Optimization of whey extraction methods for Milk Bottom up proteomics

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Abstract

The importance of milk in the human diet and in the world's, economy is well known and it is largely due to its unique nutritive quality, complexity and richness. Milk is the key element for human nutrition as it represents the only complete source of all essential nutrients providing sugars, proteins, fats, vitamins and minerals for healthy growth. With the advent of proteomics application in clinics, milk become first choice for diagnostic application due to its easy collection and non-invasiveness.

Milk is a complex fluid whose proteome displays a diverse set of proteins of high abundance and sample preparation method is prerequisite that enables high reproducibility and reliable identification of proteins present or proteins responding to conditions such as a diet, health or genetics. Using skim milk samples from *Karan Fries* cows, we compared three whey preparation approaches: Method A(ultra-centrifugation), Method B (pH- based precipitation) and Method C (pH- based precipitation followed by ultra-centrifugation) to produce comparative SDS-Profile The better recovery was observed in method A as compare to method B&C.

Keywords: Milk, Precipitation methods, SDS-PAGE.

Introduction

Milk is a highly nutritious natural product that provides not only a rich source of amino acids to the consumer but also hundreds of bioactive peptides and proteins known to elicit health-benefitting activities. [1]

Traditionally, milk proteins are categorized into three major groups: caseins, whey proteins and milk fat globule membrane (MFGM) proteins [2]. The milk proteomics covers the identification, characterization and quantification of milk proteins and displays a diverse set of proteins of high abundance such as caseins and medium to low abundance whey proteins such as ß-lactoglobulin, lactoferrin, immunoglobulins, glycoproteins, peptide hormones, and enzymes [3].

By using a wide range of fractionation techniques, whey proteins can be separated from caseins and further processed to allow extraction and identification of the low-abundance protein fraction in milk. In this study, we optimized the whey extraction method for in-depth coverage of whey proteome which can be use as reference map for further studies. A better knowledge of the bovine milk proteome and its main drivers is a prerequisite

for the modulation of bioactive proteins in milk for human nutrition, as well as for the discovery of biomarkers that are useful in husbandry and veterinary medicine

Material and Methods

Sample Collection

Milk sample were collected on quarter basis from 25 *Karan Fries* (quarter-100) cows in summer and winter season separately. The animal selected for milk collection were in lactation period of 50-250.Briefly, teats were cleaned and disinfected using 70% ethanol (vol/vol). The initial three streams were discarded and approximately 50 mL of milk was collected into a sterile plastic tube and1mM PMSF was added and stored in -80^{0}C for further use.

Milk processing

The fat is the most variable nutritional component and contributes over half of the energy to milk. For deep proteome coverage removal of fat is prerequisite step. 50 ml of collected milk was placed a 4^{0}C for thawing and skim milk produced by centrifuging milk at 10000 x g for 20 min at 4^{0}C. The defatted milk was stored at -80^{0}C for further analysis [4].

Whey preparation

Method A (Ultra-centrifugation)

The defatted milk samples were thawed at 4^{0}C. 2ml aliquots of milk was ultra-centrifuged at 60,000 x g for 2hr at 4^{0}C so that samples had a pellet of casein micelles on the bottom, a fat layer on the top and dilapidated whey supernatant in the middle. The fat layer was removed carefully and whey was collected in a fresh Eppendorf tubes and stored at -80^0 C for further use [5].

Method B (pH-based precipitation)

The pH of milk was reduced up-to 4.2 using 1N HCL to precipitate the casein followed by filtration using muslin cloth and 1mM PMSF was added to filtrate and stored at -80^0 C.

Method C (pH-based precipitation followed by ultra-centrifugation)

2ml filtrate produced by reducing the pH-4.2 of milk using 1N HCL ultra-centrifuged at 60,000 x g for 2hr at 4^{0}C. The whey was collected in a fresh Eppendorf tubes and stored at -80^0 C for further use.

Protein assay

The protein concentrations of the skim milk and whey (1:10 dilution) were assessed in duplicate using the Quick Start Bradford Protein Assay following the manufacturer's instructions. Bovine Serum Albumin (BSA) was used a standard.

