Angiotensin-I-converting enzyme inhibitory peptides from tryptic hydrolysate of bovine α_{S2}-casein

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Abstract Angiotensin-I-converting enzyme (ACE) inhibitory activity of a tryptic digest of bovine α_{S2}-casein (α_{S2}-CN) was extensively investigated. Forty-three peptide peaks were isolated and tested. Seven casokinins (i.e. CN-derived ACE inhibitory peptides) were identified and their IC_{50} values were determined. Four peptides exhibited an IC_{50} value lower than 20 μM. Peptides α_{S2}-CN (f174–181) and α_{S2}-CN (f174–179) had IC_{50} values of 4 μM. Surprisingly, deletion of the C-terminal dipeptide of two of these casokinins did not significantly alter their inhibitory activity.

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Key words: Bovine α_{S2}-casein; Angiotensin-I-converting enzyme inhibitor; Tryptic hydrolysate; Bioactive peptide; Casokinin

1. Introduction

Angiotensin-I-converting enzyme (ACE, EC 3.4.15.1) has a key role in blood pressure regulation. It principally catalyses the conversion of angiotensin I into angiotensin II, a powerful endogenous vasoconstrictor; ACE also degrades the vasodilator bradykinin. ACE is a Zn^{2+} metallopeptidase also called peptidyl dipeptidase A because of its main specificity [1]. This enzyme anchored in the cellular membrane can appear under two isoforms: a somatic form (1277 amino acids for the mature human ACE) found in endothelial, epithelial and neuronal cells and a smaller testicular form. The somatic form contains an amino and a carboxyl domain that exhibit 60% homology, suggesting a gene duplication; these two domains have their own specific catalytic site, independently working.

Inhibitors of ACE (captopril, benazepril, enalapril, lisinopril...) are used in therapy against hypertension. ACE inhibitors reduce short-term and long-term morbidity and mortality of hypertensive patients suffering from diabetes, renal insufficiency or heart failure thanks to their renoprotective and vasculoprotective effects [2,3].

During the two last decades an increasing number of data have evidenced that milk has more functions than only energetic and nutritional ones. Some peptides released from milk proteins by gastric and/or pancreatic enzymes might act as physiological regulators [4,5]. Peptides identified in milk protein hydrolysates have putative opioid, mineral carrier, antimicrobial, antithrombotic, immunomodulator, anxiolytic or antihypertensive bioactivity [6–8]. Many ACE inhibitors have been isolated in enzymatic digest from food proteins [9] and especially from milk proteins [9–12]. For some of them, an antihypertensive activity has been characterised in vivo by measuring the decrease of systolic blood pressure in humans or in spontaneously hypertensive rats [13].

Digestive enzymes are often used to produce functional or bioactive peptides. Trypsin, which is quantitatively present in human neonates as well as in adults, appears to be appropriate to search ACE inhibitory peptides as no particular amino acid sequence seems to be required for this activity and as such peptides have already been obtained with this enzyme [14–17]. Numerous bioactive peptides have been identified in bovine milk hydrolysates, especially in α_{S1}-, β-, κ-caseins (CNs), while α_{S2}-CN was less studied because of its poor solubility and because of purification difficulties. α_{S2}-CN accounts for about 10% of the bovine CNs; variant A is widespread in *Bos taurus* [18]. To date, antimicrobial [19–20] and ACE inhibitory [21] peptides have been identified in bovine α_{S2}-CN. The latter peptides weakly inhibit ACE: α_{S2}-CN (f189–193), α_{S2}-CN (f189–197), α_{S2}-CN (f190–197) and α_{S2}-CN (f198–202) have 580, 600, 300 and 400 μM IC_{50} values respectively. They have not shown significant activity during in vivo experiments [21].

The present work was undertaken to identify and characterise, in an extensive way, the tryptic peptides from bovine α_{S2}-CN that efficiently inhibited ACE.

2. Materials and methods

2.1. Enzymes and other reagents

L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK) treated insoluble trypsin from bovine pancreas (E.C. 3.4.21.4) attached to beaded agarose and angiotensin converting enzyme from rabbit lung tissue (EC 3.4.15.1) were obtained from Sigma, (St. Louis, MO, USA). Hippuryl-His-Leu-OH (HHL) was obtained from Bachem (Bubendorf, Switzerland). Captopril was from Sigma (St. Louis, MO, USA). Peptides were synthesised by Neosystem (Strasbourg, France). Others reagents were of analytical grade.

