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Transport Across Caco-2 Cell Monolayer and Sensitivity to Hydrolysis of Two Anxiolytic Peptides from α_{s1}-Casein, α-Casozepine, and α_{s1}-Casein-(f91−97): Effect of Bile Salts

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ABSTRACT: α-Casozepine and f91−97, peptides from α_{s1}-casein, display anxiolytic activity in rats and may have to cross the intestinal epithelium to exert this central effect. We evaluated their resistance to hydrolysis by the peptidases of Caco-2 cells and their ability to cross the cell monolayer. To mimic physiological conditions, two preparations of bile salts were used in noncytotoxic concentrations: porcine bile extract and an equimolar mixture of taurocholate, cholate, and deoxycholate. The presence and composition of bile salts appeared to modulate the peptidase activities of the Caco-2 cells involved (i) in the hydrolysis of α-casozepine, leading to much higher formation of fragments f91−99, f91−98, and f91−97, and (ii) in the hydrolysis of f91−97, leading to lower degradation of this peptide. Transport of α-casozepine across Caco-2 monolayer increased significantly, in the presence of bile extract, and of fragment f91−97, in the presence of bile salts.

KEYWORDS: α-Casozepine, fragment f91−97, α_{s1}-casein, bile salts, bioavailability, Caco-2, anxiety

INTRODUCTION

α-Casozepine (α-CZP), a peptide corresponding to sequence 91−100 of the bovine α_{s1}-casein (CN), exhibits anxiolytic activity in the conditioned defensive burying paradigm,[1] in the elevated plus-maze test and in the light/dark box experiment[2] after intraperitoneal (ip) administration in rats. The tryptic hydrolysate of bovine α_{s1}-CN containing α-CZP was evaluated after oral administration in animals. It prevents stress-induced sleep disturbance in rats,[3] provides a positive effect on the management of anxious disorders in cats,[4] and acts as an antidepressant in dogs.[5] Moreover, this encapsulated hydrolysate was efficient in humans since the increase in blood pressure induced by stress and the plasmatic cortisol concentration were significantly lower in treated subjects than in subjects provided with placebo treatment.[6] Likewise, oral intake of the encapsulated product by female volunteers significantly reduced their digestive, cardiovascular, intellectual, emotional, and social stress-related symptoms.[7]

In vitro digestion of α-CZP by pepsin and/or pancreatic proteases leads to the release of N-terminal fragments, the main product in hydrolysis media being the α_{s1}-CN-(f91−97). Surprisingly, this fragment has retained the anxiolytic activity of α-CZP, as has been shown in rats with three behavioral validated tests measuring anxiety.[2] As proposed by these authors, the anxiolytic activity after oral administration of the α_{s1}-CN tryptic hydrolysate may be due to α-CZP but also to its proteolytic fragment, α_{s1}-CN-(f91−97).

The pharmacological profile of α-CZP leads us to hypothesize that this peptide might play a role as an external ligand of the benzodiazepine site of the γ-aminobutyric acid type A receptor ($GABA_A$) since it competes with flunitrazepam for this site.[1] To act in vivo in the central nervous system, the active peptide must (i) resist hydrolysis by brush border peptidases, (ii) be absorbed across the intestinal epithelium (IE) without any degradation, and (iii) be transported into the bloodstream and cross the blood−brain barrier.

Peptidases in the brush−border membrane limit the bioavailability of small peptides by their proteolytic activity, therefore limiting their absorption across the intestinal mucosa. Brush−border peptidases are mainly active against tri-, tetra-, and higher peptides, while intracellular peptidases predominantly hydrolyze dipeptides.[8]

The potential mechanisms of transfer of peptides across the IE have not been clearly established, but many systems are known to transport endogenous as well as exogenous peptides across the plasma membrane of mammalian cells. The best known is the intestinal transporter, H^+-coupled PEPT1, widely expressed in IE, which can carry only di- and tripeptides.[9] Three other pathways have been recognized for transepithelial peptide absorption: the transcellular pathway (transcellular passive diffusion or transcytosis via endocytic uptake), the paracellular pathway (between the cells via tight junctions), and the transport across Peyer's patches.[10]

Using in vitro or in vivo models, various examples of biologically active peptides have been reported to cross the IE: isracidin,[11] hexarelin,[12] peptide HLPLP from β-CN[13] peptide GGYR,[14] octreotide, a synthetic analogue of somatostatin,[15] human β-casomorphin-5 and -7,[16] and 17-residue peptide of bovine β-CN.[17] Nevertheless, peptides and peptidomimetics typically show poor and variable oral bioavailability.[8] To improve the bioavailability of these compounds, some artificial enhancers of permeation have been studied as follows: polyamines, fatty acids, chitosan and its derivatives, and steroidal detergents.[18]

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Bile salts, considered as steroidal detergents, are present under physiological conditions in the small intestine and have been reported to increase epithelial paracellular permeability by modulating tight junctions.[19] However, bile salts display potential local cytotoxic effects.[20]

In the present study, we used a human colon epithelial cancer Caco-2 cell line as the experimental model of human intestinal absorption of peptides. The aim of this work was (i) to determine nontoxic conditions for the culture of the Caco-2 cell monolayer in the presence of two preparations of bile salts (porcine bile extract and an equimolar mixture of three bile salts, sodium taurocholate, sodium cholate, and sodium deoxycholate) and (ii) to investigate the hydrolysis and transport across the Caco-2 monolayer of α-CZP and α_{s1}-CN-(f91−97) under the defined conditions.

MATERIALS AND METHODS

Cell Culture Media and Chemicals. Dulbecco's modified Eagle's minimal essential medium (DMEM with glucose 4500 mg/L), heat-inactivated fetal calf serum (FCS), nonessential amino acids (NEAA), Hank's balanced salt solutions (HBSS), and penicillin−streptomycin (PS) were purchased from Invitrogen (Cergy-Pontoise, France). Cell culture inserts with polycarbonate membranes (pore sizes, 0.4 μm; effective area, 4.2 cm^2) were obtained from Millipore (Saint-Quentin-en-Yvelines, France). Plastic dishes, plates, and white 96-well plates for fluorimetric measurements were purchased from Dutscher (Brumath, France).

