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RESEARCH PAPER

Peptidome Analysis of Human Milk β-Casein *in vitro* Neonatal Digestive Model by Matrix-assisted Laser Desorption/Ionization-Time-of-Flight Tandem Mass Spectrometry

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Abstract: Human milk β-casein was digested *in vitro* by simulated neonatal digestive model to explore the peptidome of human β-casein digested in neonate by matrix-assisted laser desorption/ionization-time-of-flight tandem mass spectrometry (MALDI-TOF/TOF). The accelerating voltage was 20 kV. The laser wavelength was 337 nm and the frequency was 200 Hz. Ion extraction delay was 330 ns. Final mass spectra were produced by averaging 2000 laser shots taken at five different positions within each spot. The peptides scanning range is from *m*/*z* 500 to *m*/*z* 5000. A total of 26 peptides were identified in the molecular weight range of 1000–4000 Da, and no bioactive peptides were found. Instead, we found nine bioactive peptide precursors which include four ACE (Angiotensin converting enzyme) inhibitory peptide precursors, two CPP (Caseinophosphopeptide) precursors, two antioxidant peptide precursor and one immune active peptide precursor. Most of the bioactive peptide precursors might be further digested into biologically active peptides by proteases according to the present cleavage sites.

Key Words: β-Casein; Neonate; *In vitro* digestion; Peptidome; Matrix-assisted laser desorption/ionization-time-of-flight tandem mass spectrometry

1 Introduction

Peptidome refers to all the endogenous peptides from organ, tissue, cellular and body fluids, including bioactive peptides with special functions and peptides from proteins[1–3]. Peptidomics is usually used to explore the components, functions, variations and relationships of peptides in a peptidome[4,5], and has been applied in many studies such as peptidome of human body fluids. Human milk is regarded as the best food for neonates as it is rich in many proteins and bioactive peptides, and 30% to 35% of which is β-casein[6–8], a highest proportion of casein in human milk[9,10]. Although some researches on the identification and structure analysis of human β-casein have been reported[11–13], little is known about the peptidome of human β-casein. Recent studies focused on the separation, identification and biological functions of the digestive products of human β-casein[14–17], but there has been no report on the peptidome of digestive products of human β-casein, which can provide reference for the development of formula that is more suitable for Chinese infants and young

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children. The digestive system of infant (≤ 12 months) especially that of neonate (≤ 28 days) is not mature with strong metabolism but low enzyme activity. The activity and secretion of pepsin of newborn infants are low and in proportion to the age of infant. The activity and secretion of trypsin of infants at the age of 3–24 months are lower than that of adults[18,19]. Based on the widely used *in vitro* digestive model for infant[20] and according to the characteristics of neonate, we established a more suitable *in vitro* neonatal digestive model for newborns. Human *β*-casein was digested in the model to analyze the peptidome of the digested products by matrix-assisted laser desorption/ionization-time-of-flight tandem mass spectrometry (MALDI-TOF/TOF).

2 Experimental

2.1 Instrument and reagents

The samples were centrifuged on GL-21M high-speed centrifuge (Shanghai Institute of Mechanical Centrifugal, China). Delta 320 pH meter and AL204 electronic balance were from Mettler-Toledo, Germany). Freeze dryers were from Shanghai Medical Instrument Factory, China. DYY-10C electrophoresis apparatus and transfer membrane apparatus were purchased from Beijing Liuyi instrument factory, China. Gel imaging system (Bio-RAD, USA), AKTA purifer 100 protein purification system (GE Health, USA), and AB4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, USA) were also used in the experiment.

DEAE-Sepharose Fast Flow and ziptip C_{18} was purchased from GE Health, USA. Milk samples, fresh human milk samples were obtained from ten Chinese volunteer lactating mothers. Pepsin, trypsin and urea were from Sigma, USA. Prestained protein marker (Thermo scientific, USA), 30% polyacrylamide (Solarbio, China), Rabbit anti-human *β*-casein antibody (Abcam, USA) and Enhanced HRP-DAB chromogenic substrate kit (TIANGEN, China) were also used in the study. All the other chemicals were of analytical grade. Ultrapure water was used throughout the experiments.

2.2 Experimental method

2.2.1 Purification and characterization of human *β*-casein

According to the reported method[11], pooled milk samples was adjusted to pH 4.3 using 0.1 M HCl, then centrifuged at 15000 × g for 20 min at 4 °C. The precipitate of *β*-casein was collected and redissloved, then fractionated on AKTA purifier (GE, USA) by using DEAE-Sepharose Fast Flow column in a gradient elution mode. The collections were analyzed by 12% (*V/V*) sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) and stained with Coomassie Brilliant Blue to identify the proteins. The collected sample eluents were freeze-dried after dialysis, and then stored at –20 °C for further analysis. Western-blot was used to identify *β*-casein under a transmembrane voltage of 100 mA for 25 min. The primary antibody was 1000-times diluted rabbit anti-human *β*-casein antibody and the secondary antibody was 3000-times diluted horseradish peroxidase-labeled goat anti-rabbit antibody. After incubation at room temperature for 2 h, the membrane was stained with enhanced HRP-DAB chromogenic substrate kit

2.2.2 *In vitro* digestion of human *β*-casein

According to Johns's method[20] with minor modification, two working solutions of *β*-casein were respectively prepared by dissolving 5 mg of *β*-casein in 2 mL of PBS with pH 3.0 and pH 7.0

Simulated neonatal gastric digestion *in vitro*: *β*-Casein sample prepared by PBS at pH 3.0 was incubated with pepsin of 2400 U mg^{-1} protein at 37 °C for 2 h. After that, the sample was subjected to a boiling water bath for 5 min, and then cooled down to room temperature.

Simulated neonatal intestinal digestion *in vitro*: *β*-Casein sample prepared by PBS at pH 7.0 was incubated with trypsin of 120 U mg^{-1} protein at 37 °C for 1.5 h. After that, the sample was subjected to a boiling water bath for 5 min and then cooled down to room temperature.

Simulated neonatal gastric and intestinal digestion *in vitro*: *β*-Casein sample prepared by PBS at pH 3.0 was incubated with pepsin at 2400 U mg^{-1} protein at 37 °C for 2 h. After adjusting pH to 7.0 with 0.1 M $NaHCO_3$, trypsin was added at 120 U mg^{-1} protein, then incubated at 37 °C for 1.5 h. After that, the sample was subjected to a boiling water bath for 5 min and then cooled to room temperature.

2.2.3 Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry analysis

The accelerating voltage was set at 20 kV. The laser wavelength was 337 nm and the frequency was 200 Hz. Ion extraction delay was 330 ns. Final mass spectra were produced by averaging 2000 laser shots taken at five different positions within each spot. The peptides scanning range is from *m*/*z* 500 to *m*/*z* 5000.

3 Results and discussion

3.1 Purification and identification of *β*-casein in human milk

Human *β*-casein was purified on a DEAE-Sepharose Fast Flow column (Fig.1A). Except peaks 1 and 2 of impurity proteins, each peak was collected separately. The elution was desalted by dialysis, and analyzed by SDS-PAGE. The