2.2. Purification of bovine α_{S2}-CN

Bovine α_{S2}-CN variant A was purified by a cationic exchange chromatography followed by a hydrophobic interaction chromatography as previously described [22].

2.3. Tryptic hydrolysis

Bovine α_{S2}-CN (0.5 mg ml^{-1}) was incubated for 24 h at 37°C with immobilised trypsin (0.1 *N*α-benzoyl-L-arginine ethyl ester unit ml^{-1}) in 67 mM sodium phosphate buffer, pH 8.1. The reaction was stopped by centrifugation (2500×*g*, 6 min, 4°C).

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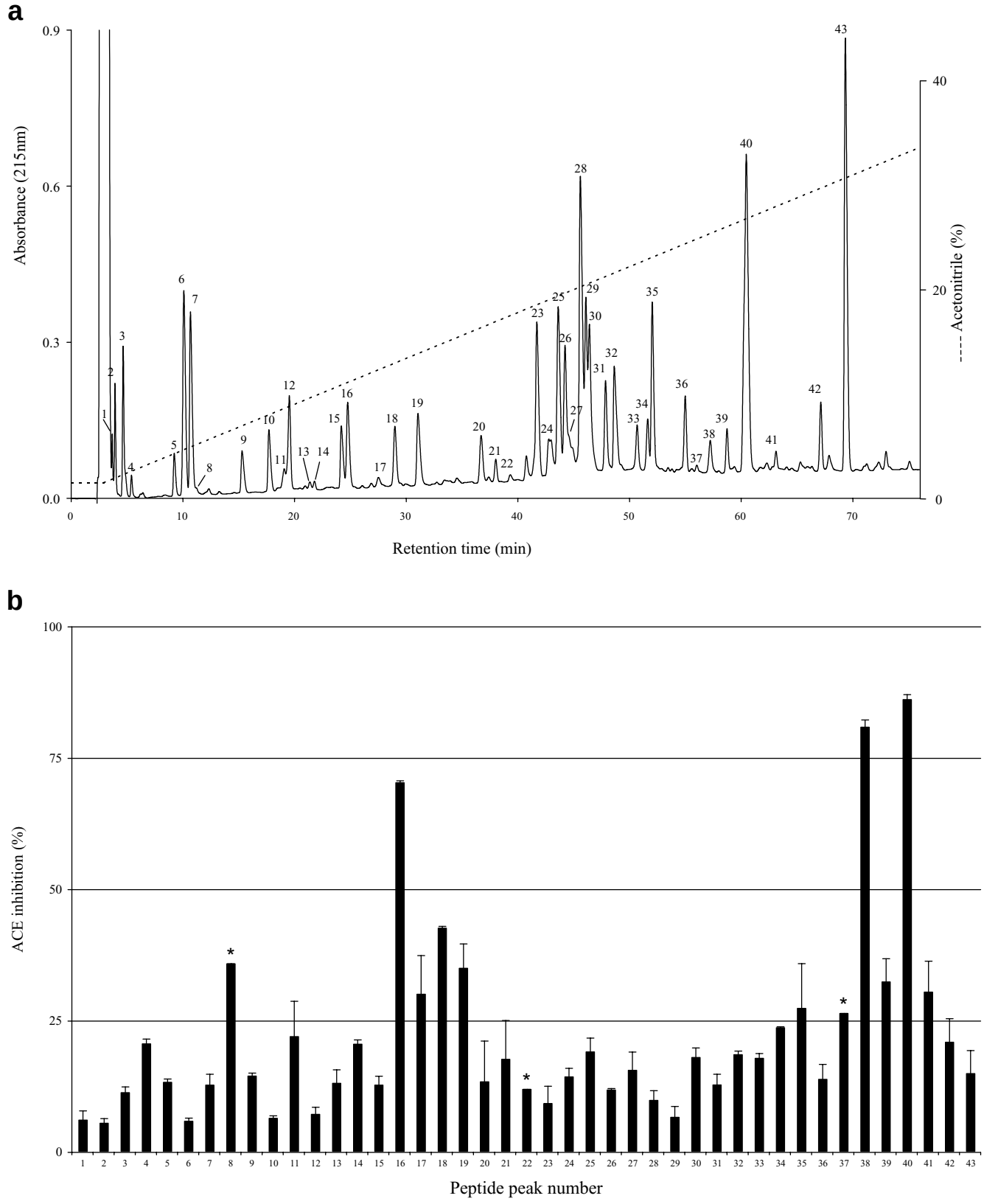

