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In Vitro Digestibility of α-Casozepine, a Benzodiazepine-like Peptide from Bovine Casein, and Biological Activity of Its Main Proteolytic Fragment

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ABSTRACT: α-Casozepine is a peptide, corresponding to the sequence 91−100 of the bovine α_{s1}-casein, displaying anxiolytic activity in the rat. The α_{s1}-casein tryptic hydrolysate containing this peptide decreases stress effects after oral administration in various species including man. Therefore, the stability of this peptide toward gastric and pancreatic proteases has been assessed by using pepsin, chymotrypsin/trypsin, Corolase PP, pepsin followed by chymotrypsin/trypsin or pepsin followed by Corolase PP. α-Casozepine was slowly degraded by chymotrypsin, much more sensitive to pepsin and Corolase PP but not completely destroyed after 4 h kinetics. The bonds in the region 91 to 95 of the α-casozepine were totally resistant to hydrolysis by all studied proteases. Surprisingly, a fragment, corresponding to the sequence 91−97 and found in all the hydrolysis media in significant amount, possessed an anxiolytic activity in three behavioral tests measuring this parameter. This peptide could participate in the *in vivo* activity of α-casozepine.

KEYWORDS: α-casozepine, simulated digestion, milk peptides, α_{s1}-casein, anxiety

INTRODUCTION

During the past three decades, it has been shown that food proteins, especially those from milk, are a potential source of biologically active peptides. These peptides are encrypted in the sequence of the parent protein and can display their activity only after release by proteolytic cleavage.[1] Such peptides can be generated from dietary proteins in three ways: (i) during digestion by gastrointestinal enzymes, (ii) during food processes by enzymes of microorganisms, (iii) by oriented *in vitro* proteolysis.[2] Numerous activities have been discovered, and the bioactive peptides may affect several body systems such as the cardiovascular, the nervous, the gastrointestinal or the immune system.[3]

Few years ago, it has been shown that a tryptic hydrolysate of bovine α_{s1}-casein displays a benzodiazepine (BDZ)-like activity. Injection of 3 mg/kg by the intraperitoneal (ip) route reduces the epileptic symptoms caused by pentylenetetrazole in rats. The same dose decreases anxiety in the elevated plus-maze (EPM) and in the conditioned defensive burying (CDB) paradigms.[4] The industrially manufactured product corresponding to the tryptic hydrolysate of bovine α_{s1}-casein has been investigated in animal and clinical studies. Oral administration of the α_{s1}-casein tryptic hydrolysate prevents stress-induced sleep disturbance in the rat.[5] In the cat, it displays positive effect in the management of anxious disorders such as social phobias[6] and it acts as selegiline in the dog.[7] This hydrolysate also exerts its biological activity in humans since the oral intake of 3 × 400 mg over two days of the encapsulated product before a stressing situation significantly decreases the blood pressure increase induced by the stress. Moreover, the plasmatic cortisol concentration decreases in the treated subjects compared to those who have taken a placebo.[8] In another study, the oral intake of the encapsulated α_{s1}-casein tryptic hydrolysate at a dose of 150 mg/day for 30 days in female volunteers significantly reduces their digestive, cardiovascular, intellectual, emotional and social stress-related symptoms.[9] Only one tryptic peptide of the hydrolysate displays the BDZ-like activity. It corresponds to the 91−100 fragment from bovine α_{s1}-casein and is named α-casozepine (α-CZP). This peptide competes *in vitro* with flunitrazepam for the BDZ site of the γ-aminobutyric acid type A receptor ($GABA_A$) with a half-maximal inhibitory concentration of 88 μM and displays a diazepam (DZP)-like profile in the CDB paradigm.[4] The conformation of α-CZP examined in a sodium dodecyl sulfate (SDS) micellar medium shows that residues 93 to 99 adopt an amphiphilic 3_{10}-helix structure initiated and terminated by an α turn. The helix structure is stabilized by ionic interactions between the guanidinium function of the arginine residue 100 and the carboxylate functions of this residue and of the glutamic residue 96. In the structure obtained in these specific experimental conditions, the two tyrosine aromatic cycles of α-CZP adopt a disposition similar to that of the aromatic rings in the crystal structure of nitrazepam, a BDZ.[10] So, the amino-terminal and carboxy-terminal parts of α-CZP might be crucial for its BDZ-like activity.

As the tryptic hydrolysate of bovine α_{s1}-casein displays a central biological activity after oral administration, it could mean that α-CZP has to reach its central target site after crossing of physiological barriers without being hydrolyzed by digestive processes. Some bioactive peptides display a resistance toward *in vitro* simulated digestion by pepsin and pancreatic extract. It has been shown that four angiotensin-I converting enzyme

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inhibitors isolated from cheese remain intact after this kind of proteolysis.[11] All these peptides contain a proline residue in carboxy-terminal and/or penultimate position, which might increase their resistance toward proteolysis. On the other hand, several results show that some bioactive peptides undergo degradation *in vivo*.[12] In these cases, three possibilities can be considered: (i) biological activity is lost or strongly decreased by proteolytic processes; (ii) biological activity is preserved or increased in proteolytic fragments; (iii) biological activity appears only after proteolytic processes of an inactive peptide. In light of results obtained in the literature, as for all identified bioactive peptides, the question arises about the minimal active sequence of α-CZP that may play a role *in vivo*.

The present study was designed to investigate the sensitivity of α-CZP toward digestion by (i) pepsin under gastric analogous conditions, (ii) trypsin, α-chymotrypsin and pancreatic extract Corolase PP under pancreatic analogous conditions, and (iii) combination of either pepsin and trypsin/α-chymotrypsin or pepsin and Corolase PP in order to simulate gastrointestinal digestion. The preferential cleavage bonds were determined from the identification of the resulting peptides by mass spectrometry. The anxiolytic effects of the main proteolytic fragment were investigated by using experimental paradigms of anxiety in rats. Among a large number of behavioral tests, which measure fearlike responses of various kinds (for review see ref 13), the validated models chosen for anxiety evaluation in rats were the CDB,[14] the EPM[15,16] and the light/dark box (LDB).[17]