SDS-PAGE

Total 20 µg of protein of whole milk and whey (Method A,B&C) was loaded on separate lanes and resolved on 4% stacking and 12% separating acrylamide-bis acrylamide gel using 100V over 3 hrs in miniVE Vertical Electrophoresis System (GE Healthcare, USA).The gels were then stained with Coomassie Brilliant blue G (Sigma, Canada) overnight, de-stained for 2 hrs. The de-stained gels were scanned by EPSON scanner (GE, Healthcare, USA).

Results and Discussion

Comparative SDS-PAGE profile

The same amount of proteins was loaded for whey prepared by different methods to produce SDS-PAGE profiles with skim milk as a reference. The electrophoretic patterns were similar from skim milk and whey, significant removal observed in high abundant proteins in whey. The whey prepared by method A (ultra-centrifugation) displayed the best resolution with the sharpest bands. In particular, method B (pH- based precipitation) showed similar efficiency for removal of high abundant proteins, but significant reduction and missing of low molecular weight proteins were also observed as compare to whey prepare by Method A and skim milk. Fig (a & b). Thus, Method A considered to be best method for whey preparation to identify whey proteome.

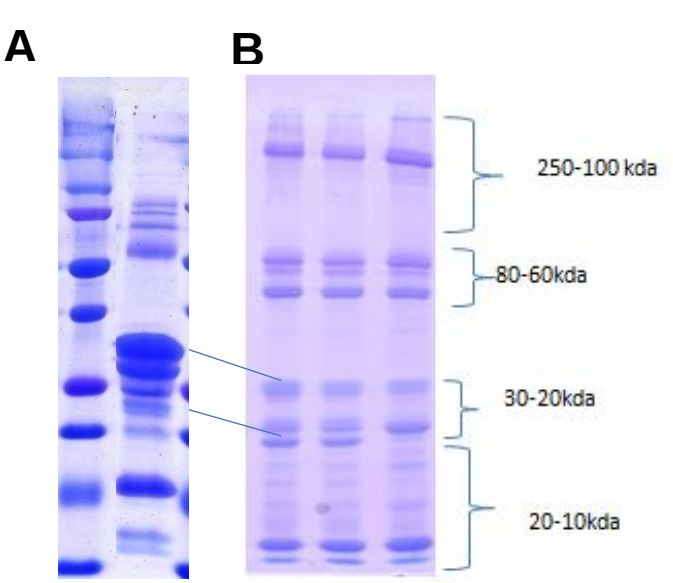

Fig A: band pattern of whole milk protein after the removal of fat
Lane 1: Marker
Lane 2: Defattened whole milk protein
Fig B: Band pattern of milk protein after casein removal by ultracentrifugation
Lane 1, 2 &3: proteins after casein removal

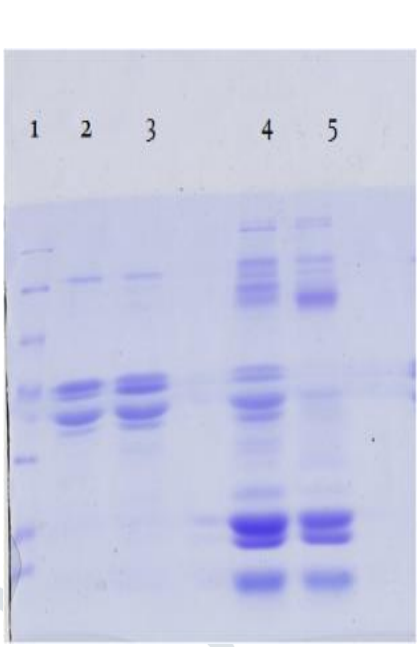

Fig C : Comparison of casein removal methods
Lane1= Protein marker
Lane2,3= removal of casein by **low pH**
Lane4,5=removal of casein by **ultracentrifugation**

Conclusion

In this study, three whey preparation methods performed on bulk milk samples from *Karan Fries* cows were compared using protein assay, SDS-PAGE. All major milk proteins such as caseins were extracted along with less abundant proteins such as whey proteins (β-lactoglobulin, α-lactalbumin, lactotransferrin), as well as minor proteins such as glycoproteins, and enzymes. The highest number of proteins bands were observed in method A as compare to other methods. However, for a proteomics-centric approach, method A offers advantages in simplicity, protein coverage and throughput and would be the preferred method for in-depth proteome coverage.

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