Fig. 1. RP-HPLC profile (a) and ACE inhibitory activity (b) of tryptic hydrolysate from bovine α_{S2}-CN. Separation was performed on a C18 column at 37°C. A quantity of 250 μg of sample (0.5 mg/ml) were injected. Flow rate was 1 ml/min. Numbers refer to peptide peaks tested. Remaining ACE activity was determined in duplicate (except *, only once) after a 60 min incubation at 37°C in a 50 mM CHES buffer, pH 8.3, containing 5 mM HHL, 350 mM NaCl, 3.33 U/l ACE and 50 μM in primary amines of potential inhibitor. Released hippuric acid was quantified at 228 nm after separation by RP-HPLC onto C18 column. Inhibition percentage was calculated by comparison with a control without peptide peak.

2.4. Peptides purification

Separation of the peptides from the tryptic digest of bovine α_{S2}-CN was performed by RP-HPLC onto a XTerra™ (Waters, Milford, MA, USA) C18 column (4.6 mm i.d.×250 mm) kept at 37°C and connected to a Waters HPLC model Alliance 2690. Samples (0.5 mg ml^{-1}) were filtered through 0.45 μm PVDF filters (Gelman Laboratory, Ann Harbor, MI, USA) and 250 μg were injected. After a 3 min isocratic elution (1.6% acetonitrile in 0.1% TFA), a linear gradient from 1.6% to 40% acetonitrile in 0.1% TFA was applied in 87 min at a flow rate of 1 ml min^{-1}. Detection was performed between 210 and 300 nm with a Waters 996 photodiode array detector. Peptides were collected and lyophilised.

2.5. Peptides quantification

Peptides were quantified with the OPA (*o*-phthaldialdehyde) method [23] adapted by Frister et al. [24] with some modifications: measurements were taken after a 3 min reaction time at room temperature and lyophilised peptides and leucine standards were solubilised in 7.5% (v/v) ethanol.

2.6. Peptide identification

Peptides from the tryptic digest have been identified by amino acid composition and mass spectrometry [22]. Sequence of the peptides selected for IC_{50} determination was checked again by Edman microsequencing with a model 476A microsequencer (Applied Biosystems, Foster City, CA, USA) before their chemical synthesis.

2.7. Determination of ACE inhibition

The method used was adapted from Cushman and Cheung [25]: ethyl-acetate extraction of hippuric acid was replaced by its RP-HPLC separation. A quantity of 140 μl of 50 mM CHES buffer, pH 8.3, containing 5 mM HHL, 350 mM NaCl and appropriate concentrations of α_{S2}-CN peptide dissolved in 5% (v/v) EtOH was preincubated 10 min at 37°C. 10 μl of ACE (3.33 U l^{-1}) was added and the mixture was incubated 60 min at 37°C. A control was realised without α_{S2}-CN peptide. The reaction was stopped with captopril, trisodic EDTA and trifluoroacetic acid at 5 μM, 1 mM and 0.067% (v/v) final concentrations respectively. Released hippuric acid was quantified by RP-HPLC onto a Symmetry® (Waters, Milford, MA, USA) C18 column (2.1 mm i.d.×150 mm) kept at 37°C. The column was connected to a Waters HPLC model Alliance 2690. Samples were filtered through 0.45 μm PVDF filters and 40 μl were injected. A linear gradient from 13 to 50% acetonitrile in 0.1% TFA was applied in 7 min, then 99% acetonitrile in 0.1% TFA was reached in 0.5 min; this proportion was maintained during 1.5 min. Flow rate was 0.25 ml min^{-1}. Detection was performed at 228 nm with a Waters 996 photodiode array detector.

ACE inhibitory activity of each chromatographic peptide peak from the hydrolysate was tested twice at 50 μM primary amines to discriminate the most efficient peptides.

