as diluents, adjuvants, excipients, or vehicles with which the

peptide is administered. Suitable appropriate excipients include, but are not limited to starch, glucose, fructose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol, and the like. Such nutritional supplements may be used to combat a wide range of conditions associated with hypertension or with the excess reactive oxygen species, and they help maintain health or a healthy lifestyle to the mammal, preferably human. The food composition also has properties for preventing hypertension in mammals, preferably human beings.

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In a further preferred embodiment, the first composition of the invention is a fermented composition.

Incorporation of active hydrolysates to fermented milks implies that these peptides have to survive in the presence of the lactic acid bacteria (LAB) because of their cell-associated proteinases/peptidases systems that could further hydrolyse the bioactive sequences, even during refrigerated storage. The compositions of the present inventions have enhanced stability under various storage conditions, and shows antihypertensive activity.

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The resistance of these peptides to processing conditions, i.e. drying, homogenization and pasteurization, and their stability in yoghurts stored at 4 °C were also evaluated. The examples of the present invention shows that active peptides SEQ ID NO:1 and SEQ ID NO: 9 were stable during the processes of atomization, homogenization and pasteurization, and were also stable during refrigerated storage.

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In another preferred embodiment the first composition of the invention is a dairy product.

In a further preferred embodiment, the first composition of the invention is a pharmaceutical composition comprising the peptide of the invention or a pharmaceutically acceptable derivative thereof and a pharmaceutically

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acceptable carrier, binder and/or auxiliary material. Said peptide or derivative thereof is preferably present in the composition in an effective amount to reduce hypertension in mammals. The carrier materials, binders and/or auxiliary materials must be pharmaceutically and pharmacologically tolerable, so that they can be combined with the other components of the formulation or preparation and do not exert adverse effects on the organism treated. The pharmaceutical compositions can be in the form of single doses. The compositions are prepared according to methods known in the field of pharmacology. The appropriate quantities of active substances suitable for administration may vary as a function of the particular field therapy. Generally, the active substance concentration in a single-dose formulation is 1% to 95% of the total formulation. Preferably, the peptide of the invention is orally administered to a mammal in a dose above 0.5-10 mg/day. The compositions of the invention may conveniently comprise the peptide of the invention incorporated into a solid, liquid or aqueous carrier medium and the pharmaceutical compositions may be in forms suitable for topical, enteral, oral, rectal, vaginal, or parenteral (e.g. intravenous, subcutaneous, intramuscular or intravascular) administration to the mammal, and preferably human body. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. Suitable pharmaceutical excipients include starch, glucose, fructose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol, and the like. The compositions of the present invention are however especially suited to the oral administration. For oral administration, the pharmaceutical compositions of the invention may be formulated using conventional pharmaceutical carriers and excipients, e.g. in the form of tablets, coated tablets, syrups, capsules, pills, powders, suspensions, emulsions, sprays, etc. The pharmaceutical compositions of the invention may of course contain further ingredients, such as for example

conventional pharmaceutical formulation aids, e.g. emulsifiers, extenders, flavours, colouring agents, surfactants, pH adjusting agents, ointment bases, gelling agents, propellants, stabilizers and the like. In a preferred embodiment the pharmaceutical compositions may also contain other physiologically active agents or therapeutic ingredients. It will be understood that the therapeutic dose of each active ingredient administered in accordance with the present invention will vary depending upon the particular active ingredient employed (including the peptide of the invention), the mode by which the active ingredient is to be administered, and the condition or disorder to be treated.

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The examples of the present invention shows that both peptides, also the peptide of the invention exerted potent antihypertensive activity in spontaneously hypertensive rats (SHR) orally administered at a dose of 5 mg kg⁻¹ of body weight, being similar to that found for VPP at the same dose (Pripp, 2008. *Food & Nutritional Research*, 52, from foodandnutritionresearch.net/index.php/fnr/article/view/1641/1525).

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Their potent ACE-inhibitory activity *in vitro* and their antihypertensive effects *in vivo* that were similar to that found for the peptide VPP orally administered in SHR at the same dose (5 mg kg⁻¹ of body weight) (Contreras et al., 2009. *International Dairy Journal*, 19, 566-573).

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In order to develop an ingredient containing both active peptides, the first objective was to optimize the hydrolytic process at food grade conditions and at the laboratory, previous to the scaling up. In general, the ACE-inhibitory activity of the casein hydrolysates depends on the enzyme/substrate ratio and time. It was found higher inhibition potency by casein hydrolysis with the lowest enzyme/substrate ratio, in agreement with Guo *et al.*, 2009 (*Food Chemistry*, 114, 328-333) for whey protein hydrolyzed with a preparation of proteinases from *Lactobacillus helveticus* LB13, although other authors have reported different results (for example, Van der Ven *et al.*, 2002. *International Dairy Journal*, 12, 813-820). In any case, the IC₅₀ values of the casein hydrolyzed with

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pepsin ranged between 13.0-30.6 μg of protein mL^{-1} that are comparable to the ACE-inhibitory activity reported for other casein hydrolysates (Contreras *et al.*, 2009. *International Dairy Journal*, 19, 566-573; Gómez-Ruiz *et al.*, 2007 *Electrophoresis*, 28, 4202-4211; López-Expósito *et al.*, 2007. *Lait*, 87, 241-249; Miguel *et al.*, 2009. *Food Chemistry*, 112, 211-214; Yamamoto *et al.*, 1994. *Journal of Dairy Science*, 77, 917-922). Although IC_{50} has been used as indicator to assess the ACE-inhibitory potency of the hydrolysates, in this study it was complemented by the evaluation of the formation of the active peptides RYLGY (SEQ ID NO: 1) and AYFYPEL (SEQ ID NO: 9) during hydrolysis by using an accurate quantitative method. As it is shown in literature, ACE could be inhibited *in vitro* by a long number of peptidic sequences (for example, see reviews of Guang and Phillips, 2009, López-Fandiño *et al.*, 2006, Vercruyssen, van Camp, & Smagghe, 2005), but there are also many possible ACE substrates in a hydrolysate because the primary activity of ACE is to cleave the C-terminal dipeptide of oligopeptides. Thus, IC_{50} value could not correlate adequately with the content of specific active peptides (Bütikofer, Meyer, Sieber & Wechsler, 2007; Quirós, Chichón, Recio, & López-Fandiño, 2007). In fact, it can be concluded from the examples of the present invention that the hydrolysis of casein with a low enzyme/substrate ratio leads to a significant increase of the content of the active peptides RYLGY (SEQ ID NO: 1) and AYFYPEL (SEQ ID NO: 9), whereas the ACE-inhibitory activity slightly varied.

Then, in a further preferred embodiment, the first composition of the invention is an oral dosage form. In a more preferred embodiment, the oral dosage form is selected from the group comprising tablets, capsules, caplets, slurries, sachets, suspensions, chewing gum, and powder formulation that may be dissolved in a liquid. In an even more preferred embodiment, the oral dosage form is a suspension. In another more preferred embodiment, the oral dosage form is a powder formulation that may be dissolved in a liquid. In an even more preferred embodiment, the liquid is water, milk, juice, or yogurt.

A further aspect the invention relates to the use of the first composition of the

invention for mitigating hypertension.

A further aspect the invention relates to the first composition of the invention for use as a medicament, or to the use of the first composition of the invention for the manufacture of a medicament. A preferred embodiment of this aspect of the invention, relates to the first composition of the invention for use in the treatment or prophylaxis of hypertension, stroke, coronary disease, myocardial infarction, metabolic syndrome, peripheral vascular disease or abdominal aortic aneurysm in mammals, or to the use of the first composition of the invention in the manufacture of a medicament for the treatment or prophylaxis of hypertension, stroke, coronary disease, myocardial infarction, metabolic syndrome, peripheral vascular disease or abdominal aortic aneurysm in mammals including a human. In another preferred embodiment of this aspect of the invention, the mammal is a human.

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Another preferred embodiment of this aspect of the invention, relates to the first composition of the invention for use in the treatment of a disease involving oxidation in mammals including a human, or to the use of the first composition of the invention in the manufacture of a medicament for the treatment or prophylaxis of a disease involving oxidation in mammals including a human. In another preferred embodiment of this aspect of the invention, the disease involving oxidation is selected from the list comprising: osteoarthritis, rheumatoid arthritis, ischemia, cataract, corneal pathology, glaucoma, retinal degeneration, vitreal degeneration, cancer, immune deficiency, hyperimmunity, autoimmunity, neurodegeneration, aging, Alzheimer's disease, Huntington's disease, Machado-Joseph disease, multiple sclerosis, muscular dystrophy, Parkinson's disease, senility, muscular atrophy, stroke, hepatopathies, systemic lupus erythematosus, mixed connective tissue disease, multiple sclerosis, and diabetes.

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A further aspect the invention relates to the first composition of the invention for use in the treatment or prophylaxis of hypertension, stroke, coronary disease,

myocardial infarction, metabolic syndrome, peripheral vascular disease or abdominal aortic aneurysm in a mammal, by oral administration, wherein said treatment or prevention comprising administering to said mammal an specific amount of the peptide of the invention per day. In another preferred
5 embodiment of this aspect of the invention, the amount of the peptide of the invention is between 0.06 to 0.08 mg for kg of body weight of the mammal.

In a further preferred embodiment, the amount of the peptide of the invention in the composition is between 0,5 mg and 10 mg. In a more preferred
10 embodiment, the amount of the peptide of the invention is between 1,0 mg and 5,0 mg. In an even more preferred embodiment, the amount of the peptide of the invention is between 1,5 mg and 2,0 mg.

In a further preferred embodiment, the first composition of the invention also
15 comprises the peptide, hereinafter second peptide of the invention, comprising the amino acid sequence of SEQ ID NO: 9. In a more preferred embodiment, the amino acid sequence of the peptide consists of SEQ ID NO: 9. In a further preferred embodiment, the amount of the peptide comprising the amino acid sequence of SEQ ID NO: 9 is between 1,5 mg and 15 mg. In a more preferred
20 embodiment, the amount of the peptide comprising the amino acid sequence of SEQ ID NO: 9 is between 2,0 mg and 10 mg. In an even more preferred embodiment, the amount of the peptide comprising the amino acid sequence of SEQ ID NO: 9 is between 2,5 mg and 3,5 mg.

25 As used herein, "mammal" includes any organism within class *Mammalia*, superrclass *Gnathostomata*, subphylum *Craniata*, phylum *Chordata*, kingdom *Metazoa*, and superkingdom *Eukaryota*.

In the context of the present specification, the term "medicament" refers to any
30 substance or combination of substances which may be used in or administered to mammals or human beings either with a view to restoring, correcting or

modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis.

As defined here, an "active substance", "pharmaceutically active substance",
5 "active ingredient", "active pharmaceutical ingredient" or "therapeutic ingredient"
refers to any substance or mixture of substances that are intended to furnish
pharmacological activity or other direct effect in the diagnosis, cure, mitigation,
treatment, or prevention of disease or medical condition, or to affect the
structure and the function of the body. The peptide of the invention is a
10 bioactive peptide, a natural or functional ingredient with antihypertensive
activity.

The term "bioactive peptide" refers to any peptide that has biological activity. In
particular, such bioactivity includes a capacity to bind to a receptor molecule, or
15 a capacity to enhance or impair enzyme catalysis, cellular signaling,
transcription, translation, or other cellular processes. The term "peptide" refers
to a molecule having at least two amino acid residues bonded together through
one or more peptide bond(s).

20 Another aspect of the invention relates to the use of a peptide comprising the
amino acid sequence of SEQ ID NO: 1, hereinafter peptide of the invention, as
antioxidant. In one embodiment, the amino acid sequence consisting essentially
of the amino acid sequence of SEQ ID NO:1. In another embodiment, the amino
acid sequence consisting of the amino acid sequence of SEQ ID NO: 1.