α-CZP (YLGYLEQLLR) and peptide f91−97 (YLGYLEQ), respectively, corresponding to regions 91−100 and 91−97 of the mature chain of bovine α_{s1}-CN (UniProtKB/Swiss-Prot P02662), were synthesized by Genospher Biotechnologies (Paris, France). 1,6-Diphenyl-1,3,5-hexatriene (DPH), fluorescein isothiocyanate dextran-labeled 4000 (FD-4), tetrahydrofuran, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Two preparations of bile salts were studied as follows: (i) porcine bile extract, named porcine bile extract (EBS) (Sigma-Aldrich, B8631), is the dried fraction from hog bile, which is soluble in 80% alcohol—this product contains hyodeoxycholic acid (1−5%), deoxycholic acid (0.5−7%), cholic acid (0.5−2%), glycodeoxycholic acid (10−15%), and taurodeoxycholic acid (3−9%)—and (ii) the equimolar mixture of three bile salts, sodium taurocholate, sodium cholate, and sodium deoxycholate (Sigma-Aldrich), named BSM. Lactate dehydrogenase (LDH) kits were purchased from Roche Diagnostics (Meylan, France).

Determination of Critical Micelle Concentration (CMC) for EBS and Bile Salt Mixture. For EBS and BSM, the CMC was determined as described by Catalioto et al.[21] Solutions of EBS (10 g/L) or BSM (mixture of 10 mM of each bile salt leading to an overall bile salt concentration of 30 mM) were prepared in 37 °C prewarmed HBSS and were incubated for 30 min at the same temperature. After it was stirred, the solution was diluted to obtain testing concentrations (0, 0.7, 1.3, 2.0, 2.7, 3.3, and 4.0 mM concentration of each bile salt in BSM and 0, 2, 4, 6, 8, and 10 g/L of EBS). An 100 μL aliquot of each concentration was transferred to a 96-well plate and mixed with 10 μL of 50 μM DPH in HBSS. The 96-well plate was incubated with shaking for 60 min at room temperature in the dark. Fluorescence was measured with a Synergy HT microplate reader, Biotek Instrument GmbH (Colmar, France), at an excitation wavelength of 350 nm and an emission wavelength of 451 nm. The change in fluorescence of DPH vs EBS or BSM concentration should allow the determination of the CMC.

Caco-2 Cell Monolayer Culture and Peptide Transport Studies. The Caco-2 TC7 cells obtained from Dr. Monique Rousset (UMRS 872, Inserm, Paris) were cultured in DMEM supplemented with 20% heat-inactivated FCS, 1% PS, and 1% NEAA. They were incubated at 37 °C in a humidified atmosphere with 10% CO_2. The cells used in the studies had been passaged between 30 and 40 times.

Measurements of peptide transport with Caco-2 cell monolayer were performed in triplicate, and the concentrations of EBS and BSM used corresponded to nontoxic conditions evaluated by transepithelial electrical resistance (TEER), FD-4 permeability, and LDH measurements. The Caco-2 cells were seeded in cell culture inserts with polycarbonate membranes at a cell density of 6×10^4 cells/cm^2. The cells were incubated in six-well culture plates at 150 rpm, 37 °C, in a humidified atmosphere containing 10% CO_2, and the medium was changed daily. The confluent monolayer was used between 21 and 23 days after seeding. The TEER was measured, and the Caco-2 cell monolayer was rinsed three times with 1 mL of HBSS. The cells were incubated for 30 min with no bile salts or with EBS (1 g/L) or BSM (1.0 or 1.5 mM) in the apical compartment and HBSS in the basolateral compartment. After incubation, the solutions in both compartments were removed, and the peptide solution was added as follows: 2 mL of a 300 μM solution of α-CZP or fragment f91−97 prepared in HBSS, with or without bile salts at the previous concentrations in the apical compartment, and 2 mL of HBSS in the basolateral compartment. Aliquots were taken from the apical and basolateral compartments after 30, 60, and 120 min of incubation and replaced by the same volume of fresh HBSS. The peptide concentration in aliquots was determined by RP-HPLC (see below), leading to the rate of peptide hydrolysis in the apical compartment and to the rate of transport from apical to basolateral compartment.

At the end of the experiments, the cells were washed three times with 1 mL of HBSS, and fresh culture medium was added to the apical and basolateral compartments. The TEER was then measured as described below.

Caco-2 Cell Monolayer Permeability and Viability Measurements. *Measurement of Transepithelial Electrical Resistance.* The integrity of the Caco-2 cell monolayer was checked under culture conditions by measuring the TEER with a Millicell-ERS Ohm Meter (Millipore, Billerica, MA) twice a week during culture, just before, immediately after, and 2, 4, and 20 h after the peptide transport studies. An increase to 350 Ω cm^2 of the TEER indicated a confluent cell monolayer with tight junctions.

FD-4 Permeability. The apparent permeability of the Caco-2 cell monolayer was measured using FD-4 at 375 μg/mL in HBSS under the same conditions, with or without bile salts, as described for peptide transport. Aliquots were taken after 30, 60, and 120 min from the basolateral compartment only, and the fluorescence was measured with a Synergy HT microplate reader at an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

Measurement by LDH Activity of Cell Viability 24 h after Bile Salt Treatment. The LDH activity is linked to mitochondrial activity, and the release of this enzyme into the medium is related to cell death. One day after the end of the transport studies, the cells were rinsed with HBSS. The cell-containing inserts were incubated with triton X-100 solution (2% v/v in HBSS) to lyse all viable cells and to obtain the maximum amount of LDH activity. This activity was measured as described by Roche Diagnostics. Results were expressed as the percentage of viable cells after treatment with or without bile salts. Cells treated without bile salts were considered as 100% viable, and 100% of LDH activity was measured after cell lysis. Tests were performed in triplicate.

RP-HPLC Analysis of Peptides in the Samples. Chromatographic analysis of the peptides was performed on a LiChrospher C_{18} column (150 mm × 2 mm, 5 μm particle size, 10 nm porosity, Cluzeau, France) with a W600 HPLC system (Waters, Guyancourt, France) coupled online with a UV detector 2487 (Waters). Linear gradient elution was applied for 80 min from 5 to 50% of acetonitrile containing 0.1% (v/v) TFA in water, at a flow rate of 0.2 mL/min. The load volume was 100 μL. Detection was recorded at 215 nm for maximum sensitivity.