MATERIALS AND METHODS

Peptides. α-Casozepine (α-CZP), f91−100 (^{91}YLGYLEQLLR^{100}) of the mature chain of bovine α_{s1}-casein (UniProtKB/Swiss-Prot P02662) and the heptapeptide corresponding to f91−97 were synthesized by PolyPeptide Group (Strasbourg, France). The contaminants and salts were removed by a purification step on Plus C_{18} Sep-Pak cartridges (Waters, Guyancourt, France) as follows: the cartridges were conditioned with 10 mL of water/acetonitrile (ACN) 10:90 (v/v) and equilibrated with 10 mL of water/ACN 90:10 (v/v) with a flow of about 1 mL/min. A volume of 2 mL of peptide solution at 2 mg/mL in water/ACN 80:20 (v/v) for α-CZP or 85:15 (v/v) for f91−97 containing 0.1% (v/v) trifluoroacetic acid (TFA) was loaded on the cartridge. The cartridges were then washed with 2 mL of previously exposed solvents according to the peptide. The peptide containing fraction was eluted with 3 mL of water/ACN 10:90 (v/v) and lyophilized. The purity of the peptide was checked by RP-HPLC on an analytical LichroCart C_{18} column (250 × 4 mm, 5 μm particule size, 10 nm porosity; Merck, Darmstadt, Germany) connected to the Alliance HPLC unit (Waters). Each purified sample was loaded onto the column. Solvent A contained 0.1% (v/v) TFA in water, and solvent B contained 0.1% (v/v) TFA in ACN. After an isocratic step with 5% of solvent B in solvent A for 5 min, a linear gradient from 5% to 40% of solvent B in solvent A was applied for 70 min with a flow rate of 1 mL/min at 30 °C. Detection was monitored with a photodiode array detector 996 (Waters) between 200 and 300 nm. The purity calculated for both peptides from the chromatogram obtained at 215 nm was higher than 99%.

***In Vitro* Digestion of α-Casozepine by Gastric or Pancreatic Proteases.** α-CZP was solubilized at 150 μg/mL in 10 mM HCl or in 10 mM acetate buffer for the peptic hydrolysis at pH 2.0 or at pH 4.0 respectively and in 100 mM sodium phosphate buffer, pH 8.0 for the hydrolysis with pure pancreatic proteases and Corolase PP. All the pH values were adjusted at 37 °C after peptide solubilization. For peptic hydrolysis, pepsin A (EC 3.4.23.1) from porcine gastric mucosa (P6887; Sigma, Saint-Quentin Fallavier, France) was added at an enzyme:substrate (E:S) ratio of 1:200 (w/w). For hydrolysis with pancreatic protease, either trypsin (EC 3.4.21.4; T1426; Sigma) or α-chymotrypsin (EC 3.4.21.1; C3142; Sigma) from bovine pancreas was added at an E:S ratio of 1:50 (w/w). Corolase PP (AB Enzymes GmbH, Darmstadt, Germany), an enzyme extract from porcine pancreas, was added at an E:S ratio of 1:100 (w/w). Phenylalanine was added in all the hydrolysis media at a final concentration of 36 μM as an internal standard. The solutions were stirred at 37 °C, and an aliquot of 200 μL was removed immediately and at 5, 15, 30, 60, 120, and 240 min. Each time, in the case of peptic hydrolysis, the reaction was stopped by addition of 200 μL of 100 mM sodium phosphate buffer, pH 8.3, which raised the pH to a final value of about 7.8. The pepsin from pig gastric mucosa displays no activity at pH 7.[18] In the case of hydrolysis with trypsin, α-chymotrypsin or Corolase PP, reaction was stopped by heating at 95 °C during 15 min. All the experiments were done in duplicate. The samples were stored at −20 °C until analysis.

***In Vitro* Digestion of α-Casozepine by Pepsin Followed by Trypsin/α-Chymotrypsin or Corolase PP.** In a first step, α-CZP was solubilized at 150 μg/mL in 10 mM HCl, pH 2.0 and pepsin A was added at an E:S ratio of 1:200 (w/w). The solution was stirred at 37 °C. After 120 min of hydrolysis, an aliquot of 200 μL was taken to assess hydrolysis by pepsin. The peptic reaction was stopped in the aliquot by adding 200 μL of sodium phosphate 100 mM buffer, pH 8.3 and in the reaction medium by addition of one volume of the same buffer containing trypsin and α-chymotrypsin at an E:S ratio of 1:50 (w/w) each. The hydrolysis medium contained also phenylalanine at a final concentration of 36 μM as an internal standard. Hydrolysis by pancreatic enzymes then continued for 240 min at 37 °C. An aliquot of 200 μL was removed immediately and at 5, 15, 30, 60, 120, and 240 min, and the reaction was stopped by heating the sample at 95 °C for 15 min. The same experiment was conducted by using Corolase PP at an E:S ratio of 1:100 (w/w) instead of trypsin and α-chymotrypsin. The experiments were done in duplicate, and the samples were stored at −20 °C until analysis.

RP-HPLC Analysis of Hydrolysis Products. Remaining α-CZP and its hydrolysis products were analyzed by RP-HPLC as described above. Before analysis, a proportion of 5% (v/v) of ACN was added in the aliquots collected at different times. A volume of 100 μL of each sample was loaded onto the C_{18} column. Peaks were detected between 200 and 300 nm. The peaks corresponding to aromatic peptides (peptides containing one or two residues of tyrosine) were integrated at 280 nm, and the area was multiplied by 2 in the case of peptides with only one residue of tyrosine. Following this, the areas were corrected according to the area at 257 nm of the peak corresponding to phenylalanine used as internal standard. The reference area of the peak corresponding to phenylalanine is taken at 0 min of hydrolysis for each type of hydrolysis. The percentages of residual α-CZP and of the aromatic amino-terminal peptides released from α-CZP were calculated by comparing the corrected area of their peak at 280 nm with that of the initial α-CZP (0 min of hydrolysis). Results were expressed as the mean ± standard deviation ($n = 2$). No determination of their proportion in the hydrolysis medium was done for the nonaromatic peptides.