IC_{50} values were determined with synthetic peptides. Peptides were first tested in duplicate between 0.1 μM and 250–500 μM to approximate their IC_{50} value, and then tested in triplicate at an optimised range of concentrations.

3. Results and discussion

The entire hydrolysate was first separated in 10 HPLC fractions, which were tested for ACE inhibitory activity. As seven of the 10 fractions presented a 20–80% ACE inhibition at 100 μM primary amines (data not shown), an extensive study was performed on about 40 collected HPLC peptide peaks (Fig. 1a) that were tested at 50 μM primary amines. Results of ACE inhibition are presented in Fig. 1b. Three peaks (16, 38 and 40) produced an ACE inhibition higher than 70%. Eight other peaks (8, 17, 18, 19, 35, 37, 39, 41) led to more than 25% inhibition. The sequence of peptides α_{S2}-CN (f182–184) (peak 16), α_{S2}-CN (f25–32) (peak 18), α_{S2}-CN (f81–91) (peak 35), α_{S2}-CN (f174–179) (peak 38), α_{S2}-CN (f81–89) (peak 39), α_{S2}-CN (f174–181) and α_{S2}-CN (f92–98) (peak 40) was validated by Edman degradation. IC_{50} values were determined with corresponding purified synthetic peptides. C-terminal α_{S2}-CN (f206–207) (peak 19) was not synthesised as it also corresponds to β-Lg (f102–103) which has an IC_{50} value of 122 μM [26].

The methodology used was validated with known inhibitors, captopril, α_{S1}-CN (f23–34) and α_{S1}-CN (f194–199) (Table 1). The IC_{50} values of the synthetic peptides, determined after logarithmic linearisation (Fig. 2), were in agreement with the percentage of inhibition obtained at 50 μM primary amines with natural peptides (Table 1). These peptides were more active than those described by Maeno et al. [21], which have IC_{50} values higher than 300 μM. Moreover, two of these peptides had an IC_{50} value lower than 5 μM, namely α_{S2}-CN (f174–181) and α_{S2}-CN (f174–179), and two others had an IC_{50} value of about 15 μM, namely α_{S2}-CN (f92–98) and α_{S2}-CN (f182–184). These four peptides are among the more active peptides obtained by an in vitro enzymatic hydrolysis of milk proteins since their IC_{50} value is lower than 20 μM [12].

Four of the eight active α_{S2}-CN peptides (α_{S2}-CN (f81–89), α_{S2}-CN (f92–98), α_{S2}-CN (f174–179) and α_{S2}-CN (f182–184)) resulted from a chymotryptic-like cleavage. Chymotryptic residual activity has often been reported during tryptic hydrolysis [27]. Peptides with an aromatic residue at C-terminal position would be better inhibitors of ACE than those with a basic one [28]. Nevertheless, numerous peptides resulting from specific tryptic activity have been identified and total chymotryptic hydrolysates do not systematically appear to be more active than tryptic ones [29]. Guanidino or ε-amino

Table 1
ACE inhibitory activity of bovine α_{S2}-CN-derived peptides

Number[a]	Inhibitor	Inhibition (%)[b]	Amino acid sequence	IC_{50} (μM)	Bibliographic IC_{50} (μM)
	Captopril	nd		0.022	0.023 [25], 0.018 [47], 0.007 [48]
	α_{S1}-CN (f23–34)	nd	FFVAPFPEVFGK	18	77 [49]
	α_{S1}-CN(f194–199)	nd	TTMPLW	12	16 [50]
18	α_{S2}-CN (f25–32)	42.5	NMAINPSK	60	
39	α_{S2}-CN (f 81–89)	32.2	ALNEINQFY	219	
35	α_{S2}-CN (f 81–91)	27.2	ALNEINQFYQK	264	
40	α_{S2}-CN (f 92–98)	86.0[c]	FPQYLQY	14	
38	α_{S2}-CN (f 174–179)	82.7	FALPQY	4.3	
40	α_{S2}-CN (f 174–181)	86.0[c]	FALPQYLK	4.3	
16	α_{S2}-CN (f 182–184)	70.2	TVY	15	
19	α_{S2}-CN (f 206–207)	34.8	YL	nd	122 [26]

nd, not determined.
[a]HPLC peak number in Fig. 1a.
[b]Determined with [primary amines] = 50 μM.
[c]Inhibition due to the mixture of α_{S2}-CN (f 92–98) and α_{S2}-CN (f 174–181) in the HPLC peak number 40.