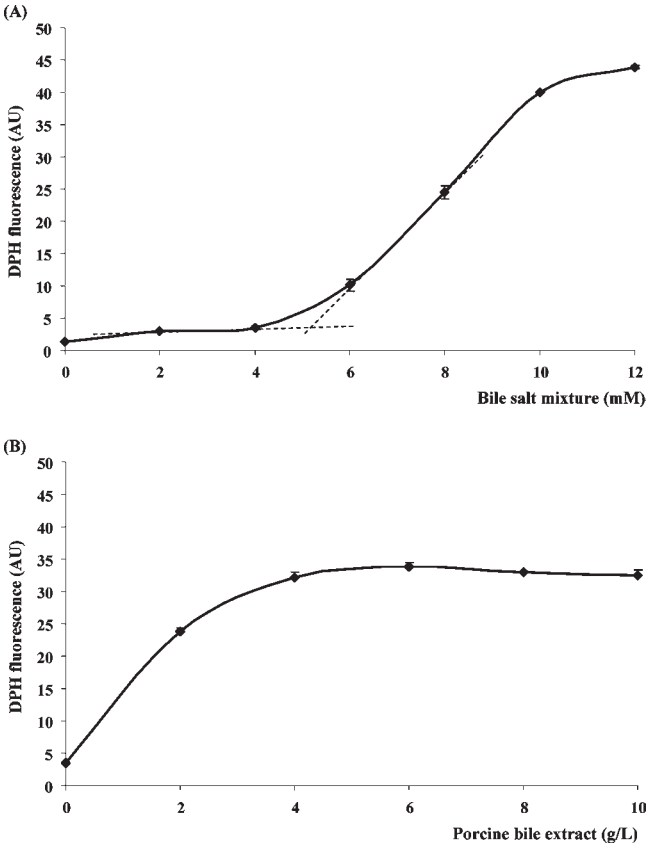

Figure 1. Variation of fluorescence intensity of DPH at an excitation wavelength of 350 nm and an emission wavelength of 451 nm vs concentration of (A) bile salt mixture (mM) and (B) EBS (g/L). The intersection of the dotted lines represents the CMC. Data are expressed as means ± SEMs (n = 3).

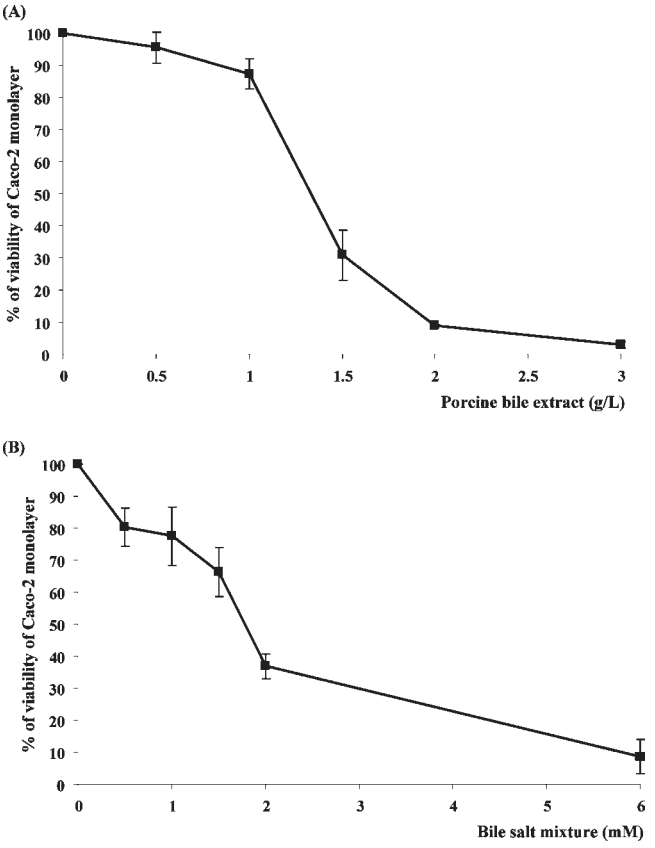

Figure 2. Effect of apical exposure to (A) EBS (EBS in g/L) and (B) bile salt mixture (BSM in mM) on viability of Caco-2 monolayer cells measured by a change in LDH activity. Data are expressed as means ± SEMs (n = 3).

The amounts of α-CZP, fragment f91−97, and the hydrolysis products were calculated from their area and their calculated molar extinction coefficient at 215 nm. The reference area for α-CZP and fragment f91−97 was that obtained under the initial conditions. The peak areas were divided by the corresponding molar extinction coefficient (ε) for each hydrolysis time. The values obtained were compared to that obtained for the α-CZP or fragment f91−97 alone under the initial conditions. The molar extinction coefficients were calculated as follows:

$$\varepsilon_{215\text{nm}}^{\text{peptide}} = x \times \varepsilon_{215\text{nm}}^{\text{Tyr}} + y \times \varepsilon_{215\text{nm}}^{\text{peptide bond}} \quad (1)$$

where x is the number of tyrosine residues and y is the number of peptide bonds in the peptide. The value of ε at 215 nm for a residue of tyrosine was 10000 and 7000 M^{-1} cm^{-1} for a peptide bond.[22]

RP-HPLC/ESI-MS Analysis of Hydrolysis Products. Characterization of hydrolysis products from α-CZP or fragment f91−97 was carried out by RP-HPLC/ESI-MS as previously described.[2]

Data Analysis of Permeability of Peptides and FD-4. The apparent permeability coefficient (P_{app}) was calculated from the following equation:

$$P_{app} = (dQ/dt) \times 1/(A \times C_0) \quad (2)$$

where A is the surface area (cm^2) of the cell monolayer, C_0 is the initial concentration of the analyzed compound, and the ratio (dQ/dt) is the change in total amount of the compound in basolateral side over incubation time, that is, the slope of the amount of tested compound in basolateral side vs time (30, 60, and 120 min).

Statistical Analysis. Hydrolysis kinetics of α-CZP and fragment f91−97 and kinetics of release of hydrolysis products were analyzed using a one-way repeated measures analysis of variance (ANOVA) followed by Tukey's test. The statistical analysis was performed using the mixed procedure of SAS 9.1 (SAS Institute Inc., Cary, NC). Results were expressed as the mean ± standard error of the mean (SEM). Differences were considered statistically significant when $p < 0.05$.

RESULTS

CMC. The CMC for BSM (equimolar mixture of sodium taurocholate, sodium cholate, and sodium deoxycholate), determined with DPH as fluorescent probe, was approximately 5.5 mM (Figure 1A). For EBS, CMC could not be determined, and it would have been interesting to test levels below 2 g/L for this treatment (Figure 1B).

Effect of Bile Salts on Viability and Permeability of Caco-2 Cell Monolayer: LDH Activity, TEER Measurement, and FD-4 Permeability. The effect of the two types of bile salt, EBS and BSM, on Caco-2 cell monolayer viability was examined by measuring LDH activity (Figure 2). The LDH activity at 100% of viability, measured after cell lysis, was stable up to 1 g/L EBS. The percentage viability of the cells dramatically decreased above 1 g/L, reaching approximately 2% for 3 g/L EBS (Figure 2A). The viability of the cells in the presence of BSM slightly decreased between 0.5 and 1.5 mM and fell above 1.5 mM. The percentage viability was under 10% with 6 mM BSM (Figure 2B).

