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Original article

Casein hydrolysates as a source of antimicrobial, antioxidant and antihypertensive peptides

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Abstract – The aim of this work was to investigate the presence of antioxidant and ACE-inhibitory activity in ovine α_{s2}-casein and bovine κ-casein hydrolysates with antibacterial activity. Several peptides which had been previously identified in these hydrolysates were selected in order to fulfil certain structural requirements and they were chemically synthesised to evaluate their antioxidant and ACE-inhibitory activity. Hydrolysates of ovine α_{s2}-casein and bovine κ-casein with pepsin strongly inhibited ACE activity, with IC_{50} values of 41.8 and 9.97 μmol·L^{-1}, respectively. The κ-casein hydrolysate also exhibited a significant oxygen radical absorbance capacity, seven times higher than that of Trolox. From the chemically synthesised peptides, two of them, LKKISQ and PYVRYL, both from ovine α_{s2}-casein, exerted potent ACE-inhibitory activity in the range of the most potent food-derived antihypertensive peptides described to date (IC_{50} values of 2.6 and 2.4 μmol·L^{-1}, respectively). The latter sequence, corresponding to the C-terminal hexapeptide of the ovine α_{s2}-casein molecule, also had antioxidant activity. The activity found is discussed in relation to the peptide sequences.

α_{s2}-casein / κ-casein / antibacterial activity / antioxidant activity / angiotensin-converting enzyme-inhibitory activity / bioactive peptide

摘要 – 来源于酪蛋白水解物的抗菌、抗氧化和抗高血压肽。本研究目的是调查源于绵羊奶 α_{s2}-酪蛋白和牛奶 κ-酪蛋白水解物是否具有抗菌、抗氧化和 ACE 抑制活性。根据前期的研究结果, 在绵羊奶α_{s2}-酪蛋白和牛奶 κ-酪蛋白的水解物中选择了几种已经确定具有生物活性的肽, 为了从结构上解释这些肽的生物活性, 通过化学方法合成了这几种肽, 并研究了化学合成肽的抗氧化和 ACE 抑制活性。绵羊奶 α_{s2}-酪蛋白和牛奶 κ-酪蛋白经胃蛋白酶水解后, 水解物具有非常强的 ACE 抑制活性, 其 IC_{50}(半抑制浓度) 值分别为 41.8 和 9.97 μmol·L^{-1}。 κ-酪蛋白的水解物呈现了较高的抗氧化能力, 其抗氧化能力指数是Trolox (定量标准) 的7倍。在化学合成的肽中, LKKISQ和PYVRYL两种肽显现出潜在的 ACE 抑制活性, 这两种肽均是基于绵羊奶 α_{s2}-酪蛋白水解物的氨基酸序列而合成的肽, 两种肽的 IC_{50} 值分别在 2.6 和 2.4 μmol·L^{-1}, 其半抑制浓度在许多食源性抗高血压肽的范围之内。此外, 具有f (203–208) 氨基酸序列的六肽PYVRYL还具有抗氧化能力。本文还讨论了肽的活性与羧氨酸序列的关系。

α_{s2}-酪蛋白 / κ-酪蛋白 / 抗菌活性 / 抗氧化活性 / 血管紧缩素转移酶抑制活性 / 生物活性肽

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Résumé – Les hydrolysats de caséine comme source de peptides antimicrobiens, antioxydants et anti-hypertensifs. Le but de cette étude était de rechercher la présence d'activité antioxydante et inhibitrice de l'enzyme de conversion de l'angiotensine (ACE) dans des hydrolysats de caséine α_{s2} ovine et de caséine κ bovine, ayant une activité antibactérienne. Plusieurs peptides, qui avaient été identifiés antérieurement dans ces hydrolysats, ont été sélectionnés pour répondre à certaines conditions structurales et ont été synthétisés chimiquement pour évaluer l'activité antioxydante et inhibitrice de l'ACE. Les hydrolysats pepsiques de caséine α_{s2} ovine et de caséine κ bovine inhibaient fortement l'ACE avec des valeurs d'IC_{50} de 41,8 et 9,97 $\mu mol \cdot L^{-1}$, respectivement. L'hydrolysat de caséine κ bovine montrait également une capacité importante d'absorption de radicaux oxygène, 7 fois plus élevée que celle du Trolox. Pour les peptides synthétisés chimiquement, deux d'entre eux, LKKISQ et PYVRYL, provenant l'un et l'autre de caséine α_{s2} ovine, manifestaient une forte activité inhibitrice de l'ACE dans la gamme des peptides antihypertensifs issus des aliments les plus puissants décrits jusqu'à présent (valeur d'IC_{50} de 2,6 et 2,4 respectivement). La dernière séquence correspondant à l'hexapeptide C-terminal de la caséine α_{s2} ovine, avait également une activité antioxydante. L'activité trouvée est discutée en relation avec les séquences peptidiques.