25 Articles such as food, cosmetics, and medicines, that contain oils and fats,
suffer from oxidation or peroxidation of the oil and fat components caused by
atmospheric oxygen, which is the most serious problem in storage,
preservation, and processing of such articles. Unsaturated fatty acids contained
30 in oils and fats, such as linoleic and linolenic acids, are known to be particularly
prone to peroxidation by oxygen to generate lipid peroxides, free radicals, or
even carcinogenic substances. Oxidation and peroxidation cause not only

coloration, discoloration, denaturalization, abnormal odor, or decrease in effective nutritional value of the articles, but also generation of toxic substances, which results in deterioration of product quality. In another aspect, the current invention is a composition, hereinafter second composition of the invention,
5 comprising:

a) a product ingredient subject to oxidation; and

b) an antioxidative peptide comprising an amino acid sequence of SEQ ID NO:1 present in an amount effective for reducing oxidation of the product ingredient subject to oxidation.

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"Effective for reducing" means that the reduction in oxidation observed with samples treated with the antioxidative peptide of the current invention is statistically significant when compared to the results using controls (i.e., samples without addition of the antioxidative peptide of the invention). This
15 statistical significance can be calculated and determined by methods well known in the art. For example, statistical significance can be determined by utilizing a T-test and a 90%, or preferably a 95%, probability cut-off value.

Then, the second composition of the invention could be an stabilized product.
20 As used herein "stabilized" products are products that are resistant to oxidative stress that occurs upon product storage over time. This resistance to oxidative stress may result in increased maximum storage time, shelf-life, or expiration dating, or increased consistency of taste and/or other organoleptic properties over time, as compared to products containing the same product ingredient
25 subject to oxidation without the antioxidative peptides of the current invention.

Product ingredients subject to oxidation typically include products that are foods, cosmetics, pharmaceuticals, or medical diagnostics. However, the present invention can include any commercial product ingredient that is subject
30 to oxidation. In one embodiment of this aspect of the invention, the product ingredient subject to oxidation is a food ingredient and the product is a food. In another embodiment, the product ingredient subject to oxidation is a cosmetic

component and the product is a cosmetic. In another embodiment, the product ingredient subject to oxidation is a medical diagnostic component and the product is a medical diagnostic. In another embodiment, the product ingredient subject to oxidation is an active ingredient, active substance, or a pharmaceutically active substance, and the product is a pharmaceutical product, a pharmaceutical composition and/or a medicament.

In the present invention, all of the technical and scientific terms are of the same meaning as that commonly understood by an expert in the field to which the invention pertains. Throughout the description and the claims, the word "comprises" and the variations thereof are not intended to exclude other technical features, components or steps. For the person skilled in the art, other objects, advantages and characteristics of the invention will be implied in part from the description and in part from the practice of the invention. The following examples and drawings are provided for illustrative purposes and are not intended to be limiting of the present invention.

DESCRIPTION OF THE DRAWINGS

Fig. 1. (A) Fractionation by RP-HPLC at semi-preparative scale of the 3000 Da-permeate obtained from the peptic hydrolysate of bovine casein. Collected fractions are termed with F followed with a number (F1-F8). (B) Angiotensin-converting enzyme-inhibitory activity, expressed as IC_{50} value, of the collected fractions from the semi-preparative RP-HPLC system. For the IC_{50} determination purposes, protein content of the fractions collected from the HPLC was estimated by the bicinchoninic acid assay. Data are expressed as mean \pm SEM for a minimum of three experiments.

Fig. 2. (A) Tandem mass spectrum of the singly charged ion m/z 905.5 and (B) of singly charged ion m/z 902.5, both included in fraction 5. Following sequence interpretation and data base searching, the peptides were identified as α_{s1} -

casein f(24-31) and α_{s1} -casein f(143-149), respectively. The sequences of these peptides are displayed with the fragment ions observed in the spectra.

Fig. 3. Change in systolic blood pressure (SBP) after oral administration of different peptides (5 mg kg⁻¹). Data are expressed as mean \pm SEM for a minimum of four animals (n=4-8). Control groups received the same volume of water. Unpaired student's t-test was used to compare groups, **p*< 0.05, ***p*< 0.01 vs control group.

Fig. 4. (A) the releasing of the peptide RYLGY was clearly increased up by the hydrolysis time, but the amounts of this peptide did not varied greatly with the enzyme/substrate ratio. In general, the content of the peptide AYFYPEL was slightly influenced by the hydrolysis time, and seems to be diminished after 8 h of hydrolysis (B). In addition, the estimated amount of AYFYPEL were higher with food grade pepsin, and as well as with a low enzyme/substrate ratio. The ACE-inhibitory activity of the commercial casein hydrolysates is shown in Figure 4C. The lower IC₅₀ values, that are the higher ACE-inhibitory activities, were obtained by casein hydrolysis with food grade pepsin and with a low enzyme/substrate ratio, contributing to minimize the costs of production. The latter values ranged between 13 and 17.3 μ g of protein mL⁻¹, indicating a potent ACE-inhibitory activity.

Fig. 5. Effect of milk casein hydrolysed product (MCHP) on (a) systolic blood pressure (SBP) and (b) diastolic blood pressure (DBP) on spontaneously hypertensive rats (SHR) during treatment. SHR drank tap water (Control) or 800 mg/kg per day of hydrolysate in the drinking water (MCHP). Data are mean values \pm S.E.M. for 8 rats. * *p*<0.05 versus control.

Fig. 6. Cumulative concentration-response curves to acetylcholine (Ach) in precontracted aorta (a) and mesenteric (b) rings from spontaneously hypertensive rats (SHR). SHR drank tap water (Control) or 800 mg/kg per day

of hydrolisate in the drinking water (**MCHP**). Data are mean values \pm S.E.M. for 4-6 rats. * $p < 0.05$ versus control.

Fig. 7. Cumulative concentration-response curves to phenilephrine (PE) in aorta rings from spontaneously hypertensive rats (SHR). SHR drank tap water (**Control**) or 800 mg/kg per day of hydrolisate in the drinking water (**MCHP**). Data are mean values \pm S.E.M. for 4-6 rats. * $p < 0.05$ versus control.

Fig. 8. Effect of long-term intake of milk casein hydrolisated product (MCHP) on left ventricular hypertrophy. **Control**, animals receiving tap water and **MCHP** animal receiving 800 mg/kg per day of hydrolisate in the drinking water. Data are mean values \pm S.E.M. for 8 rats. * $p < 0.05$ versus control.

Fig. 9. Effect of long-term intake of milk casein hydrolisated product (MCHP) on interstitial fibrosis of heart histological sections stained with Sirius red. **Control**, animals receiving tap water and **MCHP** animal receiving 800 mg/kg per day of hydrolisate in the drinking water. Data are mean values \pm S.E.M. for 8 rats. * $p < 0.05$ versus control.

Fig. 10. western blotting analysis carried out in aorta homogenates showed an increase in eNOS expression in treated animals.

The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

EXAMPLES

EXAMPLE 1: ANTIOXIDANT AND ACE-INHIBITORY ACTIVITY *IN VITRO* AND ANTIHYPERTENSIVE ACTIVITY *IN VIVO*.***Production of Casein Hydrolysates***

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A 0.5% (w/v) aqueous solution of isoelectric casein was adjusted to pH 2.0-2.5 with 1 M HCl and digested with 3.7% (w/w of substrate) porcine pepsin A (Sigma, St. Louis, MO, USA) for 24 h at 37°C in an orbital shaker at 150 rpm. Pepsin was added at the beginning and after 4 h of hydrolysis. Aliquots of the hydrolysate were taken during hydrolysis at 1, 2, 3, 4, 5, 6, 7 and 24 h. The reaction was terminated by heating at 80 °C for 15 min and the pH was adjusted to 7.0 by addition of 1 M NaOH. The hydrolysates were centrifuged at 16.000 x g for 15 min and the supernatants removed. The protein concentration of hydrolysates was determined by Kjeldahl method and the ACE-inhibitory activity was tested.

10
15***Isolation of ACE-Inhibitory Peptides from the Peptic Hydrolysate***

20 The 3-h peptic hydrolysate was subjected to ultrafiltration through a hydrophilic 3000 Da cut-off membrane (Millipore Corporation, Bedford, MA, USA) with a stirred ultrafiltration cell (model 8400, Millipore). The permeate from this ultrafiltration step was subjected to semipreparative RP-HPLC on a Waters Series 600 HPLC, equipped with Millennium 3.2 Software for data acquisition
25 (Waters Corporation, Mildford, MA, USA) as described in Quirós et al., 2007(*Dairy Journal* 17: 33-41), but elution was performed with a linear gradient of solvent B in A from 0% to 21% B in 30 min, 21% to 35% in 40 min and to 35% to 70% in 5 min at 30°C and a flow rate of 4 mL min⁻¹. Solvent A was a mixture of water-trifluoroacetic acid (1000:1) and solvent B contained a mixture
30 of acetonitrile-trifluoroacetic acid (1000:0.8). Fractions from the HPLC system were freeze-dried and kept at -20 °C until use.

Peptide sequencing by RP-HPLC-MS/MS

RP-HPLC-MS/MS analysis of the active fractions was performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-
5 line to an Esquire 3000 quadrupole ion trap (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ionisation source, as described in Hernández-Ledesma *et al.*, 2005 (*Journal of Agriculture and Food Chemistry* 53: 588-593).

10 Measurement of ACE-Inhibitory Activity

ACE-inhibitory activity was measured by Cushman & Cheung 1971 (*Biochemical Pharmacology*, 20, 1637-1648) spectrophotometric assay with some
15 modifications, as reported by (Quirós *et al.*, 2007). For this purpose, protein concentration of the aliquots of the pepsin hydrolysate was determined by the Kjeldahl method. The protein content of the fractions collected from HPLC was estimated by the bicinchoninic acid assay (Pierce, Rockford, IL, USA) using bovine serum albumin, as standard, and peptide concentration of the chemically
20 synthesized peptides was based in the dry weight of the peptides. Chemical synthesized peptides were purchased from GenScript Corp. (Piscataway, NJ, USA).

Measurement of Antioxidant Activity

25 The ORAC-FL assay was based on the method proposed by Ou *et al.* 2001 (*Journal of Agriculture and Food Chemistry*, 49, 4619-4626) and modified by Dávalos *et al.* 2004 (*Journal of Agriculture and Food Chemistry*, 52, 48-54).

Measurement of Blood Pressure

30

All procedures were carried out in accordance with conventional guidelines for experimentation with animals. Twelve-week old male SHR rats were used

(Janvier, France); they were housed in groups of four per cage in a regulated environment with a 12 h light/dark cycle in a standard experimental laboratory of the Animal Experimentation Service of the Salamanca University (N^oPAE SA001). The animals were deprived of solid diet 12 hours before experiments
5 began but received a nutritive solution of 8% sucrose in 0.2% NaCl to avoid excessive dehydration. The rats received a single dose of the synthesized peptides or zofenopril (5 mg kg⁻¹) dissolved in ultrapure water and the control group received the same volume of water. Systolic blood pressure (SBP) was measured in awake rats with an automated multichannel system, using the tail-
10 cuff method with a photoelectric sensor (Niprem 546, Cibertec SA, Spain) as described in Guerrero *et al.*, 2003 (*Journal of Cardiovascular Pharmacology* 42: 348-355). SBP was measured before administration, and also at 2, 4, 6, 8, 10 and 24 hours post-administration. The changes in SBP were expressed as the differences before and after administration of the different products. Unpaired
15 student's t-test was used to compare groups.

Results

Evolution of ACE-Inhibitory Activity during Casein Hydrolysis

20 The progress of the hydrolysis reaction of casein with pepsin was monitored by taking samples at different intervals. The water-soluble fractions of the hydrolysates were analyzed by HPLC-MS and the ACE-inhibitory activity and protein content were determined. As shown in Table 1, hydrolysis of caseins
25 with pepsin under our conditions occurred rapidly, giving a protein content in the soluble fraction of 0.45% (w/v) after 1 hour of hydrolysis. The ACE-inhibitory activity of the soluble fraction increased during the first 3 hours of hydrolysis and the lowest value for the IC₅₀ value (22.19 µg mL⁻¹), i.e., the maximum activity, was reached after 3 hours hydrolysis. This IC₅₀ value was comparable
30 to the activity found in other previously reported antihypertensive protein hydrolysates obtained by enzymatic digestion or by fermentation (Miguel *et al.*,

2004. *Journal of Food Protection* 67: 1914-1920; Muguerza *et al.*, 2006. *International Dairy Journal* 16: 61-69).