Results obtained for TEER measurements are represented in Figure 3A,B. The TEER value measured at the end of the experiments, using concentrations of 1 g/L EBS and 1 mM BSM, decreased significantly. After removal of bile salts at the end of the transport studies, the TEER recovered its initial value of approximately 300 Ω cm^2 within 20 h. With higher concentrations of EBS (above 1 g/L) or BSM (above 1 mM), both bile salt preparations induced a rapid drop, without a return to the initial value after 20 h.

The apparent permeability coefficient (P_{app}) of Caco-2 cell monolayers was determined using FD-4 as fluorescent probe (Table 1). The value of P_{app} slightly increased up to 2 mM for BSM (7.1×10^{-6}) and 1.5 g/L for EBS (3.3×10^{-5}). Above these concentrations, the P_{app} strongly increased from 1.8×10^{-6} ($P_{app(0)}$ without bile salts) to 3.1×10^{-4} ($P_{app(bile\ salts)}$ in the presence of 6 mM BSM). Similarly, P_{app} increased from 7.8×10^{-7} for cells in medium free of bile salts to 1.8×10^{-4} for cells in the presence of 3 g/L EBS. The ratio $P_{app(bile\ salts)}/P_{app(0)}$ was ca. 170 for BSM at 6 mM and 233 for EBS at 3 g/L.

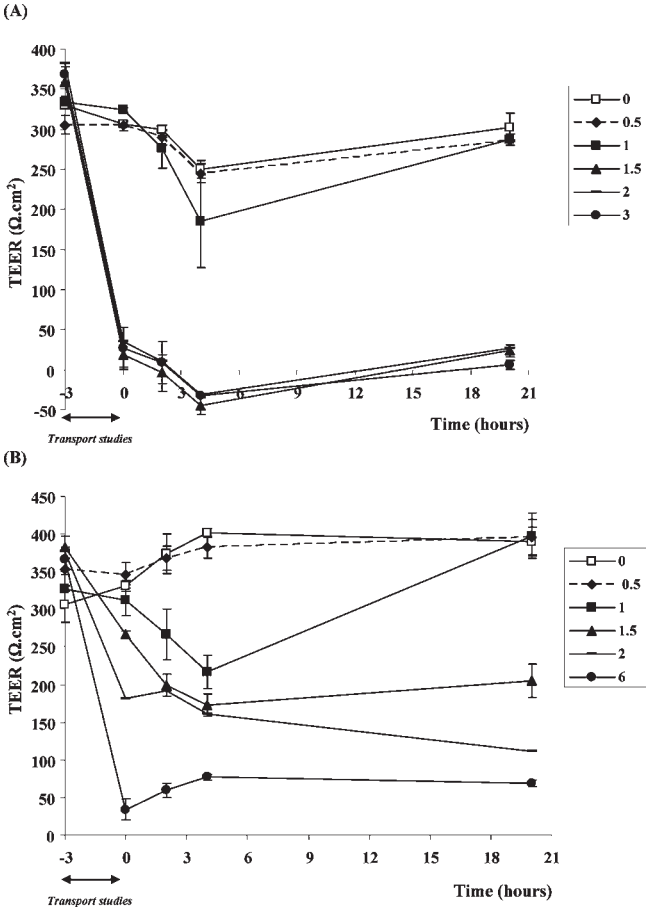

Figure 3. Variation of TEER of Caco-2 monolayer as a function of apical exposure at different concentrations of (A) EBS (in g/L) or (B) bile salt mixture (BSM in mM). Data are expressed as means ± SEMs ($n = 3$).

Impact of Bile Salts on Hydrolysis and Transport of α-CZP and Fragment f91−97 Using the Caco-2 Monolayer Model. The hydrolysis profile, along with the transfer of α-CZP and fragment f91−97 in the absence of bile salts, was compared at an EBS concentration of 1 g/L (corresponding to the concentration that maintained the viability of Caco-2 monolayer cells) and at BSM concentrations of 1.0 and 1.5 mM (which had a slight impact on this viability).

Hydrolysis of α-CZP and Fragment f91−97 on the Apical Side of a Caco-2 Cell Monolayer. *Hydrolysis of α-CZP.* No hydrolysis of α-CZP was measured on the basolateral side of the Caco-2 cell monolayer (data not shown). Disappearance profiles of α-CZP on the apical side of Caco-2 cell monolayer were almost similar, regardless of the experimental conditions used (Figure 4). In the absence of bile salts or in the presence of 1 g/L EBS, ca. 50% of the initial quantity of α-CZP remained at 30 min. After 120 min of incubation, 17% of the initial amount of α-CZP was still present in the medium without bile salts, 4% in the presence of 1 g/L EBS, and 10 and 1% in the presence of 1.0 and 1.5 mM BSM, respectively.

The disappearance of α-CZP on the apical side of Caco-2 cell monolayer was due to its hydrolysis into smaller peptides.

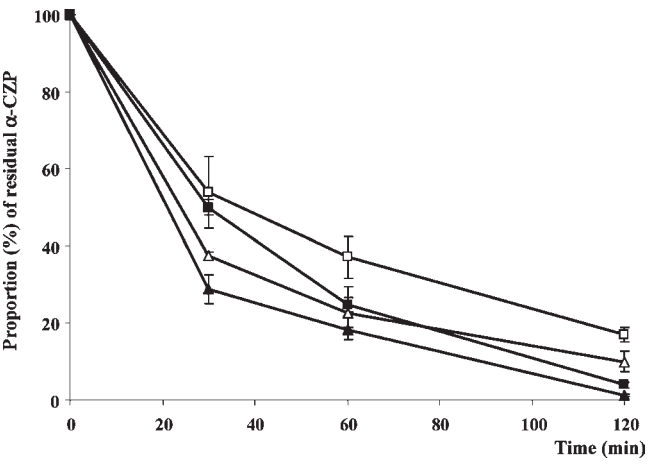

Figure 4. Disappearance kinetics of α-CZP (300 μM) in the apical side of Caco-2 monolayer without (□) or with bile salts, 1.0 mM BSM (△) or 1.5 mM BSM (▲), or with EBS, 1 g/L EBS (■). Data are expressed as means ± SEMs ($n = 3$).

