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Short communication: Tryptic β-casein hydrolysate modulates enteric nervous system development in primary culture

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ABSTRACT

The intestinal tract of the newborn is particularly sensitive to gastrointestinal disorders, such as infantile diarrhea or necrotizing colitis. Perinatal development of the gut also encompasses the maturation of the enteric nervous system (ENS), a main regulator of intestinal motility and barrier functions. It was recently shown that ENS maturation can be enhanced by nutritional factors to improve intestinal maturation. Bioactivity of milk proteins is often latent, requiring the release of bioactive peptides from inactive native proteins. Several casein-derived hydrolysates presenting immunomodulatory properties have been described recently. Furthermore, accumulating data indicate that milk-derived hydrolysate can enhance gut maturation and enrichment of milk formula with such hydrolysates has recently been proposed. However, the capability of milk-derived bioactive hydrolysate to target ENS maturation has not been analyzed so far. We, therefore, investigated the potential of a recently described tryptic β-casein hydrolysate to modulate ENS growth parameters in an in vitro model of rat primary culture of ENS. Rat primary cultures of ENS were incubated with a bioactive tryptic β-casein hydrolysate and compared with untreated controls or to cultures treated with native β-casein or a Prolyve β-casein hydrolysate (Lyven, Colombelles, France). Differentiation of enteric neurons and enteric glial cells, and establishment of enteric neural network were analyzed using immunohistochemistry and quantitative PCR. Effect of tryptic β-casein hydrolysate on bone morphogenetic proteins (BMP)/Smad pathway, an essential regulator of ENS development, was further assessed using quantitative PCR and immunochemistry. Tryptic β-casein hydrolysate stimulated neurite outgrowth and simultaneously modulated the formation of enteric ganglia-like structures, whereas native β-casein or Prolyve β-casein hydrolysate did not. Additionally, treatment with tryptic bioactive β-casein hydrolysate increased the expression of the glial marker glial fibrillary acidic protein and induced profound modifications of enteric glial cells morphology. Finally, expression of BMP2 and BMP4 and activation of Smad1/5 was altered after treatment with tryptic bioactive β-casein hydrolysate. Our data suggests that this milk-derived bioactive hydrolysate modulates ENS maturation through the regulation of BMP/Smad-signaling pathway. This study supports the need for further investigation on the influence of milk-derived bioactive peptides on ENS and intestinal maturation in vivo.
Key words: milk-derived bioactive peptide, enteric nervous system, bone morphogenetic proteins, neurotrophic

Short Communication

During the first weeks of life, dramatic maturational changes occur in the intestine of the newborn. Impairment of this process may lead to the development of important gastrointestinal disorders in infants, such as infantile diarrhea or necrotizing colitis, or increased risks of developing intestinal inflammatory disorders later in life, such as in inflammatory bowel diseases. Within the intestinal tract, the enteric nervous system (**ENS**) is a main regulator of intestinal motility and barrier functions (Neunlist et al., 2013; Furness, 2012). Maturation of the ENS is characterized by the acquisition of a differentiated phenotype by enteric neurons (de Vries et al., 2010) and enteric glial cells (**EGC**; Cossais et al., 2016) and represents an essential step for the establishment of enteric functions (de Vries et al., 2010).

Although in part regulated by genetic factors, such as members of the bone morphogenetic proteins (**BMP**) 2 and 4 (Chalazonitis and Kessler, 2012), it was recently shown that ENS maturation can be enhanced by nutritional factors, including milk-derived factors. This highlights the potential to target the ENS to improve intestinal maturation (Fichter et al., 2011; Suply et al., 2012).

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Many peptides generated by the proteolysis of milk proteins demonstrate biological activities, which are not observable with the undigested native proteins (Meisel, 2005). Interest for such bioactive peptides has rapidly grown in the recent years, and supplementation of formula with bioactive milk-derived hydrolysate was recently proposed (Lönnerdal, 2014; Raikos and Dassios, 2014). However, capability of such hydrolysates to target ENS maturation has never been investigated. Therefore, we analyzed the potential of a recently characterized bovine tryptic β-CN hydrolysate presenting anti-inflammatory properties (Altmann et al., 2016) to nutritionally target ENS development.

