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Interaction between dietary bioactive peptides of short length and bile salts in submicellar or micellar state

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ABSTRACT

Bile salts act as steroidal detergents in the gut, and could also interact with peptides and improve their bioavailability, although the mechanism is unclear. The occurrence of direct interaction between milk bioactive peptides, Ile-Asn-Tyr-Trp, Leu-Asp-Gln-Trp, and Leu-Gln-Lys-Trp, and different bile salts in the submicellar or micellar state was investigated by intrinsic fluorescence measurement and dynamic light scattering, above the critical micellar concentration, the latter being determined by isothermal titration calorimetry. The peptides form aggregates, spontaneously. In the presence of bile salts, some released peptide monomers were bound to the micellar surface. The lack of hydrogen bonding involving the C12—OH group of the steroid skeleton, and the acidic function of some bile salts, might promote the interaction with the peptides, as could the lack of the C12—OH group, rather than that of the C7—OH group. At submicellar concentrations, sodium taurochenodeoxycholate and taurodeoxycholate readily interacted with the most hydrophobic peptide Ile-Asn-Tyr-Trp.

1. Introduction

Biological activities of peptides have been widely studied during the two last decades. Thus, several milk-derived bioactive peptides are nowadays commercially available in functional foods but also in ingredients (Mills, Ross, Hill, Fitzgerald, & Stanton, 2011). To prove their effectiveness *in vivo*, there is a growing interest in the study of the bioavailability of such dietary bioactive peptides in the gastrointestinal tract. In this context, bile salts, as steroidal detergents, may help bioactive peptides to reach their targets in their active form during the postprandial phase in the gut. Indeed, they increase epithelial paracellular permeability by modulating tight junctions, and therefore facilitate the transport of drugs and other nutrients through the intestinal barrier (Mukaizawa et al., 2009). Cakir-Kiefer et al. (2011) have shown, for instance, that the bile salts protect the α-casozepine, an α_{s1}-casein decapeptide exhibiting an anxiolytic activity, against hydrolysis by intestinal peptidases and also improve the transport of α-casozepine across the Caco-2 cell monolayer. On the other hand, Kramer et al. (1994) have described how the covalent attachment of peptide to bile salt increases uptake of the peptide by ileal brush-border membrane vesicles *via* the specific intestinal absorption pathway for bile acids.

Bile salts are components derived from the cholesterol molecule which display an amphipathic complex structure: the convex side of their cyclopentanoperhydrophenanthrene nucleus (steroid skeleton) is hydrophobic, with the presence of hydrogen and methyl groups, whereas the concave side is hydrophilic due to the presence of hydroxyl groups. Bile salts also possess a lateral chain that may be conjugated with taurine or glycine (Bloch & Watkins, 1978; Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). Bile salts can be categorized according to the number and position of hydroxyl groups but also according to the presence or absence of conjugation. Due to the spatial geometry of the

Abbreviations: CMC, critical micellar concentration; DLS, dynamic light scattering; EMI, emission fluorescence intensity; ITC, isothermal titration calorimetry; NaC, sodium cholate; NaCDC, sodium chenodeoxycholate; NaDC, sodium deoxycholate; NaGC, sodium glycocholate; NaTC, sodium taurocholate; NaTCDC, sodium taurochenodeoxycholate; NaTDC, sodium taurodeoxycholate; pI, isoelectric point; TBS, Tris-buffered saline; Tris, trishydroxymethylaminomethane.

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steroid skeleton, the bile salts can form micelles with a low aggregation number. The morphology of these micelles is complex and still under study (e.g. Posa & Sebenji, 2014). Below the critical micellar concentration (CMC), the bile salts are in monomeric form, whereas in conditions neighbouring CMC, small aggregates begin to form primary micelles (Madenci & Egelhaaf, 2010). Bile salts are secreted in the liver and transported *via* the bile into the intestine, where they improve digestion, for instance by emulsifying products from the enzymatic breakdown of dietary fats by forming mixed micelles with the poorly-soluble nutrients which will be further transported through the intestinal barrier (Verde & Frenkel, 2010). They also play a major role in enzymatic lipolysis by interacting with the colipase, an amphiphilic polypeptide of 10 kDa that anchors the pancreatic lipase to the surface of lipid droplets in the intestine. The hydrophilic side of the colipase interacts with the lipase, whereas its hydrophobic side is a common binding site for lipid droplets and bile salts (Kerfelec et al., 2008).

The enterohepatic circulation of bile salts contributes to the regulation of cholesterol homeostasis. Many studies have reported the hypocholesterolemic effects of proteins. Peptides released by proteolysis and exhibiting high bile salt binding capacity could inhibit the reabsorption of bile salts in the ileum leading to a decrease in the blood cholesterol level. For instance, a β-lactoglobulin-derived pentapeptide (Ile^{71}-Ile-Ala-Glu-Lys^{75}) has been identified as having a hypocholesterolemic effect according to an unclear mechanism but likely involving bile salt binding properties (Nagaoka et al., 2001). More recently, some authors have reported the ability of peptide hydrolysates to inhibit bile salt re-absorption in the ileum, and therefore to lower cholesterol levels in the bloodstream (Perez-Galvez, García-Moreno, Morales-Medina, Guadix, & Guadix, 2015). This would also be directly linked to their capacity to bind bile salts. However, the interactions on a molecular scale between bile salt and small peptides are still unclear.