Table 1. Effect of Apical Side Exposure to Different Concentrations of Bile Salt Mixture (BSM, mM) or EBS (g/L) on Apparent Permeability Coefficient (P_{app}) of FD-4 (375 μg/mL) in Caco-2 Monolayer (Each Value Represents the Mean ± SEM, $n = 3$)[a]

[BSM] (mM)	0	0.5	1.0	1.5	2.0	6.0
P_{app} ($\times 10^{-6}$ cm/min)	1.8 ± 0.5	2.4 ± 0.6	3.2 ± 0.5	4.4 ± 0.9	7.1 ± 1.7	310.0 ± 8.9
$P_{app(BSM)}/P_{app(0)}$	1.0	1.3	1.7	2.4	3.8	169.4
[EBS] (g/L)	0	0.5	1.0	1.5	2.0	3.0
P_{app} ($\times 10^{-6}$ cm/min)	0.78 ± 0.3	1.7 ± 0.4	1.4 ± 0.3	33.0 ± 2.7	84.0 ± 6.8	180.0 ± 7.9
$P_{app(EBS)}/P_{app(0)}$	1.0	2.1	1.8	42.7	107.7	232.2

[a] $P_{app(0)}$ without bile salts.

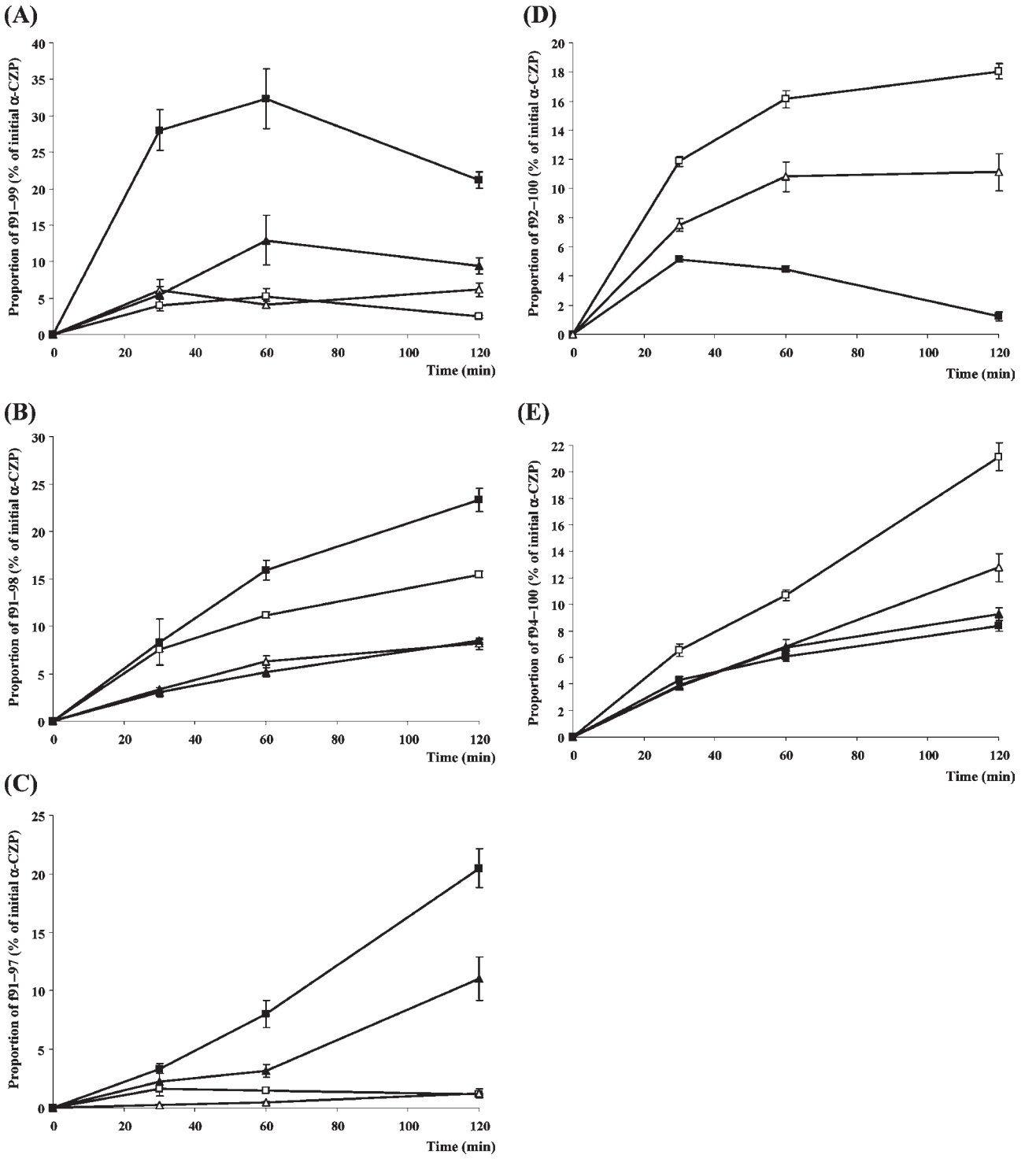

Figure 5. Release kinetics of peptide fragments (A) f91−99, (B) f91−98, (C) f91−97, (D) f92−100, and (E) f94−100 from α-CZP (300 μM) in the apical side of a Caco-2 monolayer without (□) or with bile salts, 1.0 mM BSM (△) or 1.5 mM BSM (▲), or with EBS, 1 g/L EBS (■). Data are expressed as means ± SEMs (n = 3).

The main peptides were identified by RP-HPLC/ESI-MS and corresponded to amino-terminal fragments ^{91}YLGYLEQLL^{99}, ^{91}YLGYLEQL^{98}, and ^{91}YLGYLEQ^{97} (the numbering refers to the position of the residue in the mature chain of bovine α_{s1}-CN) of α-CZP. Two other main peptides were found in the media, ^{92}LGYLEQLLR^{100} and ^{94}YLEQLLR^{100}. The proportion of each hydrolysis product differed according to the experimental conditions (Figure 5).