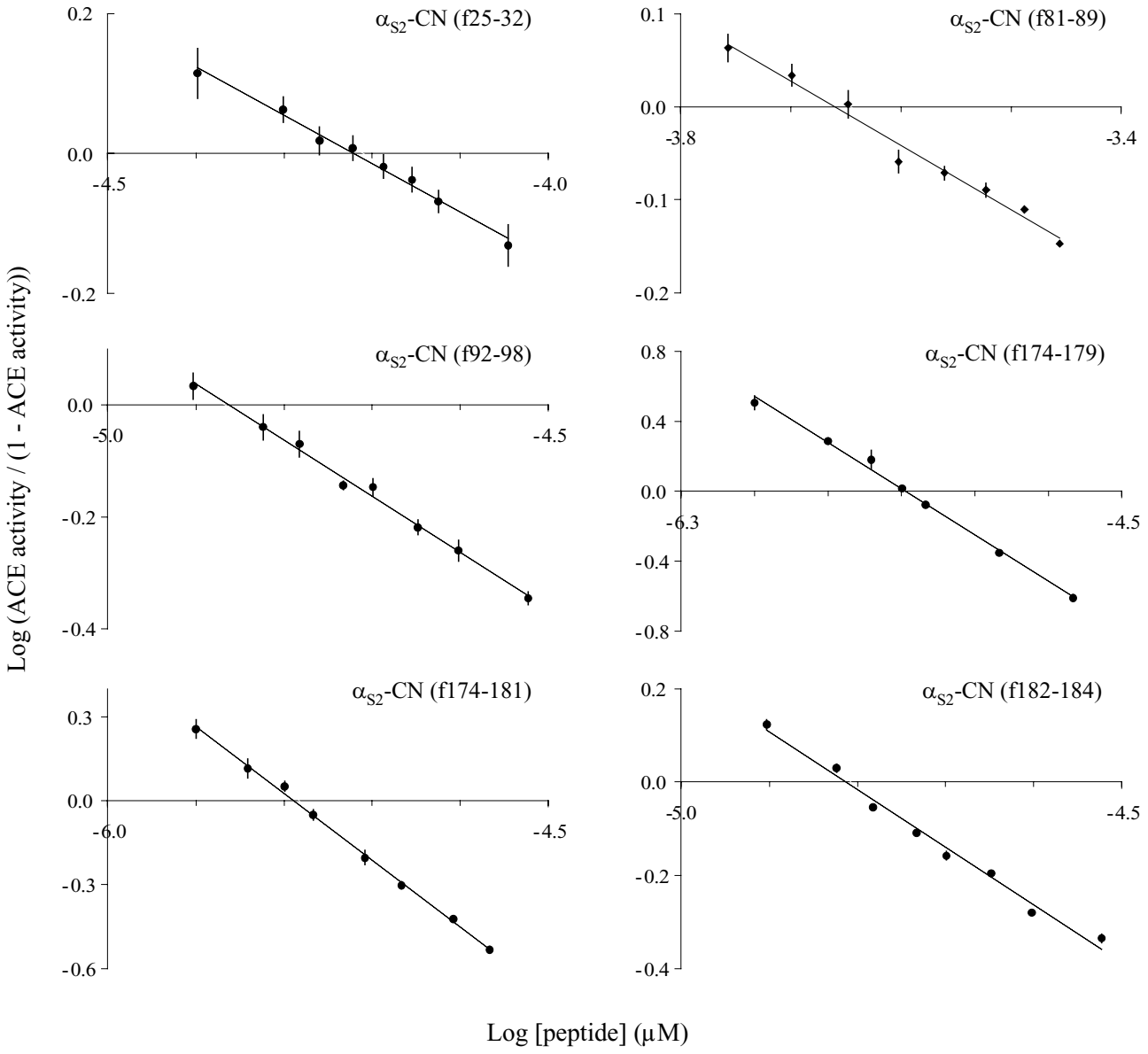

Fig. 2. Determination of the IC_{50} value of the more active ACE inhibitors obtained after tryptic hydrolysis of bovine α_{S2}-CN. IC_{50} values were obtained after logarithmic linearisation. Each point represents the mean value ± S.E.M. of three independent experiments.

group of C-terminal basic residue might interact with an anionic binding site distinct from the catalytic site of ACE [12].