caséine α_{s2} / caséine κ / activité antibactérienne / activité antioxydante / enzyme de conversion de l'angiotensine / peptide bioactif

1. INTRODUCTION

Research carried out during the last 10 years has shown that both caseins and whey proteins can be an important source of biologically active peptides [11]. These peptides, which are in a latent state within the precursor protein sequence, can be released by enzymatic proteolysis [8]. Once bioactive peptides are released, they may act as regulatory compounds in the host organism with specific activities such as antihypertensive, antioxidant, antimicrobial or opioid. Many milk-derived peptides reveal multifunctional properties, i.e., specific peptide sequences may exert two or more different biological activities. For example, some regions in the primary structure of caseins contain overlapping peptide sequences, which exert different biological effects. These regions have been considered as "strategic zones" [16].

Of special interest are biologically active peptides related to cardiovascular diseases because of the high incidence of hypertension, which is estimated to affect one-third of the Western population. Among them, angiotensin-converting enzyme (ACE)-inhibitory peptides have been extensively studied during the last decade. ACE (peptidyldipeptide hydrolase, EC 3.4.15.1) catalyses the conversion of angiotensin I, an active decapeptide, into angiotensin II, an octapeptide with a potent vasoconstrictor action. Moreover, ACE catalyses the inactivation of bradykinin, which has a significant vasodilator activity. Therefore, inhibition of this enzyme results in a lowering of blood pressure [14]. Antioxidant deficiency has also been implicated in the occurrence of hypertension. Antioxidant-rich diets have been proven to reduce blood pressure in model animals [1] and in humans [6]. Moreover, some of the most potent antihypertensive compounds, such as captopril, glutation, carnosine and others, share both antioxidant and ACE-inhibitory properties [9]. This dual (ACE-inhibitory and antioxidant) activity has also been reported for egg white-derived peptides [5].

Previous research carried out in our research group has led to the identification of casein hydrolysates, mainly ovine α_{s2}-casein and bovine κ-casein hydrolysates with antibacterial properties [12, 13]. More than 30 peptides contained in these hydrolysates were sequenced by tandem mass spectrometry and some of them were chemically synthesised to assay the antibacterial activity of individual peptides. However, there are no data about the

antioxidant or the ACE-inhibitory activity of these hydrolysates or the individual peptides, although some of the identified peptides possessed structural motifs that are relevant to exerting these biological activities.

The aim of this work is to investigate the presence of other biological activities, mainly antioxidant and ACE-inhibitory activity, in the ovine α_{s2}-casein and bovine κ-casein hydrolysates with antibacterial activity and in certain peptides selected on the basis of their amino acid sequences. The activity found is discussed in relation to the structures of the peptides.

2. MATERIALS AND METHODS

2.1. Digestion of ovine α_{s2}-casein and bovine κ-casein

The hydrolysate of ovine α_{s2}-casein was prepared as previously described [12]. A 0.5% (w/v) aqueous solution of α_{s2}-casein was adjusted to pH 3.0 with 1 mol·L^{-1} HCl and digested with 3.7% (w/w of substrate) porcine pepsin A (Sigma, St. Louis, MO, USA) for 30 min at 37 °C. The reaction was terminated by heating at 80 °C for 15 min and the pH was adjusted to 7.0 by addition of 1 mol·L^{-1} NaOH. The digest was centrifuged at 16 000× g for 15 min and the supernatant was lyophilised until use. The hydrolysate of bovine κ-casein was prepared from 3 mg·mL^{-1} solution of κ-casein (Sigma) with 0.5% (w/w of substrate) porcine pepsin A (Sigma) for 2 h at 37 °C [13].

2.2. Peptide synthesis

Synthetic peptides were prepared by the conventional Fmoc solid-phase synthesis method with a 431 A peptide synthesiser (Applied Biosystems Inc., Überlingen, Germany). All the synthetic peptides were purified after synthesis by semi-preparative RP-HPLC with the conditions previously described [13].