A detailed protocol for the production of native β-CN and tryptic bioactive β-CN hydrolysate (T-hydrolysate) has been previously published (Altmann et al., 2016). In brief, α_S- and para-κ-CN were precipitated and filtrated from a 5% rennet casein solution (wt/wt; Fonterra, Auckland, New Zealand). β-Casein was precipitated by slowly warming the remaining solution to 40°C. β-Casein precipitate was collected and further solubilized in demineralized water by adjusting the pH to 7.0 (25°C) before lyophilization. The β-CN lyophilisate was composed of 83 to 85% β-CN, whereas the remaining 15% were composed essentially of κ-CN and other minor milk contents (determined by PAGE). Tryptic proteolysis of the obtained β-CN was performed and the proteolysate was fractionated by ultrafiltration using a membrane with a cutoff of 5 kDa. The 5-kDa retentate was used as bioactive hydrolysate (T-hydrolysate) in our study. In parallel, the β-CN lyophilisate was hydrolyzed with the serine alkaline protease Prolyve 1000 (Lyven, Colombelles, France), added at an enzyme-to-substrate-ratio of 30 U/g of β-CN to generate a control hydrolysate (P-hydrolysate) presenting a largely distinct peptide profile than the T-hydrolysate [Figure 1 and Table 1; see Altmann et al. (2016) for a detailed description of the T-hydrolysate). Proteolysis was performed at 50°C for 4 h while the pH was kept constant with NaOH at 7.0. The reaction was stopped by heating to 90°C for 10 min. This Prolyve proteolysate was fractionated by ultrafiltration at 40°C using a membrane with a nominal molecular weight cut-off of 5 kDa. The generated peptides were separated and identified by online HPLC coupled to an electrospray ionization mass spectrometry (HPLC-ESI-MS^n) using an ion trap mass spectrometer LTQ XL (Thermo Scientific Inc., San Jose, CA). Freeze-dried Prolyve proteolysates were dissolved in 0.1% (vol/vol) formic acid and analyzed on a reversed phase column (Hypersil Gold aQ, 3 μm, 150 × 2.1 mm, Thermo Scientific Inc.). Solvents used for the chromatographic separation were 0.1% (vol/vol) formic acid in ultrapure water (solvent A) or 0.1% (vol/vol) formic acid in acetonitrile (solvent B). A linear gradient from 3 to 60% solvent B was applied over 40 min at a flow rate of 0.2 mL/min, followed by a column wash step with 90% solvent B for 5 min and re-equilibrating at the initial conditions for at least 10 min.

Mass spectra were generated in the positive ionization mode in the full scan range (220 up to 2,000 m/z) and with a data-dependent scan with fragmentation of the 5 most intense ions (activation type = collision-induced dissociation, normalized collision energy = 35.0 eV, isolation width of 2, and an activation radio frequency of 0.250). Electrospray ionization voltage was set to 3.5 kV; capillary temperature was 275 °C. Data acquisition and processing was performed with Xcalibur version 2.0.7 SP1 (Thermo Scientific Inc.). Peptide sequences were identified by *Bos taurus*-restricted database searches (http://www.uniprot.org/uniprot/?query=reviewed:yes taxonomy:9913) with Proteome Discoverer 1.4 (Thermo Scientific Inc.) using the search algorithm SEQUEST and MASCOT.

Animal husbandry and ENS primary cultures were performed according to Chevalier et al. (2008) with minor adaptations. Procedures were approved by the ministry of Energy, Agriculture, Environment and Rural Areas of Schleswig Holstein (Kiel, Germany; agreement V312–7224.123–5). Briefly, whole intestine was collected from Sprague-Dawley rat embryos at 15.5 d old (embryonic age; Charles River, Sulzfeld, Germany). Intestines from 1 litter (7–14 embryos) were pooled together and represent 1 batch of culture. All experiments were performed on at least 3 independent batches of culture. Tissues were finely diced in PBS and further digested at 37°C for 15 min with 0.1% trypsin in Dulbecco's modified Eagle's medium-F12 medium (50/50, PAN-Biotech, Aidenbach, Germany) containing 100U/mL of penicillin and 100μg/mL of streptomycin (PAN-Biotech). Tissues were then treated with 0.01% DNaseI (Sigma-Aldrich, Munich, Germany) for 10 min at 37°C. After homogenization, cells were centrifuged at 100 × g at 4°C for 10 min, and seeded in gelatin-coated 24-wells plates (Corning Inc., Kaiserslautern, Germany) or in removable 12-well Chamber (Ibidi, Martinsried, Germany) and cultured in a humidified 5% CO_2-95% air incubator at 37°C. After 24 h, considered d 1, the medium was replaced by Dulbecco's modified Eagle's medium-F12 containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 1% of N-2 supplement (N-2 medium, Pan-Biotech). At d 2, ENS cultures were treated with T-hydrolysate, P-hydrolysate, or β-CN (each 1 mg/mL) diluted in N-2 medium or left untreated (control) for further 48 to 96 h before analysis. Control, β-CN-, or hydrolysates-

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