- 5 Table 1: Angiotensin-converting enzyme-inhibitory activity, expressed as the protein concentration required to inhibit the enzyme activity by 50% (IC₅₀) of the water-soluble fraction from the casein hydrolysates prepared with pepsin.

	Hydrolysis time	IC ₅₀ ^a (µg protein/mL)	Protein (% w/v)
10	1 h	60.85 ± 10.8	0.45
	2 h	35.27 ± 3.6	0.45
	3 h	22.19 ± 1.6	0.45
	4 h	29.65 ± 3.9	0.46
15	5 h	38.85 ± 3.7	0.47
	6 h	45.48 ± 10.1	0.46
	7 h	37.07 ± 2.8	0.47
	24 h	25.68 ± 1.6	0.47

20

Identification of ACE-Inhibitory Peptides

- 25 The water-soluble fraction of the 3h-hydrolysate with pepsin was first subjected to ultrafiltration through a 3000 Da cut-off membrane and the ACE-inhibitory activity of the permeate and the retentate was measured. The activity of the 3000 Da permeate (IC₅₀ 5.68 ± 183 0.36 µg mL⁻¹) was four times higher than that found in the total water-soluble extract (IC₅₀ 22.19 ± 1.61 µg mL⁻¹) and 40
30 times higher than the activity measured in the retentate fraction (IC₅₀ 231 ± 35 µg mL⁻¹).

The 3000 Da permeate was subjected to semi-preparative RP-HPLC. The 70-min chromatogram was divided into 8 fractions, which were collected,

concentrated by freeze drying, and then their ACE-inhibitory activity was measured (Fig. 1A and 1B). Two fractions, F3 and F5 in Fig. 1, exhibited the highest ACE-inhibitory activities, with IC_{50} values of $5.65 \pm 0.54 \mu\text{g mL}^{-1}$ and $5.51 \pm 0.60 \mu\text{g mL}^{-1}$. The other three chromatographic fractions, F2, F4 and F6, also showed notable potent ACE-inhibitory activity with IC_{50} values under $14 \mu\text{g mL}^{-1}$. All these active chromatographic fractions (F2, F3, F4, F5 and F6 in Fig. 1) were analysed by HPLC-MS/MS. As an example, Fig. 2A shows the MS/MS spectrum of the two most abundant ions in the active fraction, F5. The fragmentation spectrum of ion m/z 905.5 matched with the α_{s1} -CN-derived peptide f(21-31) with sequence SEQ ID NO: 2. This spectrum contained a major ion at m/z 588.2, which was identified as a y-type fragment ion resulting from the cleavage N-terminal to proline (y5). It is known that the presence of proline residues in a peptide has a significant effect upon the fragments observed by MS/MS, and the fragment ions-produced N-terminal to proline are over-represented in the spectra. The fragment ions corresponding to the fragmentation N-terminal to the other proline residue, i.e., b5 and y3 occurred at lower abundance than y5 although similar X-P relative bond cleavage ratios have been reported when X was alanine or phenylalanine (Breci *et al.*, 2003. *Analytical Chemistry* 75: 1963-1971). The MS/MS spectrum in Fig. 2B was also consistent with the sequence AYFYPEL that corresponds to fragment (143-149) from α_{s1} -CN. In this spectrum, the presence of proline residues and its unusual fragmentation patterns helped identification, being fragment ions y3 and b4 (corresponding to cleavage N-terminal to proline), the most prominent fragment ions.

25

The major peptide components of each active HPLC fraction were identified and these results are summarised in Table 2. A total of 44 peptide sequences were identified of which 13 peptides corresponded to β -casein (β -CN) fragments, and 19 to α_{s1} -CN fragments. The most abundant peptides in each chromatographic fraction are in bold in Table 2. Among the identified peptides, one of them had previously been described as an ACE inhibitor. More specifically, the α_{s1} -CN peptide SEQ ID NO: 3, had been found to exhibit ACE-inhibitory activity with an

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IC₅₀ value of 98 μM (Pihlanto-Leppälä *et al.*, 1998. *International Dairy Journal* 8: 325-331). Other peptides show high homology with ACE-inhibitory peptides previously described in the literature, such as, SEQ ID NO: 4 which share five residues with SEQ ID NO: 5, and have an IC₅₀ value of 82.4 μM (Quirós *et al.*, 5 2005. *Journal of Dairy Science* 88: 3480-3487). Similarly, peptide SEQ ID NO: 6 includes the sequence SEQ ID NO: 7 (IC₅₀, 52 μM) (NZ508867 A and WO9965326), and peptide LQY, found in fraction 2, corresponds to the C-terminal tripeptide of SEQ ID O: 8 (IC₅₀, 14 μM) (Tauzin *et al.*, 2002. *FEBS Letters* 531:369-374). However, the presence of these peptides with moderate 10 ACE-inhibitory activity could not explain the high activity found in the permeate from the casein hydrolysate and in the isolated fractions most abundant peptides in each chromatographic fraction are in bold in Table 2.

In vitro ACE-Inhibitory and Antioxidant Activity

15

In an attempt to identify the peptides responsible for the ACE-inhibitory activity in the 3000 Da permeate from the casein hydrolysate, several peptides, among the most abundant in each chromatographic fraction were selected on the basis of their sequences, especially the C-terminal tripeptide. A total of six peptides 20 were chemically synthesized and the ACE inhibitory, calculated as IC₅₀ value, was measured (Table 3). Because it has been reported that antioxidant activity of several antihypertensive peptides can also contribute to their activity (Duffy *et al.*, 1999. *The Lancet* 354: 2048-2049), the radical scavenging activity of these synthetic sequences was also evaluated. Three of the six peptides, with 25 sequences SEQ ID NO: 1, SEQ ID NO: 9 and SEQ ID NO: 10, exhibited potent ACE-inhibitory activity with IC₅₀ values lower than 20 μM (Table 3). Interestingly, these three peptides also had high radical scavenging activity with values at least two times higher than Trolox. The two α_{s1}-casein-derived peptides, i.e., SEQ ID NO: 1 and SEQ ID NO: 9, were among the most 30 abundant in the hydrolysate and the 3000 Da-permeate fraction obtained from it, and therefore, given their activity (IC₅₀ values of 0.71 and 6.58 μM,

respectively) and concentration, they might be responsible for the activity found in the total hydrolysate.

5

Table 2: Identification of peptides contained in the RP-HPLC fractions with angiotensin-converting enzyme-inhibitory activity.

10

^a Fractions are termed as in Fig. 1

Fraction ^a	Ion (m/z) for MS/MS (charge)	Calculated mass	Observed mass ^b	Protein Fragment	Sequence ^c
F2	675.1 (1)	674.32	674.1	β-CN f(1-5)	RELEE
	795.2 (1)	794.35	794.2	β-CN f(118-124)	PFTESQS
	763.2 (1)	762.36	762.2	α _{S1} -CN f(56-61)	DIKQME
	423.1 (1)	422.22	422.1	α _{S2} -CN f(96-98)	LQY
	804.3 (1)	803.44	803.3	κ-CN f(162-169)	VQVTSTAV
	521.2 (1)	520.27	520.2	α _{S2} -CN f(85-88)	INQF
	628.3 (1)	627.32	627.3	β-CN f(6-11)	LNVPGE
	502.2 (1)	501.28	501.2	α _{S1} -CN f(95-98)	LEQL
	423.2 (1)	422.22	422.2	α _{S1} -CN f(154-156)	YQL
F3	597.5 (1)	596.31	596.5	α _{S1} -CN f(150-153)	FRQF
	671.5 (1)	670.34	670.5	α _{S1} -CN f(90-94)	RYLGY
	973.5 (1)	972.47	972.5	α _{S2} -CN f(89-95)	YQKFPQY
	800.4 (1)	799.34	799.4	α _{S1} -CN f(173-179)	YTDAPSF
	866.4 (1)	865.37	865.4	α _{S1} -CN f(157-164)	DAYPSGAW
	940.5 (1)	939.48	939.5	α _{S1} -CN f(165-172)	YYVPLGTQ
	465.3 (1)	464.26	464.3	α _{S1} -CN f(92-95)	LGYL
	890.5 (1)	889.48	889.5	κ-CN f(138-146)	AVESTVATL
F4	787.3 (1)	786.43	786.3	β-CN f(157-163)	FPPQSVL
	784.3 (1)	783.43	783.3	α _{S1} -CN f(90-95)	RYLGYL
	1001.3 (1)	1000.51	1000.3	α _{S1} -CN f(150-156)	FRQFYQL
	662.3 (1)	661.39	661.3	α _{S1} -CN f(19-23)	NLLRF
	831.2 (1)	830.39	830.2	α _{S1} -CN f(144-149)	YFYPEL
	1198.4 (1)	1197.62	1197.4	κ-CN f(96-105)	ARHPHPLHSF
F5	957.4 (2)	1912.91	1912.8	β-CN f(141-156)	QSWMHQPHQPLPPTVM
	1422.8 (1)	1421.79	1421.8	κ-CN f(18-29)	FSDKIAKYIPIQ
	617.4 (1)	617.32	616.4	κ-CN f(53-57)	NQFLP
	1219.6 (1)	1218.63	1218.6	α _{S1} -CN f(25-35)	VAPFPEVFGKE
	729.5 (1)	728.40	728.5	β-CN f(199-205)	GPVRGPF
	902.5 (1)	901.42	901.5	α _{S1} -CN f(143-149)	AYFYPEL
	845.6 (1)	844.44	844.6	κ-CN f(51-57)	INNQFLP
	905.5 (1)	904.47	904.5	α _{S1} -CN f(24-31)	FVAPFPEV
	1063.4 (2)	2124.12	2124.0	κ-CN f(51-68)	INNQFLPYPPYAKPAAVR
F6	919.4 (2)	1835.95	1836.8	α _{S1} -CN f(24-39)	FVAPFPEVFGKEKVNE
	1215.6 (2)	2429.27	2429.2	β-CN f(59-80)	VYFPFGPIHNSLPQNIPLTQT
	1151.7 (1)	1150.69	1150.7	β-CN f(199-209)	GPVRGPFPIIV
	1094.7 (1)	1093.66	1093.7	β-CN f(200-209)	PVRGPFPIIV
	802.6 (1)	801.51	801.6	β-CN f(134-140)	HLPLPLL
	905.5 (1)	904.47	904.5	α _{S1} -CN f(25-32)	VAPFPEVF
	995.6 (1)	994.59	994.6	β-CN f(81-89)	PVVVPPFLQ
	1300.6 (1)	1299.69	1299.6	β-CNA ² f(59-70)	VYFPFGPIPNSL
	1008.6 (1)	1007.58	1007.6	κ-CN f(25-32)	YIPIQYVL
	527.2 (1)	526.35	526.2	κ-CN f(46-50)	KPVAL
	742.6 (1)	741.44	741.6	β-CN f(203-209)	GPFPPIIV
	1015.5 (1)	1014.51	1014.5	α _{S1} -CN f(142-149)	LAYFYPEL

^b Calculated monoisotopic mass

^c Amino acids are designed using one letter code. Most abundant peptides in each fraction are in bold.

5

Antihypertensive Activity of the Synthetic Peptides

The antihypertensive effect of the peptides with higher ACE-inhibitory potency was examined in SHR. Animals used for the experiment had an average basal SBP of 178±4 mmHg. Fig. 3 shows the time-course changes in SBP after oral administration of the synthetic peptides and after the administration of zofenopril (positive control). All the peptides displayed antihypertensive effects. Peptide SEQ ID NO: 1 lowered SBP in a progressive and maintained manner during most of the time of study. The maximum reduction, observed 6 hours post-administration, was 25±3 mmHg (Fig. 3). Peptide SEQ ID NO: 9 showed a significant effect but a smaller and less maintained effect than this reached with the same dose of SEQ ID NO: 1. A small and short effect was observed with SEQ ID NO: 10. The maximum antihypertensive effect of VPP (20±5 mmHg) was similar to that of SEQ ID NO: 9, but none of the peptides reached the decrease in SBP caused by zofenopril (Fig. 3).