2.3. Determination of the antioxidant activity

An Oxygen Radical Absorbance Capacity (ORAC-FL) assay was employed to evaluate the antioxidant potential of casein hydrolysates and chemically synthesised peptides. The assay was performed according to the method of Ou et al. [18] with some modifications. Briefly, fluorescein (48 nmol·L^{-1}, 2.2 mL) was premixed with the sample, Trolox (standard) or phosphate buffer (375 μL) and incubated for 30 s. This reading was fluorescence at time zero (f_0). The assay was initiated by adding 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) (143 mmol·L^{-1}, 375 μL). Mixtures were kept in a water bath at 37 °C for 30 min. Fluorescence readings (lex = 493 nm and lem = 515 nm) were taken every 5 min after AAPH addition (f_1, f_2, f_3, etc.). The fluorescence decay curve was plotted and the area under the curve calculated. Blanks were run by replacing the sample with phosphate buffer. Sample fluorescence values were corrected for the blank value.

The final ORAC-FL values were calculated by assessing the area under curve (AUC) of the fluorescence decay curve and it was expressed as Trolox equivalents as micromole per litre for the peptides and per mg for the hydrolysates. The AUC was calculated as: AUC = $0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots + f_n/f_0$, where f_0 is the initial fluorescence reading at 0 min and f_n is the fluorescence reading at time n. The relative ORAC value (Trolox equivalents) was calculated as:

ORAC value = [(AUC_{sample} − AUC_{blank}) / (AUC_{Trolox} − AUC_{blank})] × [(molarity of Trolox / molarity of sample)].

2.4. Determination of the ACE-inhibitory activity

ACE-inhibitory activity was measured by the spectrophotometric assay of Cushman and Cheung [3], with some modifications. Briefly, 40 μL of each sample was added to 0.1 mL of 0.1 mol·L^{-1} sodium borate buffer (pH 8.3) containing 0.3 mmol·L^{-1} NaCl and 5 mmol·L^{-1} HHL. ACE (2 mU) (EC 3.4.15.1; 5.1 U·mg^{-1}) (Sigma) was added and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 0.15 mL of 1 mol·L^{-1} HCl. The hippuric acid formed was extracted with ethyl acetate, heat-evaporated at 95 °C for 10 min, redissolved in distilled water and measured spectrophotometrically at 228 nm. The activity of each sample was tested in triplicate.

The ACE-inhibitory activity was calculated as the peptide concentration needed to cause 50% inhibition of the original ACE activity (IC_{50}). Protein content of each hydrolysate was determined by the Kjeldahl method with a UDK 120 analyser and a DK 20 digester, both from VELP Scientifica (Usmate, Italy).

2.5. Antibacterial assays

Antibacterial assays were performed by a modification of the method described by Pellegrini et al. [19] using microdilution trays. The microorganisms employed in the study were *Escherichia coli* ATCC 25922 from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and *Listeria innocua* CECT 910T, from the Spanish Type Culture Collection (Colección Española de Cultivos Tipo, CECT; Valencia, Spain). Single colonies of bacteria grown on trypticase soy agar plates (TSA) (*E. coli*) or brain heart agar plates (BHIA) (*L. innocua*) were inoculated in 10 mL of trypticase soy broth (TSB) or brain heart broth (BHI) and grown overnight at 37 °C. A total of 300 μL of bacterial suspension was diluted 1/50 with TSB or BHI. Bacteria were grown at 37 °C and logarithmic phase organisms were harvested at a density of $1–4 \times 10^8$ colony-forming units (cfu)·mL^{-1}. The culture was then centrifuged at $2000 \times g$ for 10 min. The pellet of bacteria was washed twice with 10 mmol·L^{-1} Na-phosphate buffer, pH 7.4, and adjusted to 10^6 cfu·mL^{-1}, approximately. A total of 50 μL of the suspension was mixed with 50 μL of the peptide solution to be investigated and with 100 μL of 2% TSB or BHI in 10 mmol·L^{-1} phosphate buffer in a sterile 96-well microplate (Greiner Labortechnik, Frickenhausen, Germany). The mixture was incubated at 37 °C for 2 h and, after the appropriate dilution in 10 mmol·L^{-1} Na-phosphate buffer, pH 7.4, 10 μL of the dilution were spotted on TSA or BHIA agar plates. Alternatively, 100 μL of the appropriate dilution were plated on TSA or BHIA plates. The plates were incubated at 37 °C for 24 h and plate counts were performed. The assays were conducted in duplicate.