Without bile salts, the main peptides found on the apical side were the fragments f92−100 and f94−100, representing approximately 39% of the initial amount of α-CZP at 120 min (Figure 5D,E). Fragments f91−99, f91−98, and f91−97

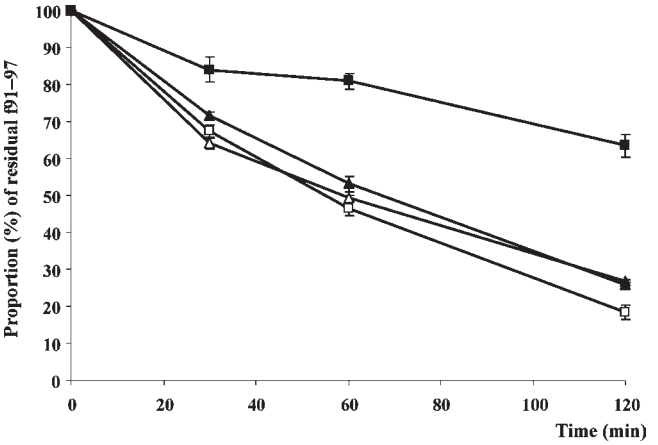

Figure 6. Disappearance kinetics of fragment f91−97 (300 μM) in the apical side of Caco-2 monolayer without (□) or with bile salts, 1.0 mM BSM (△) or 1.5 mM BSM (▲), or with EBS, 1 g/L EBS (■). Data are expressed as means ± SEMs (n = 3).

together represented less than 20% of the initial amount of α-CZP, whatever the time of the experiment (Figure 5A−C).

In the presence of 1 g/L EBS, the appearance of fragments f91−99, f91−98, and f91−97 was sequential during the experiment. After 60 min, fragments f91−99, f91−98, and f91−97 reached 33, 16, and 8% of the initial amount of α-CZP, respectively. After 120 min, they represented 22, 24, and 21% of the initial amount of α-CZP, respectively (Figure 5A−C). Fragments f92−100 and f94−100 together represented approximately 10% of the initial amount of α-CZP, whatever the time of experiment (Figure 5D,E).

In the presence of 1.5 mM BSM, fragment f91−97 represented 11% of the initial amount of α-CZP at 120 min, whereas in the presence of 1.0 mM BSM, it represented less than 2% of the initial amount of α-CZP (Figure 5C). With a concentration of 1.0 mM BSM, fragments f92−100 and f94−100 represented together approximately 24% of the initial amount of α-CZP (Figure 5D,E).

Hydrolysis of Fragment f91−97. No hydrolysis of fragment f91−97 was measured on the basolateral side of the Caco-2 cell monolayer (data not shown). A significant proportion of intact fragment f91−97 was found in the medium after 30 min under all experimental conditions (84% of initial amount of fragment f91−97 in presence of EBS and 65 and 72% for 1.0 and 1.5 mM BSM, respectively). After 120 min of incubation, 19% of the initial amount of fragment f91−97 was recovered in the medium without bile salt, approximately 27% in conditions with 1.0 or 1.5 mM BSM, and almost 65% with 1 g/L EBS (Figure 6).

Hydrolysis of fragment f91−97 on the apical side of the Caco-2 cell monolayer released some peptides, the main ones corresponding to the fragments ^{92}LGYLEQ^{97}, ^{93}GYLEQ^{97}, and ^{94}YLEQ^{97} as identified by RP-HPLC/ESI-MS (Figure 7). No amino-terminal fragment (f91−XX) produced by the hydrolysis of f91−97 was detected on the apical side of the Caco-2 cell monolayer under any of the tested conditions.

It can be also noted that no amino-terminal fragment, with less residues than f91−97, was detected in the medium obtained from hydrolysis of α-CZP.

Transport of α-CZP and Fragment f91−97 Across the Caco-2 Cell Monolayer. The transport of α-CZP and fragment f91−97 from the apical to the basolateral side of the Caco-2 cell monolayer was measured (Table 2).

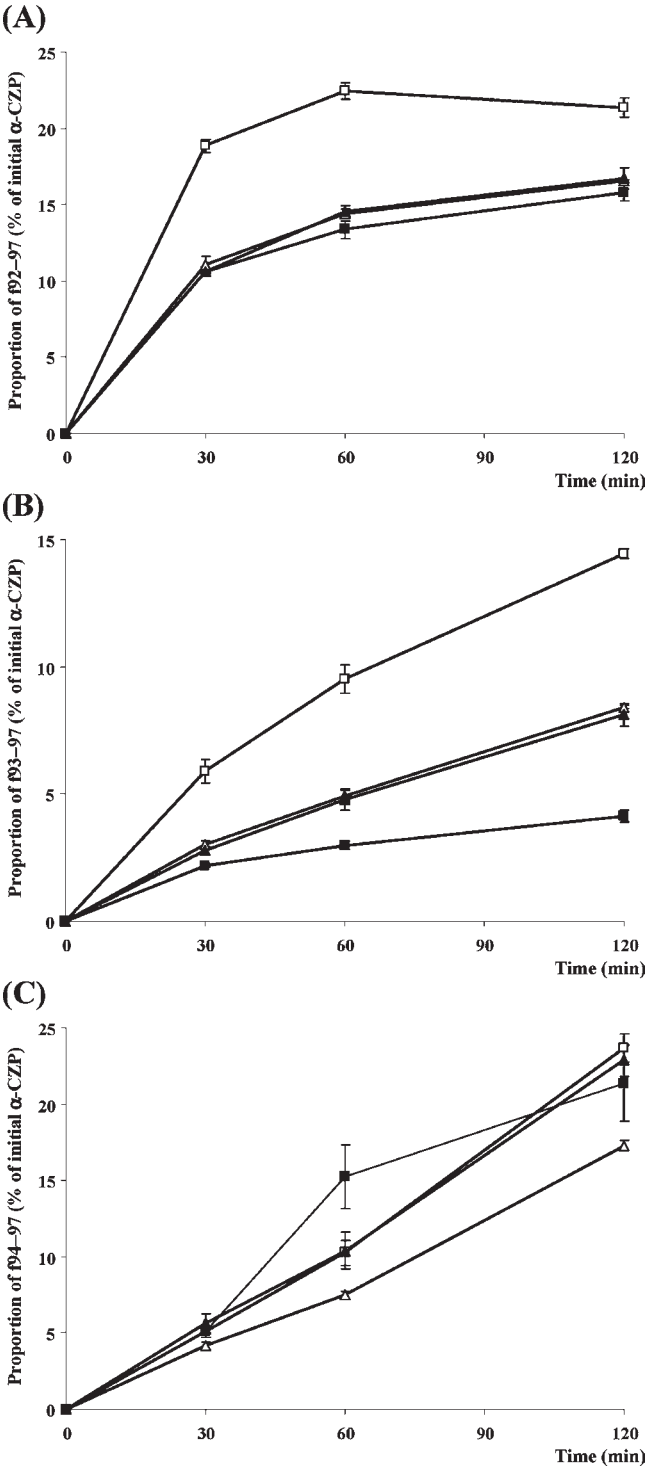

Figure 7. Release kinetics of peptide fragments (A) f92−97, (B) f93−97, and (C) f94−97 from fragment f91−97 (300 μM) in the apical side of Caco-2 monolayer without (□) or with bile salts, 1.0 mM BSM (△) or 1.5 mM BSM (▲), or with EBS, 1 g/L EBS (■). Data are expressed as means ± SEMs (n = 3).