RP-HPLC/ESI-MS Analysis of Hydrolysis Products. Analysis of hydrolysis products was carried out by RP-HPLC/ESI-MS on an API III$^+$ mass spectrometer (SCIEX, Thornhill, Ontario, Canada) as previously described[19] with some modifications. The RP-HPLC column was a narrow-bore symmetry C_{18} column (2.1 × 150 mm, 5 μm particle size, 10 nm porosity; Waters) connected to a C_{18} guard column. The elution gradient for the peptide separation was as follows: a linear gradient of solvent B in solvent A from 5% to 50% for 60 min and an isocratic step of 80% of solvent B during 2 min. Solvent A contained 0.106% (v/v) TFA in water and solvent B contained 0.1% (v/v) TFA and 80% (v/v) ACN in water. Modifications in ESI-MS analysis were as follows: orifice voltage was set between 60 and 90 V depending on

experiments; each scan was acquired over the range of m/z values from 180 to 1400 using a step size of 0.5 Da and a dwell time of 0.5 ms.

Animals for Behavioral Studies. All behavioral studies were done with six-week-old male outbred Wistar rats obtained from Charles River Laboratories (L'Arbresle, France). The animals were housed under conditions of controlled temperature (22 ± 1 °C), humidity (45 ± 5%) and artificial light (12 h light/dark cycle, lights on from 8:00 p.m. to 8:00 a.m.) with free access to fed food pellets (Global Diet 2018, Harlan, France) and water *ad libitum*. The experiments were conducted according to directive 86/609 of the council of the European Community regarding the protection of animals used for experimental purposes.

Conditioned Defensive Burying Experiment. The CDB test is an experimental paradigm designed to elicit anxiety-related behavior in rodents.[14] The experimentation was performed as previously described[20] with a modification in the shock intensity of 1 mA instead of 2 mA. The time spent to bury the probe after receiving the shock which characterizes anxiety-like behavior in this experimental situation was scored from videotapes. Behaviors were scored by a trained observer who was unaware of the experimental conditions. Thirty-two male Wistar rats weighing 180−250 g were randomly divided into four groups (8 rats per treatment). DZP (Valium, Roche, Neuilly-sur-Seine, France) was suspended, and the peptides, α-CZP and f91−97, were solubilized in 0.9% (w/v) NaCl solution. Rats were injected by the ip route at 1 mL/kg, 30 min before the test, with saline (control group), DZP (0.5 mg/kg; 1.76 μmol/kg; DZP group), α-CZP (0.7 mg/kg; 0.55 μmol/kg; α-CZP group) or f91−97 (0.5 mg/kg; 0.56 μmol/kg; f91−97 group).

Elevated Plus-Maze and Light/Dark Box Tests. To minimize the influences of possible circadian changes on behaviors, control and experimental animals were alternated for observation, being observed at the same time of day (between 9:00 a.m. and 11:00 a.m.). The experimental devices used were washed with an alcohol−water solution (5% v/v) before placement of the animal in order to avoid persistent smells left by the previous rat. The apparatuses were maintained in the same position in the room throughout the duration of the study. The animal's behavior was tape-recorded on a monitor through a video camera system, and the data was checked by an observer who was unaware of the experimental groups.

Locomotor Activity Measurements in Open Field. Since the EPM and LDB tests could be affected by changes in locomotor activity of the rodent,[21] an additional experiment in an open field (OF) was carried out with the specific aim of monitoring general motor activity[22]

The device, illuminated in red light not aversive for the animals, consists of a box (65 × 49 × 35 cm; $h \times L \times l$), divided length-wise into two units (65 × 49 × 17.5 cm) interconnected by three closed guillotine doors. One of these units is subdivided into three square units by two walls, each with a door (8 × 8 cm). Each rat was placed in the non-subdivided half of the box (65 × 49 × 17.5 cm). The floor of this compartment was covered with sawdust. Twenty-four hours after placement in the box, the rat was treated. The guillotine doors were opened one-half hour after treatment, and the rat was allowed to enter any unit of both the familiar and novel unfamiliar compartments (three square units) of the apparatus. Each rat was then observed during 10 min for its reactions to the novel unfamiliar environment. The number of familiar and unfamiliar units entered and the time spent in each compartment were recorded.

Elevated Plus-Maze Test. The EPM test was used to evaluate the animal anxiety.[15,16] This test creates an approach−avoidance conflict between the natural desire of the rodent to explore and his fear of open and high spaces of the EPM. The EPM consisted of a cross with two opposite open arms and two opposite enclosed arms. The four arms (45 × 10 cm; $L \times l$), positioned at 90° from each other, were 75 cm above the ground. Two opposite arms were delimited by wood vertical walls (40 cm high, closed arms) whereas the two other opposite arms had unprotected edges (open arms). The central square of the maze measured 10 × 10 cm. The open arms were illuminated with white light (500 lx). The EPM test was performed immediately after the end of the OF test (i.e., 40 min after treatment). Rats were placed individually facing an open arm in the central square of the maze, and their free exploration of the apparatus was tape-recorded for 5 min. The number of entries into both open and closed arms, which provides a measure of general activity, the time spent in exploring the open and closed arms of the EPM, and the latency time before entering into a closed arm were counted. The measures that reflect anxiety levels in this test are the percentage of entries into open arm versus closed arm and the percentage of time spent in open arm versus closed arm. An entry into an arm was defined as the animal placing all four paws beyond the imaginary line separating the entrance of one arm and the central area.

Light/Dark Box Test. To determine whether treatment was associated with a change in the level of anxiety, the rats were subjected to a procedure of choice in which animals could freely choose to approach or avoid a novelty that they have found attractive or aversive. In the LDB test, this novelty-seeking behavior was evaluated as described.[17] The device of the LDB test consisted of a 65 × 49 × 35 cm ($h \times L \times l$) rectangular box divided lengthwise by a wall into two equal-sized compartments. These two compartments communicated with each other by three guillotine doors (8 × 8 cm). The familiar dark compartment, covered with sawdust, was illuminated by a low intensity white light (250 lx); the other aversive unfamiliar compartment was illuminated by a 1500 lx white light. Each rat was placed in the familiar dark compartment of the box (65 × 49 × 17.5 cm). Twenty-four hours after placement for habituation, the rat was treated. The guillotine doors were opened one-half hour after treatment, and the animal was allowed to explore the familiar and the aversive unfamiliar compartments of the apparatus. Each rat was then observed during 10 min for its reactions to the novel aversive environment. The number of familiar and unfamiliar units entered and the time spent in each compartment were recorded.