3. RESULTS AND DISCUSSION

3.1. Preparation of the hydrolysates and measurement of the antibacterial activity

Ovine α_{s2}-casein and bovine κ-casein were hydrolysed with pepsin at low pH and for different times (15 min, 30 min, 45 min and from one to six hours, taking a sample each hour), and antibacterial activity was screened against *E. coli,* which was used as the test strain. As an example, Figure 1 shows the bacterial survival after two hours of incubation in the presence of non-hydrolysed ovine α_{s2}-casein (control) and samples of the hydrolysed α_{s2}-casein withdrawn at different times when assayed at a

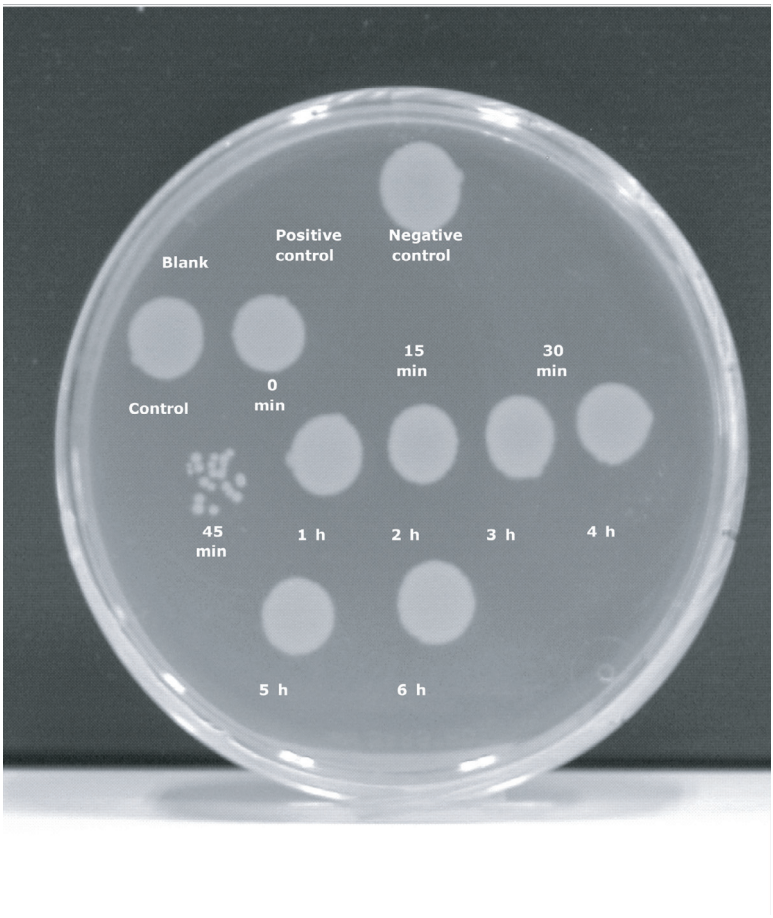

Figure 1. Antibacterial activity assay against *Escherichia coli* ATCC 25922 of the ovine α_{s2}-casein hydrolysates at different incubation times. The positive control contained 50 μL of ampicillin (75 mg·mL^{-1}), the negative control contained 50 μL of non-hydrolysed α_{s2}-casein (2.0 mg·mL^{-1}) and the control consisted of 50 μL of water.

concentration of 2.0 mg·mL^{-1}. α_{s2}-Casein hydrolysates at 15 and 30 min revealed the highest antibacterial potency against this *E. coli* strain. Serial dilutions of these two hydrolysates were also tested for antibacterial activity, proving that digestion of ovine α_{s2}-casein with pepsin for 30 min produced the most potent hydrolysate. A similar screening was performed with the κ-casein hydrolysate, but in this case, the highest effectiveness was found after 2 h of hydrolysis with pepsin (data not shown). The activity of the selected digests was also measured against *L. innocua*. While these two samples exerted a bactericidal activity against *E. coli*, the activity against *L. innocua* was lower. Hydrolysates of α_{s2}-casein and κ-casein produced a reduction of the *L. innocua* strain by 3 log and 2 log at a final concentration of the hydrolysate of 2 mg·mL^{-1}, respectively. These two hydrolysates, 30 min-α_{s2}-casein

and 2 h-κ-casein, were further used for the measurement of antioxidant and ACE-inhibitory activity.