Notably, bile salts are also able to bind compounds such as drugs, proteins, or high-molecular mass polypeptides, for which the molecular interactions have been studied in detail. For instance, the interaction of phenothiazines, anxiolytic drugs, with bile salts such as sodium cholate (NaC) and sodium deoxycholate (NaDC) has recently been investigated by the measurement of different physico-chemical parameters including CMC, surface tension, UV absorption, and intrinsic fluorescence emission. The results showed that phenothiazines can interact with these bile salts to form mixed micelles, either by electrostatic interactions involving the $(CH_3)_2NH^+$ group of the drug molecule and the carboxylate function of the lateral chain of the bile salts, or by hydrophobic interactions between the phenothiazine ring and the hydrophobic convex side of the steroid skeleton (Mahajan & Mahajan, 2012). In another study, it has been shown that oil-in-water emulsions stabilized with either β-lactoglobulin or lactoferrin interact with a complex mixture of bile salts and that the interaction capability mostly depends on the isoelectric point (pI) of the proteins (Sarkar, Horne, & Singh, 2010; Singh & Sarkar, 2011). A separate study using circular dichroism has unveiled interaction between bile salts (NaC and NaDC), either in submicellar or micellar forms, and polypeptides such as poly[Leu-Leu-Lys] and poly [Leu-Leu-Asp] displaying molecular masses ranging from 94 to 140 kDa (D'Alagni, D'Archivio, & Giglio, 1993). According to the authors, both NaC and NaDC may interact with the polypeptides either by hydrophobic interaction involving their hydrophobic side and the Leu residues or by forming hydrogen bonds between the hydroxyl groups located at C3 and C12 of the steroid skeleton and the amino function of the Lys residues.

Many short peptide sequences, that are encrypted in dietary proteins and liberated by bacterial or intestinal epithelial cell proteases, are known to play a beneficial role in human health. They display a variety of activities such as antimicrobial, antioxidative, antithrombotic, antihypertensive, anti-inflammatory, and immunomodulatory activities mainly investigated *in vitro* (Mills et al., 2011). Nevertheless, only a few clinical studies have demonstrated biological effects of such dietary peptides (e.g. the antihypertensive lactotripeptides Ile-Pro-Pro and Val-Pro-Pro; Boelsma & Kloek, 2009). To reach their target and exert their biological activity *in vivo*, the peptides of interest have to be (i) resistant toward hydrolysis by peptidases of the brush border, (ii) absorbed through the gastrointestinal barrier, and (iii) carried in the bloodstream in an active form and optionally across the blood–brain barrier.

The aim of the present study is to investigate non-covalent interactions between bioactive peptides at effective concentrations and pure bile salts at neutral pH (close to pH within both jejunum and ileum) as a function of the bile salt concentration, then to try to define whether such interactions are rather polar or hydrophobic. The tetrapeptides Ile-Asn-Tyr-Trp (INYW) and Leu-Asp-Gln-Trp (LDQW) derived from α-lactalbumin and Leu-Gln-Lys-Trp (LQKW) from β-lactoglobulin after thermolysin hydrolysis were chosen as bioactive peptide models displaying free radical scavenging activity *in vitro* (Sadat et al., 2011; Contreras, Hernandez-Ledesma, Amigo, Martin-Alvarez, & Recio, 2011). The peptide LQKW also exhibits an antihypertensive activity in spontaneously hypertensive rats (Hernandez-Ledesma, Miguel, Amigo, Aleixandre, & Recio, 2007). Several pure bile salts were selected according to their physicochemical properties: the primary bile salts (produced in the liver) NaC, sodium chenodeoxycholate (NaCDC), sodium glycocholate (NaGC), sodium taurocholate (NaTC), and sodium taurochenodeoxycholate (NaTCDC), and the secondary bile salts (generated by intestinal bacteria) NaDC and sodium taurodeoxycholate (NaTDC). The occurrence of electrostatic or hydrophobic interactions was studied by measuring of intrinsic fluorescence emission due to excitation of Trp residues, below and above the CMC of bile salts, and by dynamic light scattering (DLS) above the CMC. Beforehand, isothermal titration calorimetry (ITC) was performed to determine the CMCs of the bile salts under the experimental conditions used.

2. Materials and methods

2.1. Peptides and bile salts

Peptides LDQW (560.26 Da), LQKW (573.33 Da), and INYW (594.28 Da) were synthesized by Genosphere Biotechnologies (Paris, France). The purity of each peptide was further improved (final purity greater than 97%) by reversed-phase high-performance liquid chromatography as described in a previous study (Sadat et al., 2011). Pure bile salts (NaC, NaCDC, NaDC, NaGC, NaTC, NaTCDC, NaTDC) were purchased from Sigma−Aldrich (St. Louis, MO, USA).

2.2. Isothermal titration calorimetry (ITC)

The different bile salts (at 50 mM) were solubilized in 20 mM trishydroxymethylaminomethane/HCl (Tris/HCl) buffer, pH 7.0, containing 150 mM NaCl (Tris-buffered saline or TBS). All the ITC experiments were carried out at 310 K in TBS using a VP-ITC microcalorimeter (MicroCal, Northampton, MA, USA). During the titration, each concentrated bile salt solution was sequentially injected into a 1.428 ml reaction cell, initially containing the degassed buffer, to achieve the expected final concentrations. Each injection lasted 20 s, and intervals of 180 s between successive injections were applied. A rotating 290 ml micro-syringe permitted a constant stirring at a speed of 300 rpm. The reference power was