Rat Treatments. Sixty rats were used for the OF and EPM experiments and forty-eight rats for the LDB experiment. Animals weighing 170−220 g were randomly divided into four groups (n = 15 for OF and EPM, n = 12 for LDB) for each experiment. DZP, α-CZP and f91−97 were suspended in an aqueous solution of 1% (v/v) glycerol and 0.2% (v/v) methylcellulose. Rats were injected ip at 2 mL/kg with saline (control group), DZP (1.0 mg/kg; 3.52 μmol/kg; DZP group), α-CZP (1.0 mg/kg; 0.79 μmol/kg; α-CZP group) or f91−97 (0.7 mg/kg; 0.79 μmol/kg; f91−97 group).

Statistical Analysis. Behavioral data were analyzed by using ANOVA. When significant effects occurred, individual *post hoc* comparisons were made by Dunnett's test for the LDB test and by Tukey's test for others behavioral tests. The statistical analysis was performed using the mixed procedure of SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Results were expressed as the mean ± SEM. Differences were considered statistically significant when $p < 0.05$ in all statistical tests.

RESULTS

Peptic Hydrolysis of α-Casozepine . Hydrolysis kinetics of α-CZP with pepsin at an E:S ratio of 1:200 (w/w) was monitored at pH 2.0 (Figure 1A) and pH 4.0 (data not shown) during 4 h. The disappearance of the peptide was not statistically different at pH 4.0 compared to pH 2.0. In our conditions, α-CZP was rapidly hydrolyzed. Indeed, half of the initial quantity disappeared in 25 min. Around 14% of initial α-CZP was still present after 60 min, and around 7% of this peptide was not hydrolyzed at the end of the experiment after 240 min. Hydrolysis of the peptide by pepsin released some peptides, and the major ones were identified. They corresponded to amino-terminal fragments

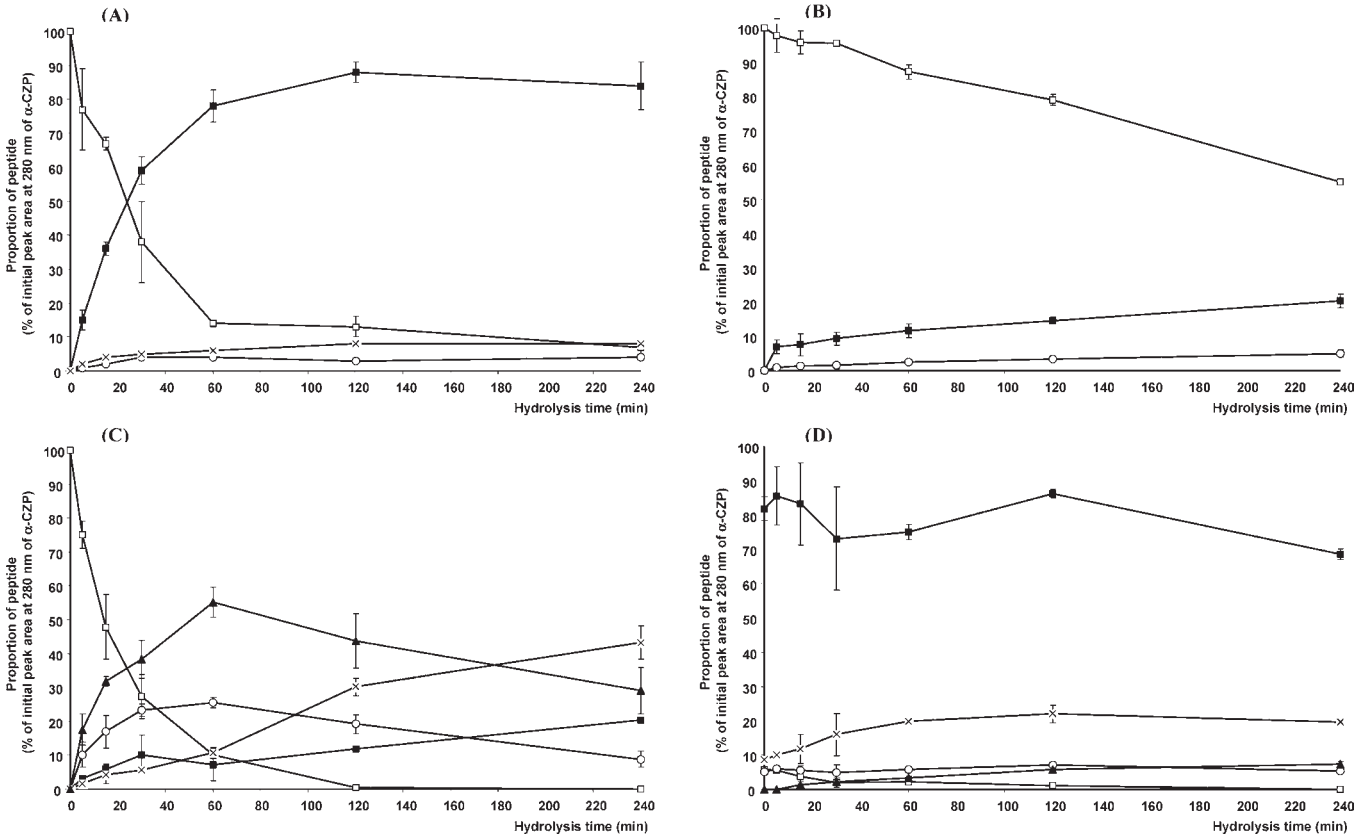

Figure 1. Kinetics of hydrolysis of α-CZP (150 μg/mL, empty square) and of the released peptides f91−95 (cross), f91−97 (filled square), f91−98 (circle) and f91−99 (triangle) at 37 °C with (A) pepsin at an E:S ratio of 1:200 (w/w) at pH 2.0, (B) α-chymotrypsin at an E:S ratio of 1:50 (w/w) at pH 8.0, (C) Corolase PP at an E:S ratio of 1:100 (w/w) at pH 8.0 or (D) pepsin at an E:S ratio of 1:200 (w/w) at pH 2.0 during 120 min followed by Corolase PP at E:S ratio of 1:100 (w/w) at pH 7.8. Data are expressed as mean ± SD (n = 2).