3.2. ACE-inhibitory and antioxidant activity of hydrolysates and synthetic peptides

Hydrolysates of ovine α_{s2}-casein and bovine κ-casein with pepsin inhibited ACE activity, while non-hydrolysed whole casein or non-hydrolysed casein fractions, i.e., α_{s2}-casein and κ-casein, did not show ACE-inhibitory activities ($IC_{50} > 1000\,\mu g \cdot mL^{-1}$). The IC_{50} values of the hydrolysates of α_{s2}-casein and κ-casein were 41.8 and 9.97 μg protein·mL^{-1}, respectively. These low IC_{50} values revealed a notable ACE-inhibitory activity as compared with other milk and milk protein hydrolysates. Vermeirssen et al. [24] reported values of IC_{50} of 398 $\mu g \cdot mL^{-1}$ after in vitro stomach digestion of a whey protein isolate, and of 40.7 $\mu g \cdot mL^{-1}$ after physiological digestion. Pepsin has also been employed for the production of ACE-inhibitory hydrolysates from individual whey proteins but these digestions yielded IC_{50} values higher than 1000 $\mu g \cdot mL^{-1}$ [20]. The most efficient inhibitors of ACE ($IC_{50} < 30\,\mu mol \cdot L^{-1}$) have been produced from caseinate and the individual major caseins, after hydrolysis by trypsin or an extracellular proteinase from *Lb. helveticus* [15, 22, 23]. This latter proteinase was used to hydrolyse α_{s1}-casein and β-casein, yielding IC_{50} values of 11 $\mu g \cdot mL^{-1}$ and 24 $\mu g \cdot mL^{-1}$ [25], which are in the range of those found in this study.

As shown in Table I, antioxidant activity of both hydrolysates was also carried out. The hydrolysates of α_{s2}-casein and κ-casein yielded ORAC-FL values of 0.78 μmol Trolox equivalents per mg of protein and 7.07 μmol Trolox equivalents per mg of protein, respectively. The ORAC-FL value of the κ-casein hydrolysate was high compared with those found in a peptic hydrolysate of egg white (ORAC-FL value of 2.78 μmol Trolox equivalents per mg of protein) [5] or in a α-lactalbumin hydrolysate (ORAC-FL value of 2.95 μmol Trolox equivalents per mg of protein) [7].

Some of the peptides, which had previously been identified in these two hydrolysates, were selected on the basis of their amino acid sequences in order to fulfil certain structural requirements, and they were chemically synthesised. Those peptides with a hydrophobic C-terminal tripeptide were selected because this is important for the ACE-inhibitory activity [2]. Other peptides were selected due to the presence of amino acid residues that are of importance for antioxidant properties. It has been reported that Trp, Tyr and Met, when tested as individual amino acids, showed the highest antioxidant activity, followed by Cys, His and Met [5, 7]. The ACE-inhibitory and the antioxidant activities of the chemically synthesised peptides were determined and are shown in Table II. Only three of the synthesised peptides exerted a remarkable ACE-inhibitory activity with IC_{50} values below 100 $\mu mol \cdot L^{-1}$, corresponding, all of them, to α_{s2}-casein fragments. One of these peptides, FALPQYLK, showed potent ACE-inhibitory activity (IC_{50} value of 11.9 $\mu mol \cdot L^{-1}$). This sequence had been previously identified by Tauzin et al. [23], who reported an IC_{50} value of 4.3 $\mu mol \cdot L^{-1}$. Differences in the IC_{50} value could be due to the method used to quantify hippuric acid. In this work, hippuric acid was evaluated spectrophotometrically, whereas Tauzin et al. [23] used a chromatographic method to quantify this product. The peptide with the sequence FALPQYLK also showed a significant oxygen radical-scavenging activity (ORAC-FL value of 1.6 μmol Trolox equivalents per μmol of

Table I. Protein content determined by the Kjeldahl method (mg protein·mL^{-1} of hydrolysate), ACE-inhibitory activity (expressed as IC_{50}) and oxygen radical absorption capacity (ORAC-FL; expressed as μmol Trolox equivalent per mg of protein) of the hydrolysates obtained from α_{s2}-casein and κ-casein digested with pepsin. For hydrolysis conditions see Materials and Methods.