25 Table 3: Angiotensin-converting enzyme-inhibitory activity and radical scavenging activity of synthetic peptides selected from those identified in the active chromatographic fractions.

30

Sequence	Protein fragment	IC ₅₀ ^a ± SE (μM)	ORAC-FL ± SEM ^b (μmol equiv Trolox/μmol peptide)
RYLGY	α_{S1}-CN f(90-94)	0.71 ± 0.08	2.829 ± 0.040
AYFYPEL	α_{S1}-CN f(143-149)	6.58 ± 0.50	3.216 ± 0.114
YQKFPQY	α _{S2} -CN f(89-95)	20.08 ± 1.25	2.033 ± 0.077
HLPLPLL	β-CN f(134-140)	34.40 ± 2.22	0.060 ± 0.002
VAPFPEVF	α _{S1} -CN f(25-32)	362.50 ± 23.56	0.046 ± 0.001
FVAPFPEV	α _{S1} -CN f(24-31)	475.89 ± 41.73	< 0.025

5 ^a IC₅₀ value corresponds to the concentration of peptide needed to inhibit 50% of the original ACE activity, expressed as mean ± standard error (n= 3)

^b Standard error of the mean (n=3)

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EXAMPLE 2: OPTIMIZATION OF THE HYDROLYSIS REACTION AND RESISTANCE OF ACTIVE PEPTIDES TO DRYING.

Material and methods

15

Production of Casein Hydrolysates

Commercial casein (Promilk 85, Arras Cedex, France) was employed to prepare the hydrolysates at laboratory scale. Briefly, casein was resuspended at 6% (w/v) in water, the suspensions was acidified with HCl and digested with pepsin for laboratory use (Sigma, St. Louis, MO, USA) or with food grade pepsin (Biocatalysts, Cardiff, UK) at different ratios. Pepsin was added twice at the beginning and after 3 h of hydrolysis. In some experiments, aliquots of the hydrolysates were taken at different times (1-8 h). Finally, after the inactivation of the enzyme, the hydrolysates were centrifuged in the laboratory, the supernatants collected and dehydrated.

10

Moreover, in some cases, an additive was incorporated during the casein hydrolysis process. All samples were kept at -20°C until analysis.

The protein content of the samples was determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA) using bovine serum albumin, as standard, or by Kjeldahl method according to the IDF Standard 20B norm.

Protein content, moisture and mineral content

The protein concentration of powdered hydrolysate was determined by Kjeldahl method. Moisture was determined by drying for 3 h in an oven at 100-105°C (International Dairy Federation 26:1964). Mineral composition (Na, K, Mg, Ca and Zn) was determined by atomic absorption spectroscopy.

Measurement of ACE-inhibitory activity

ACE inhibitory activity was measured by fluorescence using the method of Sentandreu & Toldrá (2006. *Food Chemistry*, 97, 546-554) and modified by Quirós and co-workers (Quirós *et al.*, 2009. *Peptides*, 30, 1848-1853). The ACE inhibitory activity of the samples was expressed as IC₅₀ (protein concentration required to inhibit the original ACE activity by 50%) and was determined by triplicate.

30

Measurement of free amino-groups content

The free amino-groups content was measured at 420 nm (DU-800; Beckman
5 Coulter Inc., Fullerton, CA, USA), according to McKellar (1981. *Journal of Dairy
Science*, 64, 2138-2145), and was determined by duplicate or triplicate. An
external calibration curve was prepared with L-Leucine (Sigma) from 0.2 to 4.6
mM.

10 *Quantitative analysis by RP-HPLC-MS*

The amount of peptides RYLGY (SEQ ID NO: 1) and AYFYPEL (SEQ ID NO: 9)
in the samples was estimated by RP-HPLC-MS analysis using an ion trap mass
spectrometer and an external calibration curve, as previously described
15 Contreras et al. (2010). The six point external calibration curve was prepared
with synthetic peptides (purchased from GenScript, Piscataway, NJ, USA) and
at concentrations ranged from 1 to 40 $\mu\text{g mL}^{-1}$ of each peptide. The spray- or
freeze-dried products were dissolved in Milli-Q water (Millipore, Bedford, MA,
USA) at 5 mg mL^{-1} . The liquid samples and the WSEs from fermented milks
20 were diluted 1: 12 and 1:8, respectively.

Measurement of Blood Pressure

All procedures were carried out in accordance with conventional guidelines for
25 experimentation with animals. Twelve-week old male SHR rats were used
(Janvier, Le Genest Saint Isle, France); the animals' housing and maintenance
before the experiments were described previously (Contreras *et al.*, 2009.
International Dairy Journal, 19, 566-573). The rats received, by oral
administration, using a canula, the following powdered products dissolved in
30 ultrapure water: a casein hydrolysate (300 mg), its 3 kDa-permeate (200-400
mg), and a final casein hydrolyzed ingredient (400-800 mg). The control group
received the same volume of water. Systolic blood pressure (SBP) was

measured in awake rats with an automated multichannel system, using the tail-cuff method with a photoelectric sensor (Niprem 546, Cibertec SA, Spain) as previously described (Guerrero *et al.*, 2003. *Journal of Cardiovascular Pharmacology*, 42, 348-355). SBP was measured before administration, and also at different times post-administration. The changes in SBP were expressed as the differences before and after administration of the different products.

Statistical methods

Quantitative data, IC₅₀ and free amino-groups content are represented as mean \pm SD. To evaluate the differences and compare the means, one-sided analysis of variance (ANOVA) followed by Bonferroni or LSD multiple range tests were applied by using Statgraphics Centurion XV version 15.2 (StatPoint Technologies, Inc., Warrenton, VA, USA, www.statgraphics.com). Antihypertensive data are expressed as means \pm SEM. Statistical calculations for significant differences between the different groups were performed by Student's t test for unpaired data and *P* values of less than 0.05 was considered significant. The GraphPad Prism 4 software program (GraphPad Software Inc., San Diego, CA, USA, www.graphpad.com) was used.

20

Results

Optimization of the hydrolysis process

Some experimental variables were optimized at food grade conditions in the laboratory. Firstly, the progress of the casein hydrolysis with food grade pepsin or with pepsin for laboratory use was monitored by taking samples at different intervals. The supernatants of the hydrolysates were analyzed by HPLC-MS and the ACE-inhibitory activity determined. As shown in Figure 4A, the releasing of the peptide RYLGY (SEQ ID NO: 1) was clearly increased up by the hydrolysis time, but the amounts of this peptide did not varied greatly with the enzyme/substrate ratio. In general, the content of the peptide AYFYPEL

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(SEQ ID NO: 9) was slightly influenced by the hydrolysis time, and seems to be diminished after 8 h of hydrolysis (Figure 4B). In addition, the estimated amount of AYFYPEL (SEQ ID NO: 9) were higher with food grade pepsin, and as well as with a low enzyme/substrate ratio. The ACE-inhibitory activity of the commercial casein hydrolysates is shown in Figure 4C. The lower IC₅₀ values, that are the higher ACE-inhibitory activities, were obtained by casein hydrolysis with food grade pepsin and with a low enzyme/substrate ratio, contributing to minimize the costs of production. The latter values ranged between 13 and 17.3 µg of protein mL⁻¹, indicating a potent ACE-inhibitory activity.

10

In order to monitor the food grade production of the active peptides, casein was hydrolyzed with a low enzyme/substrate ratio and the resulting hydrolysates were freeze-dried. The concentration of the active peptides was accurately determined by HPLC-MS in dry hydrolysates. In addition, the ACE-inhibitory activity was also measured, but it was expressed as the concentration of dry hydrolysate needed to inhibit 50% of the ACE activity, assuring the potency of the entire preparations, and making more rapid the total assay because of the protein content had not to be measured. As Table 4 shown, hydrolysis time had a significant effect on the formation of the peptide RYLGY (SEQ ID NO: 1) during casein hydrolysis (1.5 mg g⁻¹ at 8 h), and the content of the peptide AYFYPEL (SEQ ID NO: 9) was also significantly increased during the first four hours of hydrolysis (approximately, 3.8 mg g⁻¹ at 4 h). The free amino-groups content is related with the extent of the casein hydrolysis. Under these conditions, the hydrolysis occurred rapidly during 1 h, increased up until 4 h, and then remained stable. The ACE-inhibitory activity varied during time, but not significantly.

25

Resistance of bioactive peptides to drying

The effect of method of drying on the active peptides content was evaluated at laboratory scale. The content of both peptides and the ACE-inhibitory activity was comparable after spray-drying and freeze-drying (Table 5).

30

Antihypertensive activity in spontaneously hypertensive rats

The antihypertensive effect of different casein hydrolysates was examined in SHR. All the animals had values of SBP over 170 mmHg, this means a clearly established hypertension that will allow us to assess the antihypertensive effect of the hydrolysates. The administration of the casein hydrolysate (300 mg/kg), its corresponding 3 kDa-permeate (200-400 mg/kg) and the final casein hydrolyzed ingredient (400-800 mg/kg) produce a significant decrease in the SBP in the SHR, reaching their maximum antihypertensive activity value, approximately 25 mmHg, at 6 h post-administration ($P < 0.05$). The SBP remained constant throughout the study in control animals.

As example, Figure 5 shows the antihypertensive activity of the 3 kDa-permeate and the final ingredient. For both products, it was observed a dose-effect relationship. The decrease in the SBP observed for the 3 kDa-permeate was more pronounced at lower doses than that caused by the final ingredient, that could be explained by the high content on active peptides RYLGY and AYFYPEL of the 3 kDa-permeate, with a total content of 9.8 mg/g, as compared with the final ingredient, with a total content of 3.0 mg/g. In addition, this latter product lowered the SBP in a progressive and maintained manner during most of the time of study. The antihypertensive effect of all casein hydrolyzed products was transient and reverted 24 h after the administration. At that moment, the values of the SBP of the SHR were, therefore, similar to the initial values.

Table 4. Content of peptides RYLGY and AYFYPEL (mg g^{-1} of dry hydrolysate) and free amino-groups ($\mu\text{mol Leu-eq mL}^{-1}$) of casein hydrolysates obtained with a low enzyme/substrate ratio and at different time (1-8 h). The ACE-inhibitory activity was expressed as IC_{50} value ($\mu\text{g of dry product mL}^{-1}$).

Hydrolysis time (h)	RYLGY content (mg g ⁻¹)	AYFYPEL content (mg g ⁻¹)	Free amino-groups content (μmol Leu-eq mL ⁻¹)	IC ₅₀ (μg of dry hydrolysate mL ⁻¹)
1	0.08 ^a ± 0.01	1.61 ^a ± 0.03	20.20 ^a ± 1.34	77.0 ^a ± 11.4
2	0.21 ^a ± 0.01	2.46 ^b ± 0.06	24.63 ^{ab} ± 0.21	63.7 ^a ± 9.5
3	0.40 ^b ± 0.01	3.04 ^c ± 0.00	23.68 ^{ab} ± 1.84	65.8 ^a ± 10.7
4	0.76 ^c ± 0.05	3.80 ^d ± 0.01	29.05 ^b ± 1.65	69.8 ^a ± 26.2
5	1.05 ^d ± 0.08	4.01 ^d ± 0.01	29.29 ^b ± 2.49	58.6 ^a ± 4.9
6	1.32 ^e ± 0.04	4.11 ^d ± 0.07	30.54 ^b ± 2.21	62.1 ^a ± 2.9
8	1.51 ^f ± 0.01	3.90 ^d ± 0.19	31.10 ^b ± 1.59	70.1 ^a ± 13.5

Data are expressed as mean ± standard deviation (n = 2); mean values within a column, with different superscript letters, are significantly different ($P < 0.05$).