Table 1. Identification by ESI-MS of Peptides Generated *in Vitro* from α-Casozepine by Pepsin, Chymotrypsin, Corolase PP or Pepsin Followed by Corolase PP (Pep + Cor)

		M_r[b] (Da)			proportion of peptide[c] (%) generated at		
peptide fragment[a]	sequence	theor	found	enzymes	60 min	120 min	240 min
f98−100	^{98}LLR^{100}	400.3	400.6	pepsin	nd[d]	nd	nd
				chymotrypsin	nd	nd	nd
				Corolase PP	nd	nd	nd
f91−95	^{91}YLGYL^{95}	627.3	627.8	pepsin	6 ± 1	8 ± 1	8 ± 1
				Corolase PP	11 ± 2	30 ± 3	43 ± 5
				Pep + Cor	20 ± 1	22 ± 3	20 ± 1
f96−100	^{96}EQLLR^{100}	657.4	656.5	Corolase PP	nd	nd	nd
f91−97	^{91}YLGYLEQ^{97}	884.4	884.7	pepsin	78 ± 5	88 ± 3	84 ± 7
				chymotrypsin	12 ± 2	15 ± 1	20 ± 2
				Corolase PP	7 ± 5	12 ± 1	20 ± 1
				Pep + Cor	75 ± 3	86 ± 1	69 ± 2
f91−98	^{91}YLGYLEQL^{98}	997.5	997.7	pepsin	4 ± 1	3 ± 1	4 ± 1
				chymotrypsin	2 ± 1	3 ± 1	5 ± 1
				Corolase PP	26 ± 2	19 ± 3	9 ± 3
				Pep + Cor	6 ± 1	7 ± 1	5 ± 1
f91−99	^{91}YLGYLEQLL^{99}	1110.6	1110.8	Corolase PP	55 ± 4	44 ± 8	29 ± 7
				Pep + Cor	3 ± 1	6 ± 1	7 ± 1

[a] Peptide fragment corresponding to the sequence of bovine α_{s1}-casein mature chain. [b] Monoisotopic mass. [c] % of initial peak area at 280 nm of α-casozepine. [d] Not detectable at 280 nm.

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f91−98, f91−97 and f91−95 of α-CZP (Table 1). The peptide f91−97 was the main breakdown product whatever the time of hydrolysis at pH 2.0 (Figure 1A). The same phenomenon was observed at pH 4.0 (data not shown). The proportion, among the aromatic peptides, of f91−97 reached a maximum of about 88 ± 3% of the initial quantity of α-CZP at 120 min, whereas the proportion of f91−95 and f91−98 reached a maximum of around 8 ± 1% and 4 ± 1% of the initial amount of α-CZP at 120 and 30 min, respectively (Figure 1A). Moreover, these amino-terminal fragments were not further hydrolyzed by pepsin, as the maximal proportion achieved was stable until the end of the peptic hydrolysis.

Hydrolysis of α-Casozepine by Pancreatic Proteases. The sensibility of α-CZP toward the pancreatic proteases, trypsin or α-chymotrypsin at an E:S ratio of 1:50 (w/w), and the pancreatic protease preparation, Corolase PP at an E:S ratio of 1:100 (w/w), was assessed. There was no significant hydrolysis of α-CZP by trypsin over a period of 240 min (data not shown).

On the other side, α-CZP was slowly hydrolyzed by α-chymotrypsin. Therefore 55 ± 1% of the initial amount of α-CZP still remains after 240 min of hydrolysis (Figure 1B). Three peptides were identified in the chymotryptic media of hydrolysis: the amino-terminal fragments f91−97 and f91−98 and the carboxy-terminal fragment f98−100, this one was also found in the peptic hydrolysates (Table 1). The proportion, among the aromatic peptides, of f91−97 reached 7 ± 2% of the initial amount of α-CZP in the first 5 min of hydrolysis, and this proportion was about 20 ± 2% of the initial amount of α-CZP after 240 min (Figure 1B). The peptide f91−98 displayed the same release kinetics as f91−97, but its proportion reached only 5 ± 1% at the end of the proteolytic reaction (Figure 1B).

Corolase PP rapidly degraded α-CZP into smaller fragments. Even if α-CZP was completely hydrolyzed at 120 min, about 50% of the peptide was still intact after 20 min of hydrolysis (Figure 1C). Six hydrolysis products were identified by RP-HPLC/ESI-MS analysis: the amino-terminal fragments f91−95, f91−97, f91−98 and f91−99, and the carboxy-terminal fragments f96−100 and f98−100 (Table 1). The fragment f99−100 and the arginine that were expected to be released by the formation of f91−98 and f91−99 respectively were not detected in our experimental conditions. The maximal proportion, among the aromatic peptides, of f91−99 and f91−98 was reached at 60 min with about 55 ± 4% and 26 ± 2% of the initial quantity of α-CZP respectively. These fragments were further hydrolyzed by the endopeptidasic and/or exopeptidasic activity of the Corolase PP leading to 29 ± 7% of the initial amount of α-CZP for f91−99 and 9 ± 3% for f91−98 after a time of hydrolysis of 240 min (Figure 1C). On the contrary, fragments f91−95 and f91−97 (Figure 1C) did not display the same kinetics since their proportion increased over the hydrolysis reaction. The proportions of f91−95 and f91−97 were about 43 ± 5% and 20 ± 1% respectively of the initial quantity of α-CZP at 240 min.

Hydrolysis of α-Casozepine by Pepsin Followed by Trypsin/α-Chymotrypsin or Corolase PP. α-CZP was subjected to a hydrolysis process in two steps that simulated the physiological sequence of proteases. The peptic hydrolysis of α-CZP at pH 2.0 led to an amount of about 10% of the initial quantity of the peptide after 120 min as it was shown in our previous experiment. The main released fragments were once again f91−97, f91−95 and f91−98. These proportions of α-CZP and its hydrolysis products did not change after hydrolysis by trypsin or chymotrysin during 240 min (data not shown).

