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RESEARCH PAPER

Study on Differences of Milk Proteins by Liquid Chromatography-Mass Spectrometer

XU Ming-Fang*, DAI Jin-Feng, XIANG Ming-Xia, CHENG Xi-Fei, ZENG Xiao-Cong, WAN Cong-Qing, ZHOU Wei-Jun

Department of Biotechnology, College of Life Science and Technology, Jinan University, Guangzhou 510632, China

Abstract: The peptide mass fingerprinting differences of whey ((*α*-La) and (*β*-Lg)) and caseins (α_s-CN, *β*-CN, *κ*-CN) components by enzymatic hydrolysis of milk protein from the Southern water buffalo compared with goat and Holstein milk were analyzed by using the LTQ Orbitrap XL mass spectrometer with high resolution. The results showed that peptide mass fingerprinting differences of *α*-La amino acid mutation in Southern water buffalo and Holstein milk whey protein were lower than that in the goat milk whey protein. However, the differences of *β*-Lg amino acid mutation in Southern water buffalo and Holstein milk whey protein were higher than that in the goat milk whey protein. In contrast, compared with the three different milk-producing species, the amino acids sequence differences of caseins (α_s-CN, *β*-CN, *κ*-CN) changed more significantly than that in the whey protein, which indicated that the peptide mass fingerprinting differences of milk protein were especially impacted by the species variety.

Key Words: Liquid chromatography-mass spectrometer; Milk proteins; Casein; Whey protein; Peptide mass fingerprinting differences

1 Introduction

The main protein from animal milk is mainly composed of casein (αs1-casein (α_{s1}-CN), α_{s2}-CN, *β*-CN and *κ*-CN), whey protein (*β*–lactoglobulin(*β*-Lg) and *α*-lactalbumin (*α*-La), immunoglobulin, bovine serum albumin). However, the synthesis and expression of milk protein showed greater difference and complexity because it was affected by a large number of genetic variation and modification after DNA translation in different animal body, breed region, species, feed, climate factors and many other factors[1]. In recent years, the analysis of protein components in milk and dairy products has mostly focused on the separation of milk protein components and detection of protein content[2,3]. However, the identification fine structure difference of peptide mass fingerprint spectrum from the complex of milk protein components still is a serious challenge[4].

As the most advanced mass spectrometer, high-resolution two-dimensional linear ion trap mass spectrometer combined with electrostatic field (LTQ Orbitrap XL) has a very high sensitivity and accuracy (up to 100 K of resolving power), and can be used to quickly scan the sample, for instance, when the resolution is set at 60 K, the scan cycle is less than 1 s. LTQ Orbitrap XL can also be used for the direct detection of compounds from complex samples.

LTQ Orbitrap XL mass spectrometry can provide reliable quantitative information of proteins for the identification of various unknown proteins and peptides sequences, the site analysis of protein phosphorylation, the determination of nucleic acids molecular weight, and the differences discrimination of proteins. In this study, the whey protein enzymatic peptides from the Southern buffalo milk were analyzed and their relative molecular mass and amino acid composition were detected. The peptides sequence was predicted by retrieving the information with Mascot software. By comparing with the known sequence of peptides information in the Pubmed database, the proteins origin from species was identified. Also the difference of casein protein

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* Corresponding author. Email: xumingfang@jnu.edu.cn
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corresponding to the amino acid sequence from bovine and goat species milk with buffalo whey were investigated to reveal the law nature of the variance of whey protein caused by genetic traits differences. Meanwhile, the fingerprint spectrum of peptide was established to confirm the genetic traits differences of whey protein under different conditions. The method provided a technical support for the rapid detection of milk protein molecular markers, and also provided a theoretical basis for the optimal selection conditions of dairy products processing and preservation and the study of biological active peptide of different components of milk protein source.

2 Experimental

2.1 Instruments and reagents

LTQ Orbitrap XL mass spectrometer (Thermo Company), RP-HPLC C18 column (Michrom Bioresources Inc.), ultrasonic cell disrupter (NingBo XinZhi Bio-Instruments, Inc.), and SCIENTZ-11D High-speed refrigerated centrifuge (Eppendorf Company) were used in the experiments.