ACE would be selective with regard to the COOH-terminal dipeptide sequence of its substrates and inhibitors, with aliphatic (V, I, A), basic (R) and aromatic (Y, F) residues being preferred in penultimate position and aromatic (W, Y, F), proline and aliphatic (I, A, L, M) residues being preferred in ultimate position [28]. Surprisingly, α_{S2}-CN (f174–181) and α_{S2}-CN (f174–179) on the one hand and α_{S2}-CN (f81–91) and α_{S2}-CN (f81–89) on the other hand had similar IC_{50} values (Table 1) despite different C-terminal dipeptides. Here, the change of the C-terminal dipeptide did not seem to have a drastic influence on the inhibition capacity. Evidence of the sequential removing of dipeptides from peptide substrate by ACE has been shown with bradykinin where two successive C-terminal dipeptides are released [30], suggesting that this phenomenon might also happen with inhibitors. The possible degradation of α_{S2}-CN (f174–181) into α_{S2}-CN (f174–179) by ACE was assayed. Only 2% of the initial quantity of α_{S2}-CN (f174–181) was hydrolysed in presence or absence of HHL (Fig. 3). Then, the inhibitory activity of α_{S2}-CN (f174–181) could not be ascribed to the release of peptide α_{S2}-CN (f174–179). This type of degradation only concerned 1 or 3% of α_{S2}-CN (f174–179) in presence or absence of HHL respectively while it reached 10 or 34% respectively for α_{S2}-CN (f92–98) despite these two peptides ended with the same C-terminal dipeptide, QY. Residues from the N-terminal side of the cleaved bond could interact with critical subsites of ACE. The weak cleavage of α_{S2}-CN (f174–179) is in agreement with the fact that ACE is drastically slowed down by the presence of a proline residue in penultimate or antepenultimate position [31].

N-terminal extension can increase the inhibition potency as shown by following IC_{50} values: IYP (61 μM), LIYP (10 μM), PLIYP (4.4 μM) or LPLP (720 μM), HLPLP (41 μM),