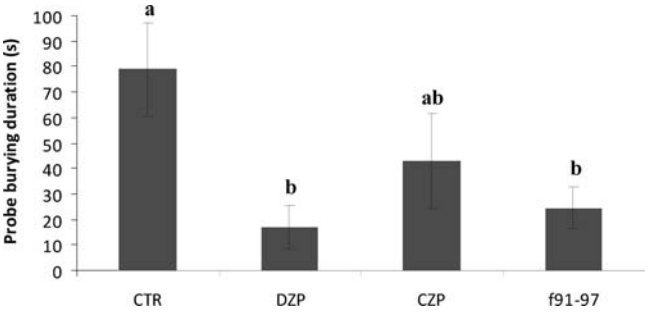

Figure 2. Effect of diazepam (DZP; 0.5 mg/kg), α-casozepine (CZP; 0.7 mg/kg) and f91−97 (0.5 mg/kg) on the duration of burying of the probe in the conditioned defensive burying test in rats. The treatments were administered by the ip route 30 min before the experiment. After receiving the electric shock, the behavior of animals was evaluated during 5 min. Data are expressed as mean ± SEM (n = 8 in each group). Two points with different letter are significantly different ($p < 0.05$).

On the other side, Corolase PP hydrolyzed totally the α-CZP not degraded by pepsin with a kinetics similar to that observed in the previous experiment conducted with only Corolase PP. Once again, two-thirds of the α-CZP was hydrolyzed in 30 min (Figure 1D). The proportions, among the aromatic peptides, of f91−97 and f91−98 were unchanged during hydrolysis by Corolase PP—with a proportion of f91−97 corresponding to about 70% of the initial quantity of α-CZP—while the proportion of f91−95 increased from 9 ± 1% to 20 ± 1% (Figure 1D). The f91−99, that was not found after peptic hydrolysis, was formed by Corolase PP by a carboxypeptidase-like activity, and its proportion reached 7 ± 1% of the initial quantity of α-CZP after 240 min (Table 1).

Pre-Evaluation of the Anxiolytic Effect of f91−97 in Conditioned Defensive Burying Test. The first test used to evaluate the anxiolytic activity of f91−97 was the CDB paradigm. The duration of the burying of the probe after receiving the shock was recorded (Figure 2). The duration of the burying of the probe differed significantly between the groups of animals treated with DZP or f91−97 and the group of animals treated with saline (at threshold of $p < 0.05$). Indeed, the animals treated with saline buried the probe for a longer time (78.9 ± 18.3 s) than the animals treated with DZP (16.8 ± 8.6 s) or f91−97 (24.4 ± 8.3 s). The decrease in burying duration observed with α-CZP-treated rats (43.0 ± 18.6 s) compared to saline group was surprisingly not significant. This preliminary result obtained with 8 rats per treatment group had to be reinforced with a larger number of animals.

Fragment f91−97 Exhibited Anxiolytic Activity in the Elevated Plus-Maze and in the Light/Dark Box. The locomotor activity of rats treated with DZP, α-CZP or f91−97 was compared to that of rats treated with vehicle in an OF with familiar and unfamiliar areas. Results expressed as percentages of entries (Figure 3A) and of time spent (Figure 3B) in each compartment showed no significant difference in the behavior of rats whatever the treatments (vehicle, DZP, α-CZP and f91−97). Then, no treatment affected significantly the animal locomotor activity.

Fragment f91−97 Exhibited Anxiolytic Activity in the Elevated Plus-Maze. The measured parameters in the EPM (Table 2) were (i) the total number of arm entries (both open and closed arms), (ii) the latency before entering into closed arms, (iii) the calculated percentage of open arm entries

versus total number of arm entries, (iv) the calculated percentage of time spent in the open arms versus total time spent in arms.

As shown in Table 2, the total number of entries in both open and closed arms of the EPM was not significantly different for animals treated with DZP, α-CZP or f91−97 compared to the control group (at $p < 0.05$). Moreover, no significant difference was observed between the treatment groups. Significant increase was noticed in the percentage of entries into the open arms between the DZP, α-CZP, f91−97-treated rat groups and the control animals (31.6 ± 4.2%). No significant difference was observed between the DZP, α-CZP and f91−97 treatments for this parameter (45.2 ± 3.8%; 46.0 ± 4.0%; 45.0 ± 5.6%, respectively).

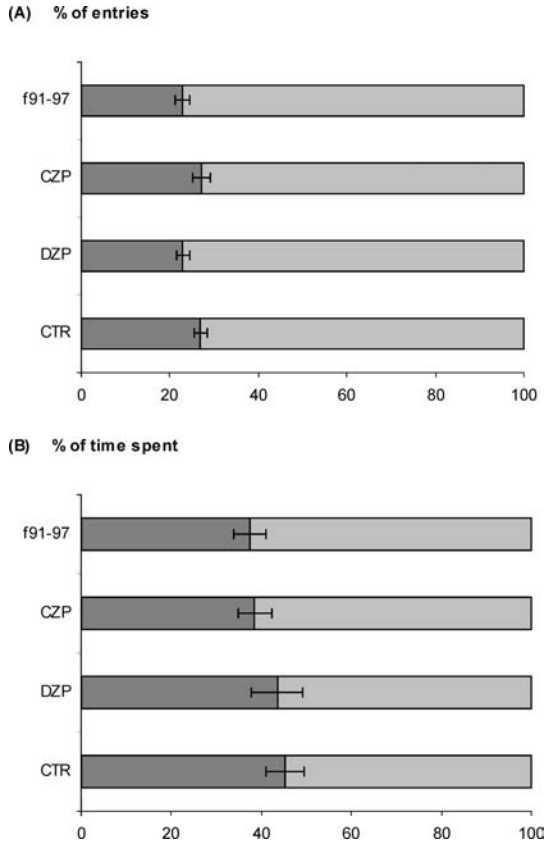

Figure 3. Locomotor activity of rats treated by diazepam (DZP; 1.0 mg/kg), α-casozepine (CZP; 1.0 mg/kg) and f91−97 (0.7 mg/kg) in an open field. (A) Percentage of entries and (B) percentage of time spent in familiar (dark gray bar) and unfamiliar (light gray bar) compartment. Thirty minutes after ip treatment, the animals were observed in the open field during 10 min. Data are expressed as mean ± SEM ($n = 15$ in each group).

Furthermore, the animals treated with one of the peptides, α-CZP or f91−97, spent a percentage of time in the open arms (47.3 ± 5.9% and 45.9 ± 5.9%, respectively) identical to that of the animals treated with DZP (54.9 ± 4.1%) and spent significantly more time in this type of arm compared to the vehicle group (20.2 ± 2.9% for vehicle; $p < 0.05$).