Acetonitrile, ammonium bicarbonate, trifluoroacetic acid, dithiothreitol, indole-3-acetic acid were purchased from Sigma,(USA) and Trypsin from Promega Corporation (USA). Southern water buffalo milk was purchased from local supermarket in Guangzhou, China. The water used in the experiments was treated by Milli-Q system.

2.2 Preparation of reagents

The solutions used in the experiment were as follows: 100 mM NH_4HCO_3 (100 mL), 25 mM NH_4HCO_3 (10 mL, containing 10 mM of DTT), decolorizing solution (containing 2.5 mL 100% acetonitrile, 1.25 mL 100 mM NH_4HCO_3, 1.25 mL H_2O), reducing agents (including 12.4 mg DTT; 2 mL 100 mM NH_4HCO_3, 6 mL H_2O), alkylating agents (including 40.69 mg IAA, 1 mL 100 mM NH_4HCO_3, 3 mL H_2O), covering solution (containing 1 mL 100 mM NH_4HCO_3, 0.4 mL 100% acetonitrile, 2.6 mL H_2O), enzymatic stock solution (2 μg trypsin was added to 150 μL of covering pre-cooling solution, –80 ºC preservation), extracting solution (containing 0.075 mL TFA, 2.01 mL 100% acetonitrile, 0.915 mL H_2O), and sample dissolved solution (containing 0.001 mL formic acid , 0.02 mL 100% acetonitrile, 0.979 mL H_2O).

2.3 Experimental methods

2.3.1 Separation of whey from buffalo milk

Fresh buffalo milk were centrifuged at 4000 rpm at 4 ºC for 30 min, removed the upper milk fat, collected the skim milk and adjusted to pH 5.0 and 4.6 with 5 M and 0.5 M HCl[5], respectively, then stored for 30 min, centrifuged at 4000 rpm at 4 ºC for 20 min, collected the supernatant, and refined the supernatant through 0.22 μm membrane filter[6].

2.3.2 Separation of casein from buffalo milk

Fresh buffalo milk were centrifuged at 4000 rpm for 20 min to remove the milk fat, and the reserved skim milk was collected and its pH was adjusted to 4.6 with 1 M HCl, and then centrifuged for 15 min at 4000 rpm. The precipitate was washed twice with distilled water and acetone, respectively, and then centrifuged for 10 min to remove the supernatant. The dried precipitate was stored in refrigerator at –20 ºC.

2.3.3 SDS-PAGE electrophoresis

The whey and casein derived from buffalo milk were isolated by sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) on 12% separated gel and 5% concentrated gel, respectively. The electrophoresis gel were analyzed with scanning optical density by using gel imaging and analysis system, and the composition and content of the isolated protein was initially determined by comparing the electrophoregram of casein derived from buffalo milk protein obtained by isoelectric precipitation.

2.3.4 Protein gel of enzymatic digestion

The SDS-PAGE protein gel obtained from casein and whey were respectively hydrolyzed with enzyme as following steps. Firstly, the target protein on SDS-PAGE gels was cut and put into labeled EP tubes. After washed twice, the gel was discolored to transparent by suitable amount of fresh decolorizing agents at 37 ºC water bath. The waste liquid in the EP tubes was abandoned, and the gel was dehydrated with 100% (*V*/*V*) acetonitrile until the color of gel changed to white. After the waste liquid was filtrated, 25 mM NH_4HCO_3 solution (containing 10 mM DTT) as the reducing agent was added, then the tubes was sealed and put in a 57 ºC bath for 1 h. After that, the sample was taken out and cooled to room temperature. Then an equal volume of alkylating agent was quickly added to the sample and the gel was placed in dark room at ambient temperature for 30 min. After dealkylation reagent was removed, 50% (*V*/*V*) acetonitrile was added. After 15 min's standing, the gel was filtrated and 25 mM NH_4HCO_3 was added. After a 5-min reaction, the gel was filtrated and then washed by 50% and 100% (*V*/*V*) acetonitrile dehydration respectively until the color of gel turn to white. The waste solution was excluded. Finally, 4 μL of trypsin enzyme solution was added into the EP tube that was put in the ice. After 30 min, the excess enzyme solution was sucked away and 20 μL covering solution was added. Then the tube was sealed and hydrolyzed for 18 h at 37 ºC. The hydrolysate was