Peptic hydrolysate	Protein content	IC_{50} [a] (μmol·L^{-1})	ORAC-FL [b]
α_{s2}-casein	2.6	41.8	0.78 ± 0.04
κ-casein	0.3	9.97	7.07 ± 0.08

[a] Peptide concentration needed to inhibit 50% original ACE activity.
[b] Mean value ± SD (n=2).

Table II. ACE-inhibitory activity (expressed as IC_{50}) and oxygen radical absorption capacity (ORAC-FL; expressed as μmol Trolox equivalent per μmol of peptide) of chemically synthesised peptides.

Protein fragment	Sequence	IC_{50}(μmol·L^{-1})	ORAC-FL [a]
κ-casein f(18–24)	FSDKIAK	113.6	0
κ-casein f(28–30)	IQY	70.4	0.55 ± 0.01
κ-casein (30–32)	YVL	>1000	0.96 ± 0.04
κ-casein f(118–121)	EIPT	>1000	0
κ-casein f(162–169)	VQVTSTAV	>1000	0
β-casein f(25–29)	RINKK	>1000	nd
β-casein f(25–28)	RINK	>1000	0
α_{s2}-casein f(174–181)	FALPQYLK	11.9	1.63 ± 0.29
α_{s2}-casein f(165–170)	LKKISQ	2.6	0
α_{s2}-casein f(203–208)	PYVRYL	2.4	1.82 ± 0.09

nd: Not determined.
[a] Mean value ± SD (n=3).

peptide). The presence of Phe and Tyr residues in this α_{s2}-casein fragment can explain the antioxidant activity found. Of special interest are the other two α_{s2}-casein fragments, LKKISQ and PYVRYL, that exhibited potent ACE-inhibitory activity with IC_{50} values of 2.6 and 2.4, respectively, close to those of the most potent peptides derived from milk proteins, such as IPP and VPP (IC_{50} values of 5 and 9 μmol·L^{-1}, respectively) [17], or β-casein f(133–138), LHLPLP (IC_{50} value of 5.5 μmol·L^{-1}) [21]. Interestingly, of these two potent ACE-inhibitors, α_{s2}-casein f(203–208), PYVRYL, showed an ORAC-FL value of 1.82 μmol Trolox equivalents per μmol of peptide. Compared with other antioxidant peptides of food origin measured by the ORAC method, this peptide has a similar antioxidant capacity to other potent antioxidant peptides found in egg-white hydrolysates and β-Lg hydrolysates [5, 7]. The antioxidant activity of this peptide is also comparable with other well-known antioxidants such as butylated hydroxyanisole (2.43 μmol Trolox per μmol), which is currently used in the food industry as a synthetic antioxidant [4]. The antioxidant activity of this peptide can be exclusively attributed to the two Tyr residues of the molecule, since the other residues of this peptide (Arg, Val, Pro, Val and Leu) did not exhibit antioxidant activity by this method (ORAC-FL value < 0.00001 μmol Trolox per μmol of amino acid). Therefore, it has to be

highlighted that this peptide possesses a dual biological activity, antioxidant and ACE-inhibitory, together with a moderate antimicrobial activity [12], and thus can be considered a multifunctional peptide. Antioxidant and ACE-inhibitory activity are both implicated in the occurrence of hypertension. Several peptides derived from food proteins, mainly from egg and milk proteins, have been demonstrated to have simultaneously antioxidant and ACE-inhibitory activity, and it has been postulated that these two activities can contribute to their antihypertensive effect in vivo [10]. Further research with spontaneously hypertensive rats is already in progress to evaluate the in vivo antihypertensive activity of these potent ACE-inhibitory and antioxidant peptides.

4. CONCLUSION

In conclusion, this report demonstrates the preparation of an ovine α_{s2}- and bovine κ-casein hydrolysate with antibacterial, ACE-inhibitory and antioxidant activities. In addition, the presence of multifunctional peptides in these hydrolysates was also demonstrated. Of special interest are the fragments f(165–170), LKKISQ, and f(203–208), PYVRYL, from ovine α_{s2}-casein, both of them with a potent ACE-inhibitory activity comparable with that of the most potent ACE-inhibitory peptides derived from milk proteins described to date. Furthermore, the fragment f(203–208) with the sequence PYVRYL was revealed to be a multifunctional peptide as it possessed antibacterial and antioxidant capacity in addition to ACE-inhibitory activity. Further work using animal models will be needed to conclude if any of these promising peptides can also exert in vivo antihypertensive activity.

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