In the absence of bile salts as well as in the presence of 1.0 or 1.5 mM BSM, the P_{app} value for α-CZP could not be calculated because Pearson's correlation coefficient of dQ/dt was not

Table 2. Effect of Apical Side Exposure to Bile Salt Mixture (1.0 and 1.5 mM BSM) or EBS (1 g/L EBS) on Apparent Permeability Coefficient (P_{app}) of α-CZP (300 μM) and Fragment f91−97 (300 μM) in Caco-2 Monolayer (Each Value Represents the Mean ± SEM, n = 3)[a]

	0	1 g/L EBS	1.0 mM BSM	1.5 mM BSM
		α-CZP		
P_{app} ($\times 10^{-6}$cm/min)	trace amounts	2.1 ± 0.7	trace amounts	trace amounts
		fragment f91−97		
P_{app} ($\times 10^{-6}$cm/min)	0.65 ± 0.6	4.4 ± 1.1	1.3 ± 0.3	9.6 ± 2.8
$P_{app(bile\ salts)}/P_{app(0)}$	1.0	6.6	2.0	14.7

[a] $P_{app(0)}$ without bile salts.

significant (the concentration of α-CZP in the basolateral side was above the detection level but below the quantification level of our analytical method). In the presence of 1 g/L EBS, the P_{app} value reached 2.1 × 10^{-6} cm/min (Table 2).

The P_{app} was measurable for fragment f91−97 under all test conditions. Without bile salt preparation, the value of P_{app} reached 6.5 × 10^{-7} cm/min, whereas it was equal to 9.6 × 10^{-6}cm/min in the presence of 1.5 mM BSM. The ratio $P_{app(bile\ salts)}/P_{app(0)}$ reached approximately 15 with 1.5 mM BSM and a value close to 7 with 1 g/L EBS (Table 2).

DISCUSSION

It has previously been shown that α-CZP and f91−97, one of its main proteolytic fragments found after pepsin, chymoptrypsin, or Corolase PP hydrolysis, display an anxiolytic activity in different behavioral tests in rats after ip administration.[1,2] These peptides appear to exert their biological activity at a central level in vivo, and the α_{s1}-CN tryptic hydrolysate containing α-CZP also displays anxiolytic activity after per os administration.[23] Consequently, to display this kind of effect, they must be able to resist hydrolysis by the peptidases of the brush border and to cross the IE without any degradation. In the present study, a Caco-2 cell monolayer was used as a model of the human IE to evaluate the resistance of both peptides to hydrolysis by the peptidases of the enterocyte and their rate of transfer across the IE. Several studies have shown that peptides containing more than three residues are poorly absorbed in the gut with less than 1% of the initial quantity able to cross the intestinal barrier.[13,17] Nevertheless, α-CZP could exert its biological activity at low concentrations since the lowest active dose after ip administration was 0.4 mg/kg (i.e., 300 nmol/kg).[1] The Caco-2 model is widely used, but the environment in which the cells are immersed does not reflect the intestinal bolus. Indeed, under physiological conditions, enterocytes are in contact with bile salts in the small intestine. In humans, these compounds are synthesized and conjugated in the live and excreted into the duodenum, at concentrations between 5 and 10 mM throughout the small intestine.[24] The pool of bile salts, composed of sodium cholate (20%), chenodeoxycholate (40%), deoxycholate (20%), and trace amounts of lithocholate and ursodeoxycholate,[25,26] varies from 2.5 to 4.0 g and undergoes 6−12 cycles of recycling per day, thus 15−48 g of bile salts flow through the liver and intestine each day. Recent studies have reported that bile salts enhance the transport of hydrophilic and hydrophobic compounds by increasing epithelial paracellular and transcellular permeability.[19,21] The nature and concentration of bile salts are major parameters as their toxic effects have specific structural requirements, for example, dihydroxy bile salts being more toxic than trihydroxy bile salts.[27] Most authors have used one bile salt or equimolar mixtures of different bile salts,[21,28] but to our knowledge, very few of them have used bile extract, which contains several bile salts at various concentrations. The use of bile extract appears to more closely mimic physiological conditions, even though its exact composition is not well-known. Consequently, two kinds of bile salt preparations were used in our study: EBS and the equimolar mixture of three bile salts (BSM) previously used by Catalioto et al.[21] as a reference.

Effects of Bile Salts on Caco-2 Cell Monolayer Integrity. The effects induced on the Caco-2 cell monolayer by EBS at 1 g/L or BSM at 1 mM were reversible 24 h after removal of bile salts. Cytotoxic and irreversible effects, on the other hand, were observed above 1.5 g/L for EBS and 1.5 mM for BSM. Catalioto et al.[21] showed a noncytotoxic effect with 3 mM BSM. In the same way, Michael et al.[29] used cholysarcosine (20 mM) or chenodeoxycholate (2.5 mM) to enhance octreotide absorption, but TEER and LDH activities showed a direct and irreversible cytotoxic effect. Finally, 10 mM cholate induces a reversible decrease of TEER and a permeation increase to mannitol.[28] In our experimental conditions, measurements of LDH release, TEER evolution, and FD-4 transport in Caco-2 monolayers exposed to EBS (1 g/L) or BSM (1 mM) indicated that the cells maintained full viability even though the barrier function was reduced and permeability increased. This demonstrated that, for selected form and concentration of bile salts, the decrease in TEER and the increase in paracellular flux of FD-4 were not linked to cytotoxicity but only to a reversible opening of tight junctions. Raimondi et al.[20] showed that bile acids modulate tight junctions through the redistribution of occludins by the activation of the receptor of the epithelial growth factor.

The α-CZP adopts a helicoidal structure, a 3_{10}-helix initiated and terminated by an α-turn, in micellar medium. The hydrophobic residues are located on one face of the helix and the hydrophilic ones on the other face, conferring amphipathic properties to the peptide.[30] Thus, the presence of micelles under physiological conditions may change the structure of the peptide potentially interacting with these micelles. Such an interaction would occur at the CMC of bile salts. Unfortunately, the toxicity of BSM close to its CMC (5.5 mM) was irreversible in our experiments, and it was impossible to define the CMC for EBS.