Moreover, once the animal was placed facing an open arm, the latency before entering into a closed arm was also measured (Table 2). Only rats treated with DZP entered after a significantly longer time in a closed arm (7.5 ± 2.2% for vehicle-treated animals vs 72.8 ± 17.0%, 27.6 ± 5.0% and 22.8 ± 2.9% for DZP, α-CZP and f91−97-treated groups, respectively).

Fragment f91−97 Exhibited Anxiolytic Activity in Light/Dark Box. The possible anxiolytic effect of f91−97 fragment was also investigated by recording the behavior of animals in LDB. Animals were monitored for (i) the total number of entries into both lighted and dark compartments, (ii) the latency before entering into the lighted compartment, (iii) the percentage of entries into the lighted compartment, (iv) the percentage of time spent in lighted compartment and (v) the number of rears in lit area (Table 3).

The percentage of entries and the time spent in lighted compartment were significantly higher in the case of DZP, α-CZP and f91−97 treated rats compared to the control group (Table 3). Moreover, the number of rears in lit area was significantly greater for animals treated with DZP, α-CZP and f91−97 compared to the control group.

DISCUSSION

α-CZP is a tryptic peptide from bovine α_{s1}-casein with BDZ-like activity. It displays an anxiolytic profile in the rat by the ip route and inhibited flunitrazepam binding on the BDZ site of $GABA_A$ receptor.[4] The bovine α_{s1}-casein tryptic hydrolysate, that contains it, shows anxiolytic effects in the rat after ip administration of 3 mg/kg[4] but also after oral administration of 15 mg/kg.[20] The industrially manufactured product in encapsulated form is orally active in the cat,[6] the dog,[7] and also in humans.[8,9] The amino-terminal tyrosine residue and the carboxy-terminal arginine residue of α-CZP seem to be key residues for its activity and a complete sequence seems hence to be essential.[10] Thus, it seems crucial that this peptide resists different biological barriers, the first of them being the digestive barrier.

Hydrolysis of α-CZP by Digestive Proteases. The resistance of α-CZP against digestion by pepsin and/or pancreatic proteases was evaluated. Physiological elements were taken into account even if a model *in vitro* cannot encompass all the parameters occurring *in vivo*. Pepsin was added at an E:S ratio of 1:200, which corresponds to the physiological concentration of this protease in the human stomach,[23] and reaction was

Table 2. Effect of Diazepam (DZP; 1.0 mg/kg), α-Casozepine (α-CZP; 1.0 mg/kg) and f91−97 (0.7 mg/kg) on Rat Behavior in the Elevated Plus Maze Test[a]

	CTR	DZP	α-CZP	f91−97
total no. of entries	8.1 ± 0.9	9.3 ± 0.9	8.1 ± 0.8	9.5 ± 1.0
latency time before entering into closed arms (s)	7.5 ± 2.2	72.8 ± 17.0*[b]	27.6 ± 5.0	22.8 ± 2.9
% of entries in open arms	31.6 ± 4.2	45.2 ± 3.8*	46.0 ± 4.0*	45.0 ± 5.6*
% of time spent in open arms	20.2 ± 2.9	54.9 ± 4.2*	47.3 ± 5.9*	45.9 ± 5.9*

[a] Data are expressed as mean ± SEM ($n = 15$ in each group). [b] (*) Significant difference compared to the CTR group ($p < 0.05$).

Table 3. Effect of Diazepam (DZP; 1.0 mg/kg), α-Casozepine (α-CZP; 1.0 mg/kg) and f91−97 (0.7 mg/kg) on Rat Behavior in the Light/Dark Box Test[a]

	CTR	DZP	α-CZP	f91−97
total no. of entries	12.8 ± 1.8	21.0 ± 3.0* [b]	18.4 ± 3.7	18.2 ± 3.7
latency (s)	124.3 ± 22.7	144.4 ± 18.5	123.9 ± 17.3	164.7 ± 24.4
% of entries in lighted compartment	32.6 ± 2.7	72.1 ± 2.8**	71.9 ± 1.9**	75.3 ± 2.2**
% of time spent in lighted compartment	7.3 ± 1.3	23.6 ± 3.5**	17.8 ± 1.1*	23.8 ± 3.1**
no. of rears in lighted compartment	16.8 ± 2.5	5.2 ± 0.7**	7.6 ± 1.2**	5.7 ± 0.9**

[a] Data are expressed as mean ± SEM (n = 12 in each group). [b] Significant difference compared to the CTR group: (*) $p < 0.05$ and (**) $p < 0.01$.

performed at pH 2.0 and 4.0. A pH of 2.0 is found in the stomach body of humans during interprandial phase, and intragastric pH reaches a pH of 4.0, even 6.0, in postmeal time,[24] because of the buffering effect of food. At both pH levels, fifty percent of α-CZP was still intact after 25 min, a time where the gastric emptying of a solid meal could normally begin.[24] Considering this fact, the resistance of the intact peptide to pancreatic proteases was also investigated. We showed that trypsin and chymotrypsin, the two major proteases of pancreatic juice, were little or not involved in the degradation of this peptide. Corolase PP was used to approach the proteolytic processes occurring in the gut. This extract contains trypsin, chymotrypsin, elastase (EC 3.4.21.36), carboxypeptidases A (EC 3.4.17.1) and B (EC 3.4.17.2), aminopeptidase activities—principally leucine-aminopeptidase—and dipeptidyl peptidase activities.[25] In the presence of these enzymes, the α-CZP was rapidly and strongly hydrolyzed, since forty percent of the peptide disappeared in the first 10 min. Considering that 30−40% of the food proteins arriving in the duodenum can be hydrolyzed into small peptides (2 to 6 residues) in only 10 min,[26] α-CZP has exhibited a certain resistance to these enzymes. It must be noted that these hydrolyses were performed at pH 8, which is optimal for enzymatic activities, whereas the pH in duodenum at the beginning of and during digestion is about 6−6.5. A pH of about 8 is only found in the distal ileum.[27] Knowing that, at a pH of 6, the activity of the bovine chymotrypsin corresponds to about 35% of the maximal activity of the enzyme,[28] resistance of the α-CZP might be greater *in vivo* in the duodenum. The hydrolysis of the α-CZP by pepsin during 2 h followed by trypsin/chymotrypsin or Corolase PP led to results similar to those obtained with the enzymes used alone.