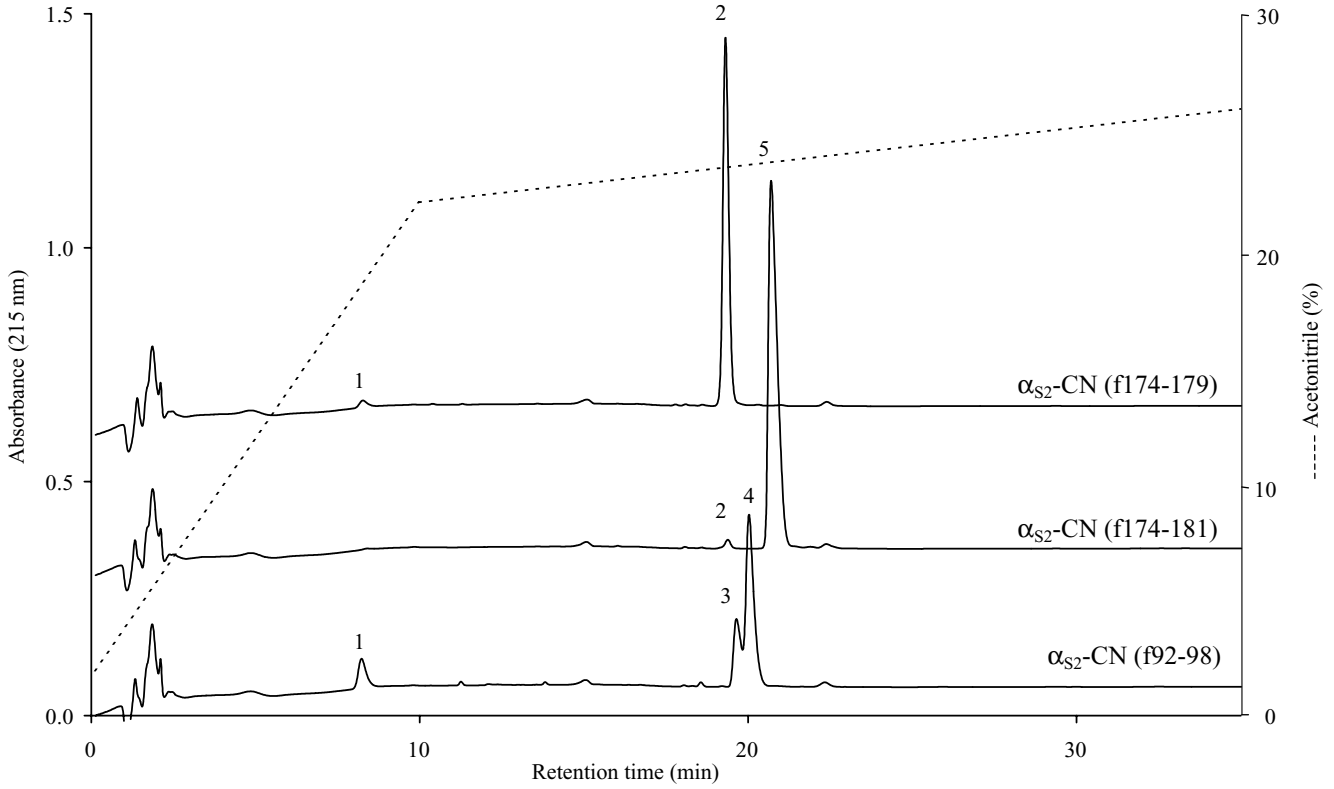

Fig. 3. Incubation of α_{S2}-CN peptides (100 μM) with ACE in assay conditions without HHL. Samples were analysed by RP-HPLC at 215 nm after separation on a Symmetry® C18 column at 37°C. 40 μl was injected. Flow rate was 0.25 ml min^{-1}. 1, QY (α_{S2}-CN (f96–98) or α_{S2}-CN (f177–179)); 2, FALPQY (α_{S2}-CN (f174–179)); 3, FPQYL (α_{S2}-CN (f92–96)); 4, FPQYLQY (α_{S2}-CN (f92–98)); 5, FALPQYLK (α_{S2}-CN (f174–181)).

LHLPLP (2.9 μM) [32]. ACE–inhibitor interactions may extend beyond the three C-terminal residues. The diversity of the inhibitors and the absence of known amino acid sequence recognised by ACE points out the complexity of the inhibitory mechanism. Several ACE inhibitors have a proline residue in C-terminal position [32], but this is not sufficient or essential to confer activity [21]. Proline residue at penultimate position is very unfavourable [28] whereas it is present at antepenultimate position in several inhibitors: YPER, AVPYPQR, LKPNM, ALPHA and here, NMAINPSK and FALPQY having IC_{50} values of 132, 15, 17, 10, 60, and 4.3 μM respectively [16,17,33].

Glutamine and tyrosine residues were present in C-terminal part of five from the seven studied peptides. QY and LQAr C-terminal motives (where Ar is an aromatic amino acid residue) have already been identified in ACE inhibitory peptides: α_{S1}-CN (f136–143) (YYPQIMQY, $IC_{50} = 24.8$ μM), α_{S1}-CN (f164–170) (NNVMLQW, $IC_{50} = 41.0$ μM) [17]. Moreover, VY contained in α_{S2}-CN (f182–184), has a 7 μM IC_{50} value [34].

Sour milks or milk protein hydrolysates have been reported to significantly decrease blood pressure in spontaneously hypertensive rats [35–42] and in hypertensive humans [43,44] after a single dose or several weeks of oral administration. Surprisingly, some of the in vivo very active peptides are weakly active in vitro (IC_{50} values comprised between 100 and 1000 μM [36]) and reciprocally. In vivo activation or loss of activity is perhaps due to further endogenous enzymatic cleavage. Interestingly, five of the seven inhibitors studied in the present work had a proline residue in various positions that could protect them from in vivo degradation. Proline is known to increase resistance to proteolysis [45,46].

Some of the ACE inhibitors identified here had IC_{50} values close to those of the most potent peptides found in milk proteins hydrolysates. Moreover, α_{S2}-CN (f174–181) and α_{S2}-CN (f174–179) were in vitro only 200 times less potent than captopril and were inhibitors for ACE but not substrate. These peptides and the entire tryptic hydrolysate need to be tested on spontaneously hypertensive rats to control if their activity is maintained in vivo.

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