Hydrolysis of α-CZP and Fragment f91−97 by Peptidases of Caco-2 Cells. Numerous brush−border peptidases have been identified in Caco-2 monolayers: membrane alanyl aminopeptidase (EC 3.4.11.2), Xaa-Pro aminopeptidase (EC 3.4.11.9), Xaa-Trp aminopeptidase (EC 3.4.11.16), neprilysin (EC 3.4.24.11), dipeptidyl-peptidase IV (EC 3.4.14.5), angiotensin converting

enzyme or peptidyl-dipeptidase A (EC 3.4.15.1), and membrane dipeptidase (3.4.13.19).[31] The Caco-2 model allowed us to assess the impact of bile salts on hydrolysis by the peptidases but did not provide any reliable answer concerning those peptides that could be physiologically generated in the gut. Indeed, some studies suggest that differentiated Caco-2 cells express an enzymatic profile similar to that of enterocytes of the fetus but not of the adult.[32]

Hydrolysis of α-CZP may imply a carboxypeptidase activity with specificity for arginine residues, since the fragment f91−99 was recovered. Nevertheless, carboxypeptidase M (EC 3.4.17.12), a membrane-bound enzyme with specificity for lysine and arginine residues, was not described in Caco-2 cells but in HT-29 cells.[31] In the same way, another carboxypeptidase activity might be involved in the release of fragments f91−98 and f91−97.

Such activities were not highlighted in the degradation studies of fragment f91−97, whose last residue is a glutamine. Aminopeptidase and/or dipeptidyl-peptidase activities seemed to be involved in a similar way in the degradation of f91−97 and α-CZP. Indeed, fragments f92−97/100 and f94−97/100 were recovered from the hydrolysis media of both peptides.

The peptidase activities of Caco-2 cells involved in the hydrolysis of α-CZP or fragment f91−97 were strongly affected by the presence of bile salts. On one hand, the hydrolysis of α-CZP led to a lower release of the carboxy-terminal fragments f92−100 and f94−100 and specifically generated the amino-terminal fragments f91−99, f91−98, and f91−97 in the presence of 1 g/L EBS. On the other hand, the resistance of fragment f91−97 toward hydrolysis was highly dependent on the presence of bile salts but also on their nature, an observation already mentioned by Bai.[33] Almost 65% of the initial amount of fragment f91−97 was recovered after 120 min in presence of 1 g/L EBS, 27% with 1 mM BSM, and only 19% without bile salts. Only carboxy-terminal fragments of f91−97 (i.e., f92−97, f93−97, and f94−97) were found on the apical side of the Caco-2 monolayer. The presence of bile salts seemed to decrease the aminopeptidase activities of the Caco-2 cells involved in the hydrolysis of α-CZP and f91−97. The hydrolysis of α-CZP by another peptidase, less affected by the bile salts, led to fragment f91−97, but this enzyme was not able to hydrolyze it. Bai[33] previously observed that bile salts can inhibit some of the peptidases of the brush border and consequently reduce intestinal degradation of peptides. In our experiments with Caco-2 cells, bile salts improved the formation or resistance of fragment f91−97 that has been previously detected in high proportion in the hydrolysis media of α-CZP by gastric and pancreatic proteases.[2] The resistance of f91−97 toward hydrolysis may be due to its protection by bile salts from peptidase-induced hydrolysis. This hypothesis is in agreement with Bai,[33] who proposed that negatively charged bile salts at pH 7.5 can interact with proteins through electrostatic and van der Waals interactions, leading to reduced accessibility to peptidases.

Transport Studies of α-CZP and Fragment f91−97. The transport mechanisms encountered in the human IE are specific transporter-mediated transport, transcellular pathway, and passive diffusion through the tight junctions (paracellular pathway). Only di- and tripeptides are transported by the H^+-coupled peptide transporter PEPT1.[9] Longer peptides may be absorbed in significant amounts but by nonmediated mechanisms[34] such as the paracellular pathway[35,36] or transcellular route.

In our experiment, the transfer of α-CZP across a Caco-2 monolayer without bile salts or in the presence of 1.0 or 1.5 mM BSM was not quantifiable (the quantification limit of α-CZP and fragment f91−97 was close to 20 nM under our experimental conditions). Conversely, the apparent permeability coefficient (P_{app}) of α-CZP reached 2.1×10^{-6} cm/min in the presence of EBS. This value was lower than the value of 4×10^{-4} cm/min obtained by Iwan et al.[16] for the β-casomorphin-7 in the Caco-2 model. This difference may be explained by culture conditions, seeding density, cell passages, and cell clones, which affect transfer, especially the paracellular pathway.[37] Differences in P_{app} value between treatments under identical culture conditions should be compared and not the absolute values of results obtained in the literature.

Without bile salts, the P_{app} of fragment f91−97 reached 6.5×10^{-7} cm/min and a maximum value ca. 15-fold higher (9.6×10^{-6} cm/min) in the presence of 1.5 mM BSM. The lack of effect of wortmanin or cytochalasin, both transcellular transport inhibitors, on the transfer of the pentapeptide HLPLP indicates that paracellular passive diffusion is likely the main mechanism of its transport across the Caco-2 monolayer.[13] The fragment f91−97 might also be transferred by paracellular diffusion. Indeed, the transcellular route does not seem the most likely given the physicochemical characteristics of this peptide. The molecular complexity of intercellular occluding junctions appears to be the rate-limiting barrier of the paracellular pathway. Bile salts may modulate both the structure and the function of occluding junctions by a cytoskeleton-dependent mechanism since tight junctions are dynamic.[20] An in vitro study of transport or hydrolysis of peptides by intestinal cells will not be representing physiological phenomena if it does not take into account the interactions of digestive tract components, including bile salts. The increased transfers observed with bile salts might allow a greater flow of molecules, exerting their action on the central nervous system, toward their target.

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ABBREVIATIONS USED

α-CZP, α-casozepine; BSM, mixture of bile salts; CN, casein; CMC, critical micellar concentration; DMEM, Dulbecco's modified Eagle's minimal essential medium; DPH, 1,6-diphenyl-1,3,5-hexatriene; EBS, porcine bile extract; FCS, fetal calf serum; FD-4, fluorescein isothiocyanate dextran-labeled 4000; HBSS, Hank's

balanced salt solution; IE, intestinal epithelium; ip, intraperitoneal; LDH, lactate dehydrogenase; NEAA, nonessential amino acids; PS, penicillin−streptomycin; TEER, transepithelial electrical resistance; TFA, trifluoroacetic acid

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