5

Table 5. Content of peptides RYLGY and AYFYPEL (mg g⁻¹ of dry product) and free amino-groups (μmol Leu eq mg⁻¹ of dry product) in the casein hydrolysates prepared with a low enzyme/substrate ratio and with an additive incorporated before casein hydrolysis or after the inactivation of the enzyme. The hydrolysates were dehydrated by spray- or freeze-drying. The ACE-inhibitory activity was expressed as IC₅₀ value (μg of dry product mL⁻¹).

10

Batch [*]	Drying method	RYLGY content (mg g ⁻¹)	AYFYPEL content (mg g ⁻¹)	Free amino-groups content (μmol Leu eq mg ⁻¹)	IC ₅₀ , μg of dry product mL ⁻¹
1	Spray-drying	0.67 ^a ± 0.04	2.44 ^a ± 0.11	± 0.304 ^a ± 0.006	126.12 ^a ± 11.71
	Freeze-drying	0.72 ^a ± 0.01	2.56 ^a ± 0.02	± 0.315 ^{ab} ± 0.006	122.88 ^a ± 21.93
2	Spray-drying	0.74 ^a ± 0.004	2.49 ^a ± 0.07	± 0.310 ^{ab} ± 0.011	131.56 ^a ± 8.08
	Freeze-drying	0.68 ^a ± 0.07	2.24 ^a ± 0.20	± 0.328 ^b ± 0.005	131.04 ^a ± 18.89

*: Data are expressed as mean ± standard deviation (n = 3); mean values within a column, with different superscript letters, are significantly different (*P* < 0.05).

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EXAMPLE 3: LONG-TERM INTAKE OF A MILK CASEIN HYDROLYSATE AFFECTS DEVELOPMENT OF HYPERTENSION AND PRODUCES CARDIOVASCULAR BENEFITS.

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The aim of this study was evaluated the effect of the long-term intake of a milk casein hydrolysate product (MCHP) on the development of hypertension in spontaneously hypertensive rats (SHR). A daily dose of 800 mg/kg body weight of MCHP was administered dissolved in drinking water during 6 weeks. Systolic and diastolic blood pressure were measured weekly by the tail cuff method. At the end of treatment, we evaluated endothelial function in aorta and mesenteric segments, left ventricular hypertrophy, eNOS expression in aorta and plasmatic angiotensin conversion enzyme (ACE) activity. The development of hypertension was attenuated in the group treated with MCHP. In addition, MCHP improved the aorta and mesenteric acetylcholine relaxations and increased the eNOS expression in aorta. Left ventricular hypertrophy decreased in treated SHR which was accompanied by a significant decrease in interstitial

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fibrosis. In conclusion, MCHP could be used as a functional food ingredient with antihypertensive activity.

Materials and Methods

5

Milk casein hydrolysate Product

The powdered casein hydrolysate was prepared by casein hydrolysis with food-grade pepsin (Biocatalysts, Cardiff, UK) as described in a previous paper
10 [Contreras *et al.*, 2009. *Int Dairy J.* 566-73.]. The resulting hydrolyzed casein was subsequently spray-dried.

General protocol in rats

15 All the above-mentioned experiments were performed according to the European Union guidelines for the ethical care of animals. Sixteen male SHR 12 weeks old (Janvier, Le Genest Saint Isle, France) were housed in groups of four rats and were maintained at a temperature of 23 °C with 12 h light/dark cycles. Rats were fed on a solid standard diet (Global Diet 2014, Harlan,
20 France) with *ad libitum* intake. They were in turn randomly divided into two groups of 8 animals, that received during 6 weeks tap water (control) or a solution of casein hydrolysate prepared to give 800 mg/kg body weight per day (MCHP). The rat body weight in both groups was recorded weekly during the experimental period. Daily intake of drinking fluids and freely accessible feed
25 was also estimated weekly.

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured in awake rats using the CODA tail-cuff blood pressure system (Kent Scientific Torrington, USA). This system utilizes volume pressure recording
30 sensor technology to measure rat tail blood pressure. Before the measurements, the rats were kept at 38 °C for 10-15 minutes to make the pulsations of the tail artery detectable. Arterial blood pressure measurements

were performed at the same time of day (between 9 a.m. and 13 p.m.) in order to avoid the influence of the circadian cycle, and the values of SBP and DBP were obtained by estimating the average reading of 8 measurements.

5 At the end of treatment animals were anaesthetised with sodium pentobarbital (60 mg/kg, i.p.) and blood samples were collected from carotid artery into tubes containing heparin as anticoagulant. After that, tissues (heart, aorta, and mesenteric bed) were harvested immediately, placed in chilled Krebs solution of the following composition (in mM): NaCl 118; KCl 4.7; CaCl₂ 2.5; KH₂PO₄ 1.2;
10 MgSO₄·7H₂O 1.2; NaHCO₃ 25 and glucose 11, pH=7.4 and each one was processed appropriately for further study.

Blood samples were centrifuged at 2500 g for 10 min at 4 °C to obtain the plasma which was kept frozen at -20 °C until analysis of ACE activity. Heart was
15 utilized to carry out hypertrophy determinations and tissue fibrosis. Vascular reactivity was evaluated in aorta and mesenteric arteries and a portion of aorta was also kept frozen until analysis of eNOS expression by western blotting.

Experiments in aorta rings

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The thoracic aorta was carefully cleaned of fat and connective tissue and cut into rings (3 mm in length) that were placed between stainless steel hooks and set up in organ chambers filled with 5 ml of Krebs solution, gassed with carbogen and kept at 37 °C. One of the hooks was fixed to the bath and the
25 other connected to an isometric force transducer (UF1, Harvard apparatus Inc., U.S.A). Force was recorded on a PC computer using Lab Chart version 3.4 software and a Power Lab/800 data acquisition system (AD Instruments, UK). All rings were allowed to equilibrate for 1 hour at a resting tension of 2 g. The Krebs solution was periodically changed and tension was reset during this
30 period. Then the vessels were exposed to phenylephrine (PE, 10⁻⁶ M) and the presence of functional endothelium was assessed by the ability of acetylcholine (ACh, 10⁻⁶ M) to induce relaxation. After a washout period, cumulative

concentration response curves to PE (10^{-8} - 3×10^{-5} M) were obtained. After precontraction with PE (10^{-6} M) and the steady maximal contraction, cumulative concentration response curves were obtained for ACh (10^{-8} - 3×10^{-5} M) or sodium nitroprusside (SNP, 10^{-8} - 10^{-5} M). Angiotensin I (Ang I, 10^{-7} M) response was also evaluated. Each curve was obtained in different rings.

Experiments in mesenteric rings

Ring segments, 2 mm in length from third branch of mesenteric bed, were mounted in a small vessel dual chamber myograph for measurement of isometric tension. Two steel wires (40 μ m diameter) were introduced through the lumen of the segments and mounted according to the method described by Mulvany and Halpern (Mulvany and Halpern, 1977. *Circ Res* 41:19-26). After a 30 min equilibration period in Krebs solution bubbled with carbogen, at 37 °C and pH=7.4, segments were stretched to their optimal lumen diameter for active tension development. After a new 30 min equilibration period the vessels were exposed to PE (10^{-5} M) and the presence of functional endothelium was assessed by the ability of ACh (10^{-6} M) to induce relaxation. After precontraction with PE (10^{-5} M) and the steady maximal contraction, cumulative concentration response curves were obtained for ACh (10^{-8} - 10^{-5} M).

Left ventricular hypertrophy

The atrium was removed from the heart and all the epicardial fat was scraped off. The right and the left ventricle were separated, regarding this interventricular septum as an integral part of the left ventricle, and this portion was weighed. Left ventricular hypertrophy index (LVH) was calculated using left ventricle weight/body weight ratio.

Left ventricular fibrosis

A transverse section of left ventricle was fixed in 4% formaldehyde, embedded in paraffin and cut into 4 μ m thick sections. Slices were stained with the

collagen-specific stain Sirius-red (Sigma-Aldrich, Spain). At least eight areas from each heart were captured using a high-resolution digital camera (Olympus DP50, Japan). The collagen was quantified using Adobe Photoshop CS2 (Microsoft), for each image the percentage of interstitial fibrosis was determined as the ratio of the collagen surface area with respect to myocardial surface area.

Determination of ACE activity

ACE activity in plasma was measured by a colorimetric commercial kit (BÜHLMANN laboratories AG, Switzerland) based in the method described by Hurst and Lowell-Smith Hurst and Lovell-Smith, 1981 (*Nature* 1970; 227:680-5). Briefly, the plasma samples were mixed with N-hippuryl-L-histidyl-L-leucine (HHL) and the mixture was incubated at 37 °C for 15 min. The reaction was stopped by addition of HCl and the hippuric acid released was then complexed with cyanuric chloride. The absorbance of this complex was measured at 382 nm. One unit of ACE activity is defined as the amount of enzyme required to release one μmol of hippuric acid per min and per L of serum at 37 °C.

Aorta western blotting

Frozen aortic samples were homogenized and centrifuged at 2000 rpm for 1 min at 4 °C in RIPA-lysis buffer; the lysates were collected and centrifuged at 10 000 rpm for 5 min at 4 °C. The supernatant was collected and the protein concentration was determined using a standard protein assay (Bio-Rad Laboratories Inc, USA). Proteins (35 μg) were eluted in Laemmli buffer, resolved by SDS-PAGE (10%), and electrophoretically transferred to nitrocellulose membranes (Whatman, Germany). The membranes were blocked with 5% nonfat dried milk in Tris-buffered saline (TBS)-Tween (0.1%) for 1 h at room temperature and subsequently exposed to a polyclonal rabbit anti-eNOS antibody (1:5000, Bio-Rad) overnight at 4 °C. Blots were washed in TBS-Tween, followed by incubation with horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:1000, Bio-Rad) for 1 h at room temperature.

Blots were developed by chemiluminescence using the ECL Western blotting system (Amersham-Pharmacia-Biotech) and exposed on X-ray films (Fuji, India). Densitometry was performed using a documentation program

(Fluorochem, Alpha Innotech Corp., USA). eNOS corresponding to a 145-kDa band was visualized with reference to molecular weight markers.

The results are expressed as the ratio between signals on the immunoblot
5 corresponding to eNOS and α -actin.

Drugs

Phenylephrine hydrochloride, acetylcholine chloride, sodium nitroprusside, and
10 angiotensin I were obtained from Sigma-Aldrich (Spain). Stock solutions of drugs were made up in ultrapure water, stored at -20 °C and appropriate dilutions were made on the day of the experiments.

Statistical analysis

15 Data are expressed as mean \pm SEM. The responses to ACh and SNP are expressed as percentages of PE contraction. Responses to PE and Ang I are expressed as mg of contraction. Statistical calculations for significant differences were performed using Student's t test. Significance was accepted at
20 $p < 0.05$.

Results

Body weight, liquid and dry food intake were increased progressively through
25 the experimental period in both control and treated groups (data not shown). Dose was selected in preliminary assays which MCHP (400 and 800 mg/kg body weight) was administered acutely and the SBP was measured several times post-administration. The maximum decrease of SBP was 11 ± 2 and 21 ± 3 mmHg respectively

30 Values of SBP and DBP from control and treated groups were similar before starting the treatment (SBP, 169 ± 2 and 167 ± 2 mmHg in control and MCHP respectively and DBP was 121 ± 1 in control and 119 ± 2 mmHg in MCHP group) but they rose gradually during the experiment. However, this increase was
35 smaller in animals that received long-term oral intake of casein hydrolysate (Fig. 5a and 5b).

Vascular responses

Aortic and mesenteric rings from treated rats showed greater relaxations to ACh than the control arteries (Fig. 6a and 6b). These results indicated an improving
5 of endothelial function.

In aorta the NTP-relaxations were not modified after MCHP intake (data not shown). A lower response to PE was observed in aortic rings from treated animals (Fig. 7). Contraction to Ang I (10^{-7} M) were similar in both groups
10 (213 ± 34 and 220 ± 25 mg in control and MCHP respectively).

Left ventricular hypertrophy and fibrosis

Rats fed with MCHP during six weeks had a significant reduction of left ventricle
15 weight compared with the control group (1062 ± 22 and 1129 ± 30 mg respectively, $p<0.05$), and this led to an improvement of the LVH index (Fig. 8). In addition, rats treated with MCHP showed a significant decrease in collagen deposition measured in left ventricle histological sections stained with Sirius-red (Fig. 9).