Peptides Generated from α-CZP by Digestive Proteases. The amino-terminal part of the α-CZP was particularly resistant to hydrolysis since no fragment corresponding to the hydrolysis of a bond in the region 91−95 was detected. Nonetheless, some bonds could be hydrolyzed in this region by pepsin as well as by chymotrypsin. Pepsin and chymotrypsin are endoproteases, and hydrolysis of bonds in small peptides may be hampered by the proximity of the terminal amino and carboxyl groups.[29] Indeed, pepsin and chymotrypsin did not cut the last bond of the peptide, $L^{99}-R^{100}$, corresponding to their specificity, but these enzymes hydrolyzed other bonds in its carboxy-terminal part. Moreover, Corolase PP, displaying aminopeptidase activities, did not degrade the amino-terminal region of α-CZP. Pepsin produced essentially f91−97 and to a lesser extent f91−95 and f91−98, all corresponding to its specificity, and chymotrypsin only f91−97 and f91−98, with a greater proportion of f91−97. None of the amino-terminal peptides generated by pepsin or chymotrypsin was subsequently hydrolyzed showing that these fragments were resistant to these proteases. Chymotrypsin has generated f91−97 by cleaving the $Q^{97}-L^{98}$ bond, which seemed not to correspond to the enzyme specificity. Nevertheless, it has been shown on the proteome of *Shewanella oneidensis* that chymotrypsin generates peptides with carboxy-terminal residue of glutamine. The cleavage at these unexpected sites was more influenced by residues in position P3, P2, P1′ and P2′ than the cleavage at expected sites.[30] Unexpected cleavage sites obtained with serine proteases may be explained by conformation of peptide substrates, and bulky P3 residue may improve extended conformation of peptides favoring hydrogen-bonding and van der Waals interactions.[31] In our case, when the glutamine residue was the P1 residue, the P3 residue corresponded to the bulky leucine residue 95. Corolase PP formed f91−95 and f91−97 by an endoproteolytic activity since the complementary carboxy-terminal peptides were found in the hydrolysis medium. The peptides f91−95 and f91−97 were not the main ones in the medium at the beginning of the hydrolysis of the α-CZP—the two main ones were f91−99 and f91−98—but their concentration increased over time. The carboxypeptidase B found in Corolase PP[25] may generate f91−99 and f91−98 may be formed from f91−99 by the carboxypeptidase A which can quickly release leucine residues but has no activity on arginine residues.[32] It must be noted that the concentration of f91−99 was always greater than that of f91−98 at all times of hydrolysis. This result might seem surprising, since the carboxypeptidase A activity contained in Corolase PP is higher than that of carboxypeptidase B,[25] but f91−98 can be proteolytically processed into f91−97 by carboxypeptidase A. So, f91−97 could be generated by the carboxypeptidases and the proteases contained in Corolase PP in proportions depending on the relative activities of these enzymes.

Anxiolytic Activity of f91−97 in Behavioral Tests . As f91−97 was the main fragment formed by pepsin, by chymotrypsin and represented a significant proportion of the peptides found in the medium after hydrolysis by Corolase PP, it could be formed in the stomach or be directly generated in the gut. Hence, we evaluated the anxiolytic activity of f91−97 using three classical anxiety-related behavioral tests despite the fact that this peptide lacked the carboxy-terminal part of the α-CZP. A pre-evaluation of the anxiolytic activity was done with the CDB test, which is hardly affected by a change in general locomotor activity of animals after various treatments.[33] Injected by the ip route at 0.56 μg/kg, f91−97 seemed to display an anxiolytic profile similar to that of DZP. In the two other tests, EPM and LDB, a change in the mobility of animals may interfere with the behavioral responses.[20] So, the general locomotor and exploratory activities were measured in OF test in a first time. No treatment significantly affected these parameters in animals. The EPM is currently employed by a large number of investigators to detect anxiolytic-like effects of drugs[34] and is considered as a validated

test of anxiety based on the antagonistic behavior of the rodents split between exploration and avoidance of height and of open space.[35] The ratio of activities of open arms relative to closed arms assessed the anxiolytic/anxiogenic effect of a compound. The LDB test is also a validated test to measure the anxiety level of laboratory rodents.[36,37] The BDZs are known to increase the level of exploration of the more aversive illuminated area.[38] In the EPM and LDB paradigms, α-CZP injected by the ip route at 0.79 μmol/kg displayed an anxiolytic profile similar to that obtained with DZP injected at 3.52 μmol/kg. Injected by the ip route at 0.79 μmol/kg, f91−97 possessed the same activity as the longer peptide. The carboxy-terminal arginine residue is crucial for maintaining the structure of α-CZP determined in SDS micellar medium, and replacement of this residue by an alanine residue leads to a drastic decrease of the *in vitro* binding of the peptide to the $GABA_A$ receptor.[10] Surprisingly, the shortened peptide, f91−97, conserves an anxiolytic activity indicating that it has retained the active structure. These results suggest that, in the decapeptide, α-CZP, the arginine residue leads to an active structure despite the steric constraints imposed by additional residues compared to the heptapeptide. The removal of carboxy-terminal residues deletes these constraints and allows the heptapeptide to adopt an active form.

Our results showed that, even if α-CZP possesses a certain resistance toward gastric and pancreatic proteases, this resistance will strongly depend on the gastric emptying time and the galenic form. Experiments were conducted with pure α-CZP, so its resistance would be greater in a hydrolysate because of the number of various peptide sequences. Nevertheless, the amount of α-CZP absorbed at the brush border might be too low to explain its high activity. However its proteolytic fragment, f91−97, displayed, after ip administration, an anxiolytic profile similar to that of α-CZP in the three different behavioral tests used. So, the centrally anxiolytic activity of the α-CZP-containing products after oral administration may be due to the α-CZP but also to its proteolytic fragment, f91−97.

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ABBREVIATIONS USED

ACN, acetonitrile; BDZ, benzodiazepine; α-CZP, α-casozepine; CDB, conditioned defensive burying; DZP, diazepam; E:S, enzyme:substrate ratio; EPM, elevated plus-maze; $GABA_A$, γ-aminobutyric acid type A; ip, intraperitoneal; LDB, light/dark box; OF, open field; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid

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