20

ACE activity

We evaluated the plasma ACE activity and we observed that values were higher in the animals that received MCHP than in the control group (104.3 ± 0.8
25 and 94.8 ± 3.7 $\mu\text{mol}/\text{min}/\text{L}$ respectively, $p<0.05$).

eNOS expression in aorta

The western blotting analysis carried out in aorta homogenates showed an
30 increase in eNOS expression in treated animals (Fig. 10).

In the present study, cardiovascular benefits of casein hydrolysate administered chronically during 6 weeks were investigated. Our findings indicate that 800 mg/kg body weight per day were able to attenuate the

progression of hypertension, to improve the endothelium-dependent relaxation and to decrease cardiac hypertrophy.

SHR are considered one of the best experimental models to evaluate antihypertensive drugs. Okamoto and Aoki developed this experimental model of hypertension, and showed that the progression of hypertension in these animals is in fact very similar to that in humans. In both cases hypertension appears at an early age, there is a family history of this pathology and it is worsened by a sodium-rich diet (Okamoto and Aoki, 1963. *Japanese Circulation Journal*, 1963 27:282-293). Several studies using SHR, have demonstrated antihypertensive properties due to long-term intake of different hydrolysates obtained from food (Sipola *et al.*, 2001. *J Physiol Pharmacol.* 2001; 52:745-54; Yang *et al.*, 2004. *British Journal of Nutrition* 92, 507 – 512; Miguel *et al.*, 2006. *Life Sci.* 2006;78(25):2960-6)

15

The study was performed in animals 12 weeks-old in which the development of hypertrophy is at its early stages. We have demonstrated that chronic administration of MCHP produced an improvement of the left ventricular hypertrophy and in the heart fibrosis. We thought that the effect of MCHP in preventing cardiac hypertrophy and collagen deposition is well correlated with its antihypertensive effect.

20

After 6 weeks of treatment the endothelium-dependent relaxation of mesenteric arteries was also improved. In our opinion, the effect on endothelial function could justify, at least in part, the antihypertensive effect of MCHP.

25

Hypersensitivity of vascular smooth muscle to α 1-adrenoceptor agonists has been suggested as an important element in the development and maintenance of hypertension (Mulvany *et al.*, 1980. *Hypertension* 2:664-71; Takata & Kato. 1996. *Life Sci.* 58:91-106]. Consistent with this, we have observed that aortic rings from control animals contracted stronger to phenylephrine than arteries from the treated ones.

30

Due to the incomplete and often unknown bioavailability of this kind of hydrolysates/preparados following oral administration, it is difficult to predict the *in vivo* enzyme inhibitory activity. It is worth mentioning that, compared with
 5 ACE inhibitory synthetic drugs, peptides possess higher *in vivo* antihypertensive activity than would be expected from their *in vitro* ACE inhibitory activity.

But in conclusion, the present study shows that MCHP could be incorporated in human foods as a possible functional ingredient with expected antihypertensive
 10 activity.

Table 6. Protein content (% w/w), moisture (% w/w), content of minerals (mg g⁻¹) and active peptides RYLGY and AYFYPEL (mg g⁻¹) of the powdered milk casein hydrolysate (MCH). Data are expressed as mean ± SD.

15

	MCH ^a
Total protein	38.82 ± 0.25 ^b
Moisture	2.92 ± 0.02 ^b 20
Calcium	10.92 ± 0.06 ^c
Magnesium	0.54 ± 0.01 ^c
Sodium	2.45 ± 0.14 ^c
Potassium	19.49 ± 0.25 ^c
Zinc	0.06 ± 0.004 ^c
RYLGY	0.71 ± 0.02 ^c
AYFYPEL	1.84 ± 0.02 ^c

CLAIMS

1. A composition comprising a peptide comprising the amino acid sequence of
5 SEQ ID NO: 1 and having up to 10 amino acids.
2. The composition according to claim 1, wherein the amino acid sequence of
the peptide has up to 8 amino acids.
- 10 3. The composition according to any of claims 1-2, wherein the amino acid
sequence of the peptide consists of SEQ ID NO: 1.
4. The composition according to any of claims 1-3, comprising a protein
hydrolysate.
- 15 5. The composition according to claim 4, wherein the protein hydrolysate is
obtained from bovine milk.
6. The composition according to any of claims 1-5, wherein the amount of the
20 peptide is between 0.06 to 0.08 mg for kg of body weight of the mammal.
7. The composition according to any of claims 1-5, wherein the amount of the
peptide is between 0.5 mg and 10 mg.
- 25 8. The composition according to claim 7, wherein the amount of the peptide is
between 1.0 mg and 5.0 mg.
9. The composition according to claim 8, wherein the amount of the peptide is
30 between 1.5 mg and 2.0 mg.

10. The composition according to any of claims 1-9, wherein said composition also comprises the peptide comprising the amino acid sequence of SEQ ID NO: 9.
- 5 11. The composition according to claim 10, wherein the amino acid sequence of the peptide consists of SEQ ID NO: 9.
12. The composition according to any of claims 10-11, wherein the amount of
10 the peptide comprising the amino acid sequence of SEQ ID NO: 9 is between 1.5 mg and 15 mg.
13. The composition according to claim 12, wherein the amount of the peptide
15 comprising the amino acid sequence of SEQ ID NO: 9 is between 2.0 mg and 10 mg.
14. The composition according to claim 13, wherein the amount of the peptide
20 comprising the amino acid sequence of SEQ ID NO: 9 is between 2.5 mg and 3.5 mg.
15. The composition according to any of claims 1-14, wherein the composition is a food composition.
16. The composition according to any of claims 1-15, wherein the composition is
25 a fermented composition.
17. The composition according to any of claims 1-16, wherein the composition is a dairy product.
- 30 18. The composition according to any of claims 1-17, wherein the composition is a pharmaceutical composition.

19. The composition according to any of claims 1-18, wherein the composition is an oral dosage form.
20. The composition according to claim 19, wherein the oral dosage form is selected from the group comprising tablets, capsules, caplets, slurries, sachets, suspensions, chewing gum, and powder formulation that may be dissolved in a liquid.
21. The composition according to claim 20, wherein the oral dosage form is a suspension.
22. The composition according to claim 20, wherein the oral dosage form is a powder formulation that may be dissolved in a liquid.
23. The composition according to claim 22, wherein the liquid is water, milk, juice, or yogurt.
24. Use of a composition according to any of claims 1-23 for mitigating hypertension.
25. A composition according to any of claims 1-23, for use as a medicament.
26. A composition according to any of claims 1-23, for use in the treatment or prevention of hypertension in a mammal.
27. A composition according to any of claims 1-23, for use in the treatment or prevention of stroke, coronary disease, myocardial infarction, metabolic syndrome, peripheral vascular disease or abdominal aortic aneurysm in a mammal.
28. A composition according to any of claims 26-27, wherein the mammal is a human.

29. A composition according to any of claims 1-23, for use in the treatment or prevention of hypertension, stroke, coronary disease, myocardial infarction, metabolic syndrome, peripheral vascular disease or abdominal aortic aneurysm in a mammal by oral administration, wherein said treatment or prevention comprising administering to said mammal the composition with the amount of the peptide or peptides as defined in any of claims 7-14, per day.

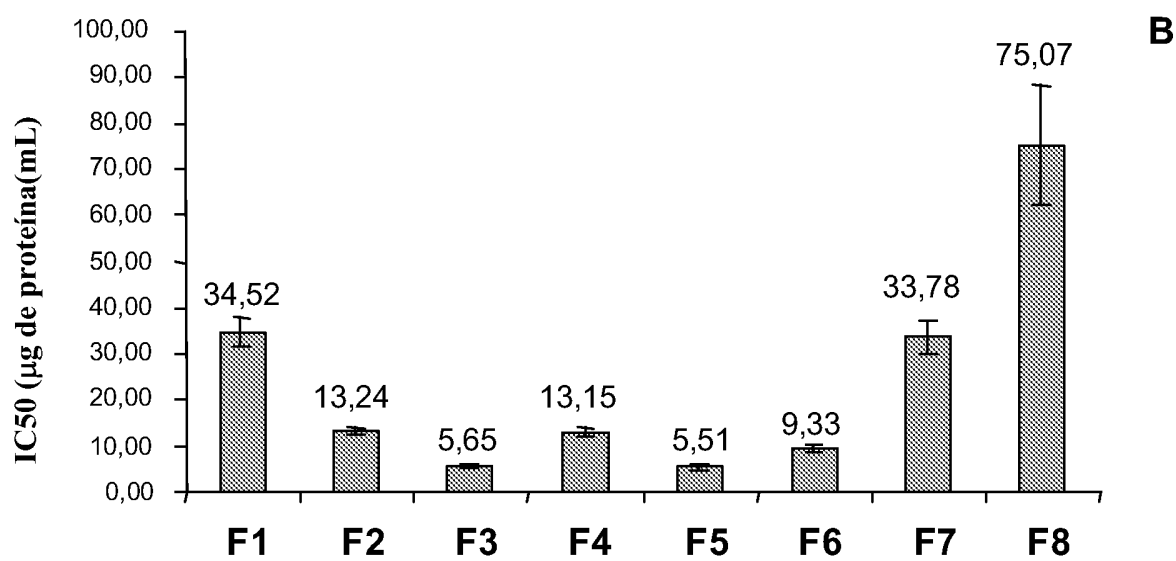
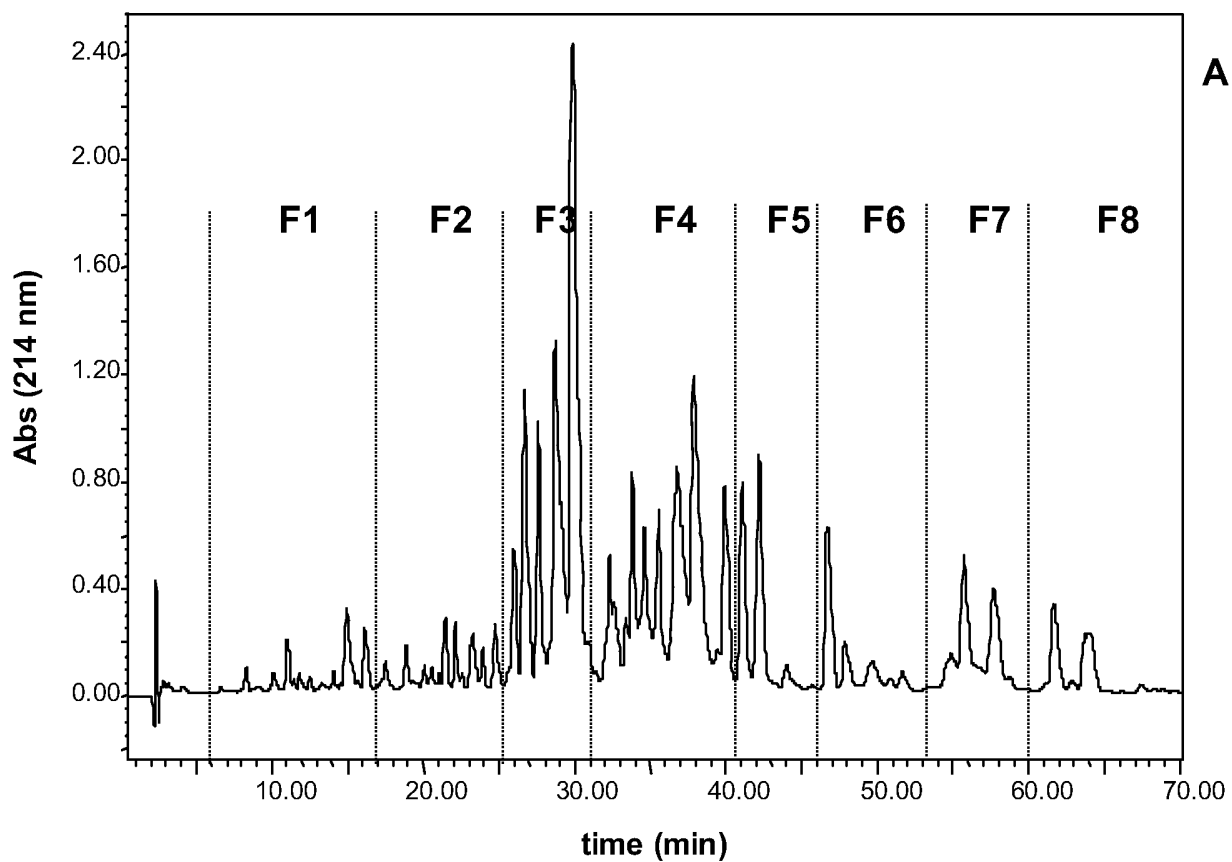


FIG. 1

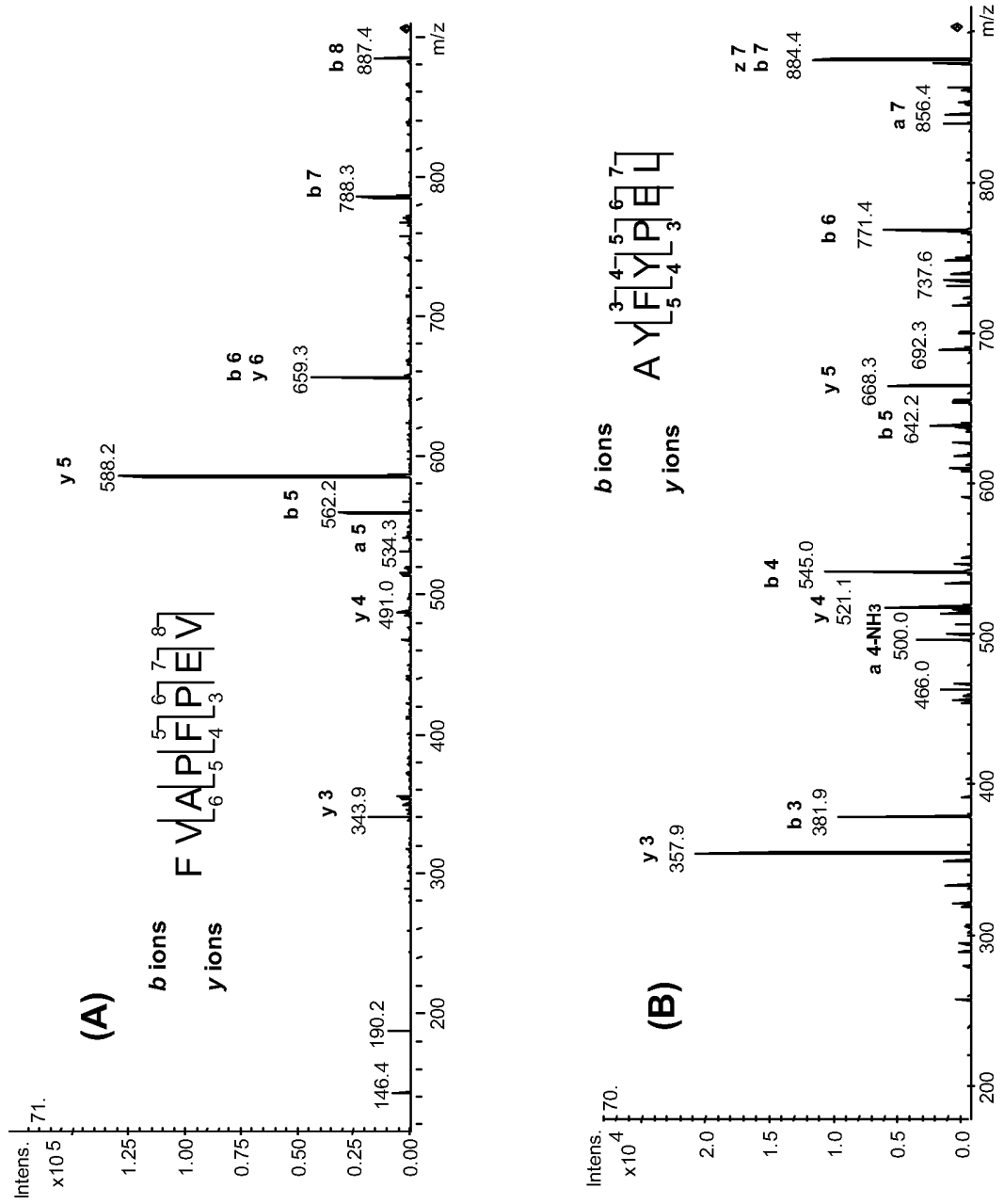


FIG. 2

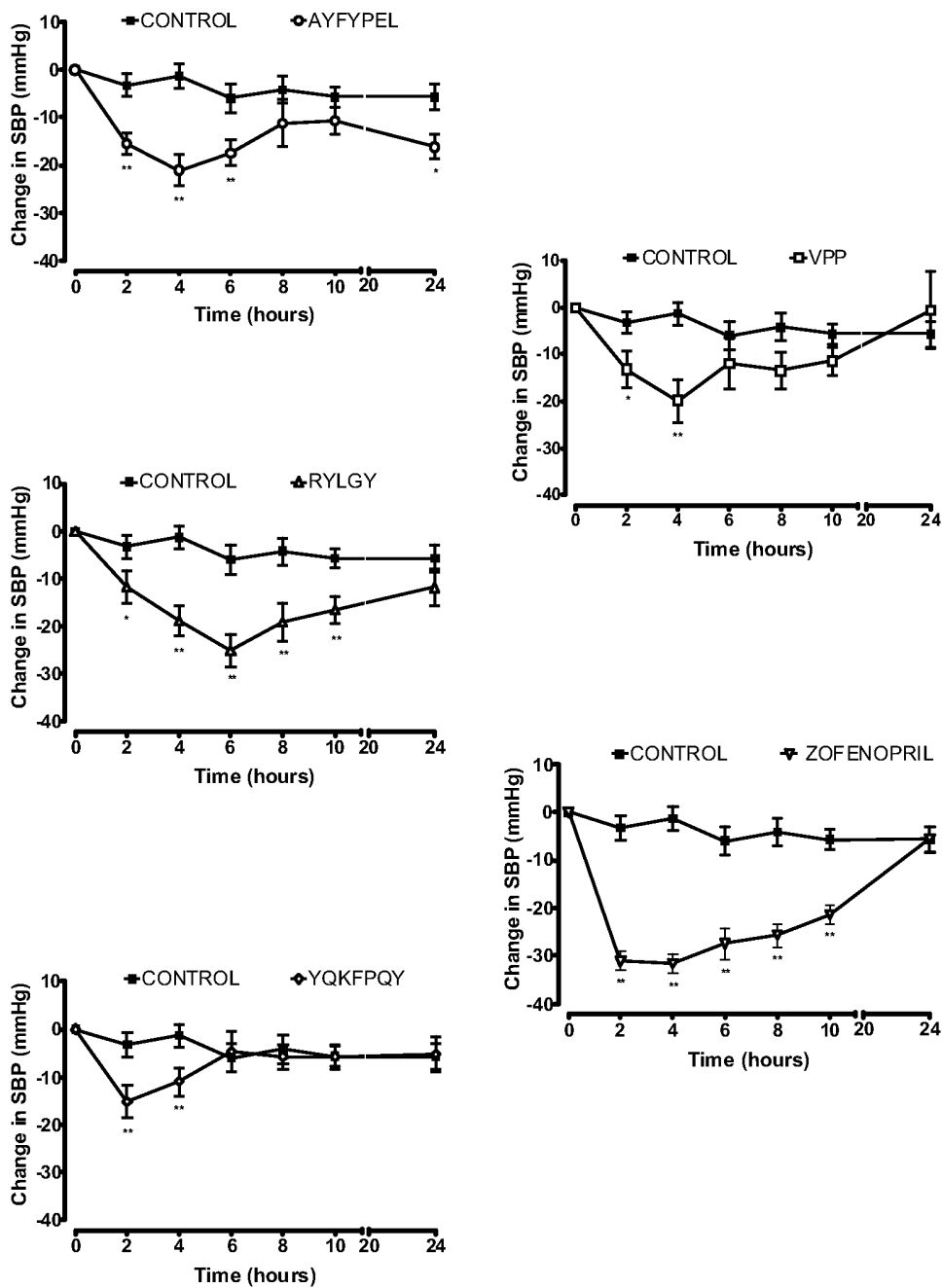


FIG. 3

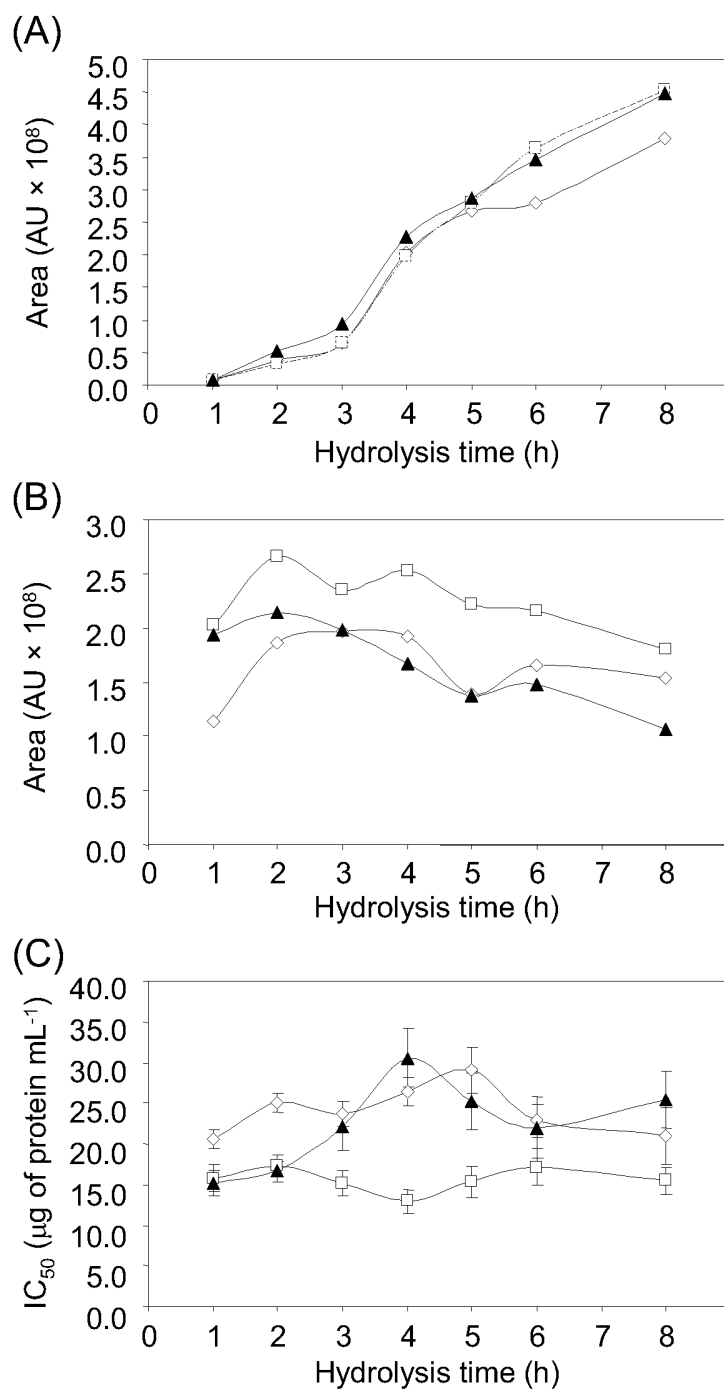


FIG. 4

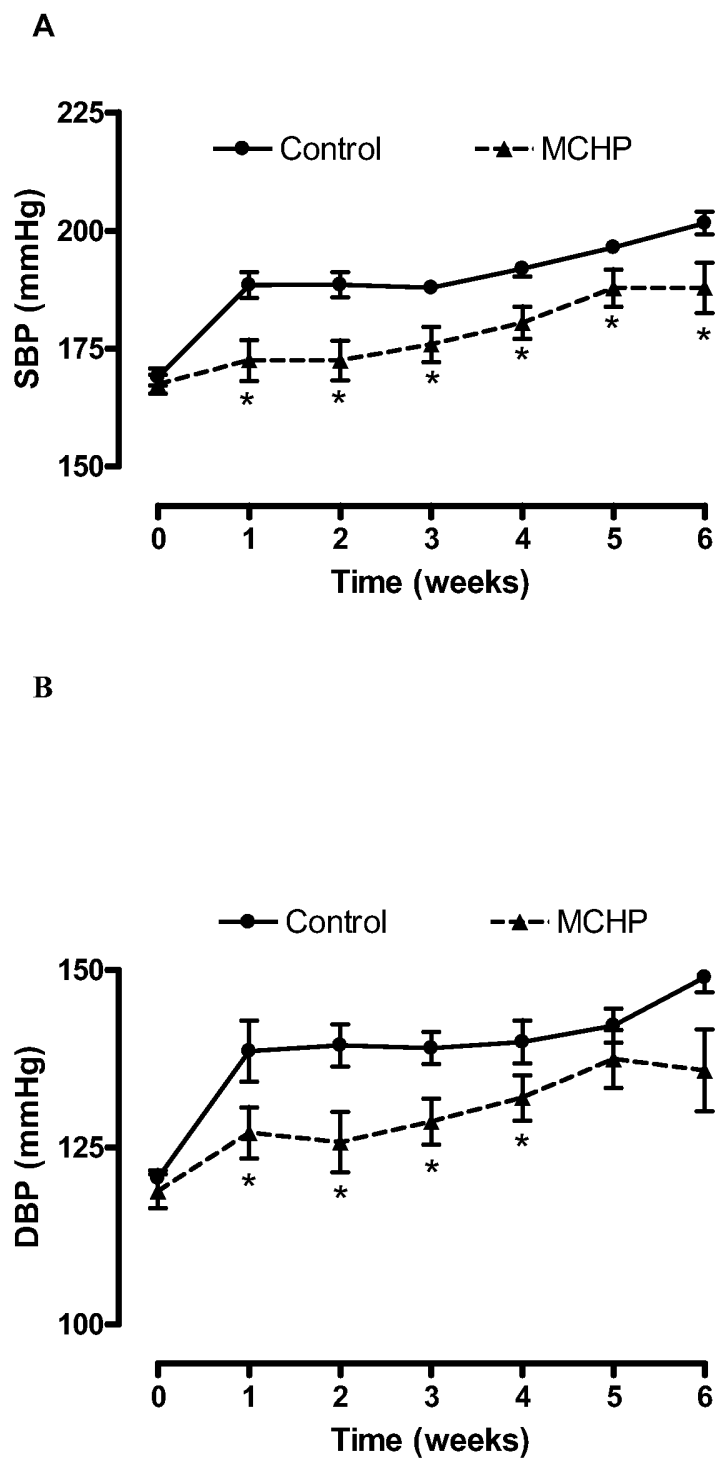
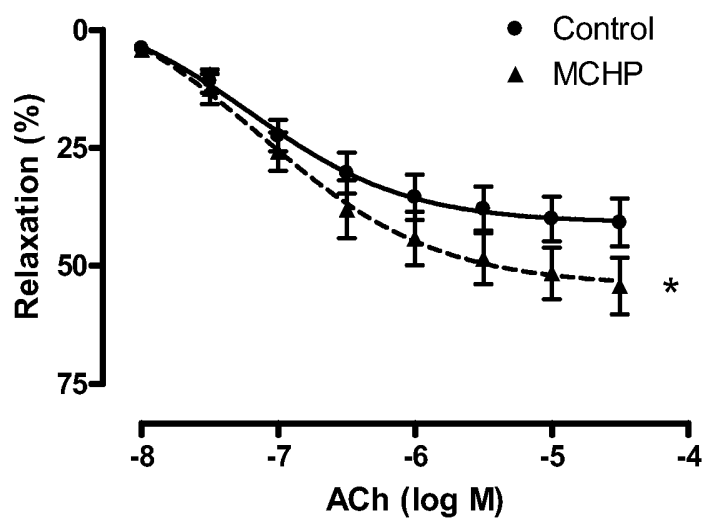


FIG. 5

A



B

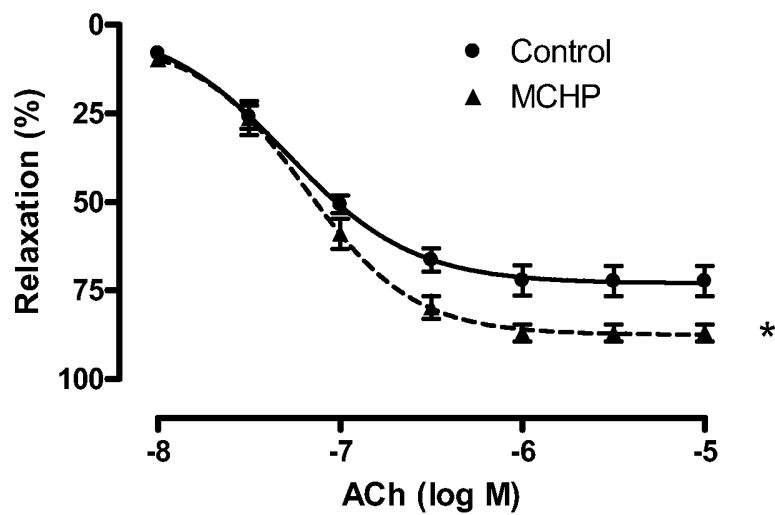


FIG. 6

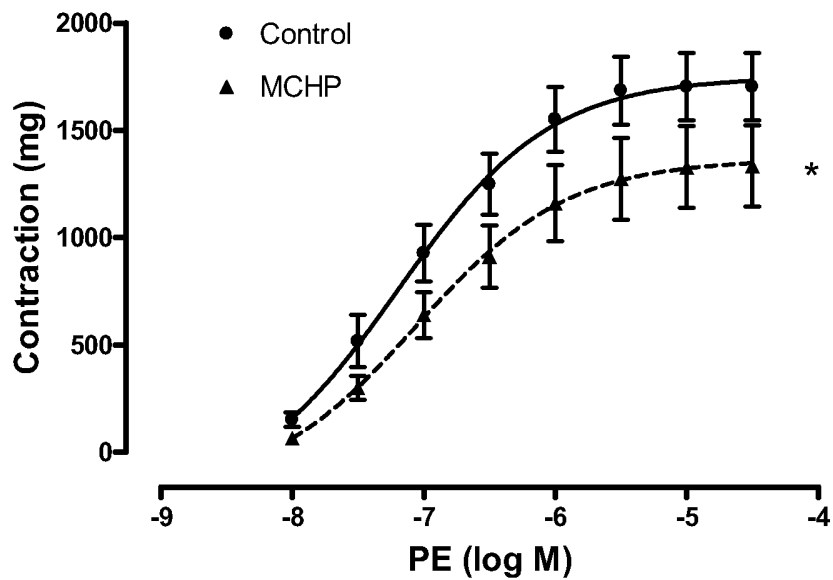


FIG. 7

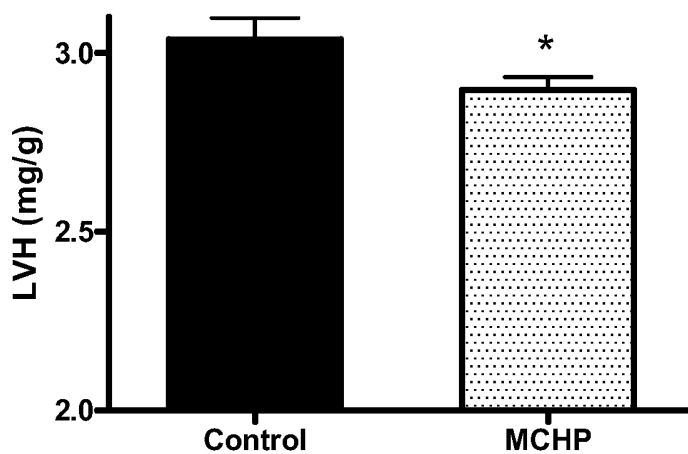


FIG. 8

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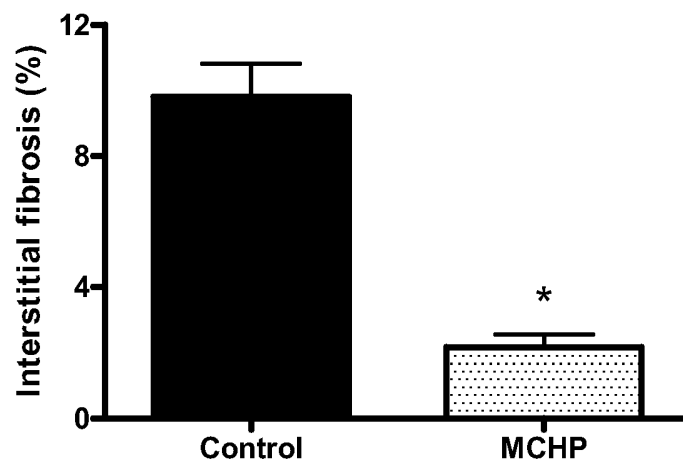
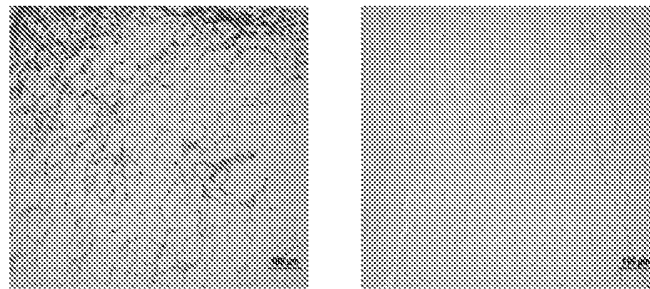


FIG. 9

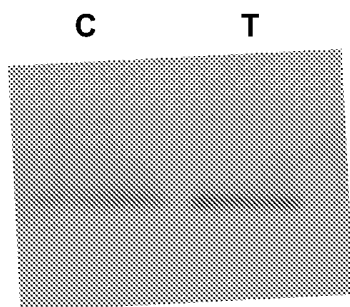


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/055924

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/01 C07K14/47
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 905 779 A (CONSEJO SUPERIOR INVESTIGACION [ES]) 2 April 2008 (2008-04-02) paragraph [0011] paragraph [0012] paragraph [0062] claims 1, 5, 10, 11, paragraph [0064]	1-14, 18, 19, 23-29
X	JAUHAINEN TIINA ET AL: "Milk peptides and blood pressure" JOURNAL OF NUTRITION, vol. 137, no. 3, Suppl. S, March 2007 (2007-03), pages 825S-829S, XP002535671 ISSN: 0022-3166 page 827S, left-hand column, paragraph 2 - right-hand column, paragraph 2 ----- -/--	1-5, 10, 11, 18, 19, 23-29

Further documents are listed in the continuation of Box C.

See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

24 June 2010

Date of mailing of the international search report

01/07/2010

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Authorized officer

Rojo Romeo, Elena

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/055924

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	JAKUB FICHNA ET AL: "Opioid peptides in cancer" CANCER AND METASTASIS REVIEWS, KLUWER ACADEMIC PUBLISHERS, DO, vol. 23, no. 3-4, 1 August 2004 (2004-08-01), pages 351-366, XP019205160 ISSN: 1573-7233 page 360, right-hand column, paragraph 2 - paragraph 3; tables 2,11	1-5,10, 11,18, 19,23, 25,26, 28,29
X	WO 2008/088472 A2 (DANISCO US INC GENENCOR DIV [US]; KUMAR MANOJ [US]; WONG DAVID [US]) 24 July 2008 (2008-07-24) paragraph [0074] - paragraph [0077]; claims 15, 20-22	1-5, 15-17, 19-23

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No PCT/EP2010/055924

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