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On-line casein micelle disruption for downstream purification of recombinant human myelin basic protein produced in the milk of transgenic cows

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ABSTRACT

Downstream purification of a model recombinant protein (human myelin basic protein) from milk of transgenic cows is described. The recombinant protein was expressed as a His tagged fusion protein in the milk of transgenic cows and was found associated with the casein micellar phase. While difficulties in obtaining good recoveries were found when employing conventional micelle disruption procedures, direct capture using the cation exchanger SP Sepharose Big Beads™ was found successful in the extraction of the recombinant protein. Early breakthrough suggested a slow release of the recombinant protein from the micelles and dictated micelle disruption in order to obtain good yields. A new approach for deconstruction of the calcium core of the casein micelles, employing the interaction between the micellar calcium and the active sites of the cation exchanger resin was developed. Milk samples were loaded to the column in aliquots with a column washing step after each aliquot. This sequential loading approach successfully liberated the recombinant protein from the micelles and was found superior to the conventional sample loading approach. It increased the recovery by more than 25%, reduced fouling due to milk components and improved the column hydrodynamic properties as compared to the conventional sample loading approach. Hardware and software modifications to the chromatography system were necessary in order to keep the whole process automated. A second purification step using a Ni^{2+} affinity column was used to isolate the recombinant protein at purity more than 90% and a recovery percentage of 78%.

1. Introduction

"Biopharming" is a new brand of farming which involves production of human protein pharmaceuticals in farm animals. After the successful production of the first transgenic (Tg) farm animals in 1985, the potential of molecular pharming has been demonstrated with several plant- and animal-derived recombinant proteins [1,2]. A wide variety of species are being used to generate proteins of therapeutic value. However, the use of large mammals as bioreactors was recognized as a novel platform to enhance the commercial development of drugs. Production is always targeted to various body fluids such as urine, blood and milk in order to facilitate harvesting of the product. The mammary gland is generally the preferred bioreactor due to its ability to carry out complex post-translational modifications in a pattern similar to that obtained by human machinery, large volumes of milk and high levels of expression. Regulatory elements derived from genes encoding abundantly expressed milk proteins were used to target expression of foreign proteins in the mammary gland [1,3–5]. Several promoters have been tested and in some cases very high levels of expression were obtained [6–11].

Once acceptable expression levels of a recombinant protein have been achieved in milk, the next step is to isolate and characterize the product. Use of as few steps as possible with the least number of denaturants is also of extreme importance in order to maintain activity and good yields. If process scale is an objective, any step that is not scaleable should be avoided even when developing bench-top scale purification [12–14]. Human proteins expressed in heterologous systems are of unpredictable nature and assessment of their activity and stability is of primary importance. Early milk collection by hormonal induction of the heifers is an advantage in order to setup a preliminary purification strategy as well as biochemical characterization of the product. However, product testing has to be carried out for each animal at different stages of lactation and over different lactation cycles to ensure production consistency. Well designed testing protocols have to be developed and integrated with the purification protocols since regulatory agencies around the world require extensive testing of recombinant products [1,2,15–19].

Bovine milk contains a few primary proteins; αs_1-casein (αs_1-CN), β-casein (β-CN), κ-casein (κ-CN), αs2-casein (αs2-

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CN), β-lactoglobulin (β-LG), α-lactalbumin (α-LA) and bovine serum albumin (BSA) with relative abundance of approximately 30:30:10:12:10:4:1. However, the milk proteome is still extremely complex due to post-translational modifications, numerous genetic variants, low abundance proteins and presence of naturally occurring proteolysis. An additional level of complexity arises from the fact that milk is a complex bio-colloid system composed of three phases; dispersed lipid phase, aqueous serum phase and casein micellar phase which contains 70–80% of milk proteins [12,20,21]. Milk with its complex nature is challenging for chromatographic processing and several approaches have been reported in order to reduce the complexity of milk prior to processing. However, serious product losses have always been the main concern. Fat globules normally cause problems by blocking packed columns. It has been reported that chromatographic processing of raw milk is possible at temperatures close to the milking temperature (35–37 °C) [22]. Under such conditions, bacterial growth and stability of the recombinant proteins over the processing time has to be considered since milk is processed normally at 4 °C [22]. Moreover, deposition of casein micelles over the whole length of chromatographic beds results in a gradual increase in back pressure and mass transfer limitations [13,14,23].

Owing to its commercial value, the milk system has been studied extensively by the dairy industry and could be considered the best characterized food protein system. A combination of physical and chemical fractionation techniques has been in use for many years [20,21]. However, most of the traditional methods are not suitable for processing milk containing recombinant proteins that are typically pH and temperature sensitive. For example, isoelectric point precipitation of caseins resulted in loss of 50% of activity and only 25% overall yield in case of tissue-type plasminogen activator separation from transgenic goat milk [6]. It has been reported also that an overall yield of 2.0–2.5% for factor IX was obtained upon using acid removal of caseins from transgenic ewe milk [24]. On the other hand, several procedures have been employed successfully in order to clarify milk samples prior to chromatographic processing and high product yields were obtained. It has been reported that more than 90% removal of caseins was achieved by deconstruction of casein micelle calcium core using EDTA then precipitation of caseins using insoluble calcium phosphate nanoparticles (CAP). This approach was claimed to be successful in the liberation of recombinant proteins that could be associated with the casein micelles [25,26]. Other approaches using additives such as arginine [27] or increasing the milk ionic strength by adding salt [28] have been reported in order to disrupt the interaction between casein micelles and valuable proteins in milk.

Myelin basic protein (MBP) represents 30% of the protein content of the myelin sheath which covers the nerves and acts as an insulator for efficient signal transduction. Multiple sclerosis is the most common cause of myelin breakdown and MBP is generally considered as the antigen responsible for autoimmunity in such disease [29–33]. The presence of MBP in the cerebrospinal fluid, at levels higher than normal (>4 ng/ml), is a marker of active inflammation and myelin breakdown. Recent research suggested that administration of neuroantigens to patients, as therapeutic vaccines, can tolerize the autoimmune response in multiple sclerosis patients [34–36]. MBP sequence reveals an unusually high percentage of lysine and arginine residues which gave MBP a very basic character (p*I* 11–12). Four molecular weight isoforms (17.2–21.5 kDa) and several charge isoforms have been identified for each of them. MBP has an open conformation which is an important factor in its role as a structural protein. However, lack of secondary structure makes MBP more susceptible to proteolysis [29–33].

A line of transgenic cows which produce the recombinant 17.2 kDa isoform of human MBP (rhMBP) as an N-terminal His tagged fusion protein in their milk has previously been generated by nuclear transfer as a model for production of protein pharmaceuticals in farm animals (manuscript in preparation). In this study, we used milk from these animals to develop a simple and reliable downstream purification protocol with potential for scale-up for rhMBP. Design of an automated purification strategy for the efficient purification of the recombinant protein from the complex milk system was the main objective. Direct capture of rhMBP from transgenic milk using SP Sepharose BB^{TM} (SPBB) was investigated. A modified sample loading procedure was developed and optimized in order to improve the dynamic capacity of product capture step.

2. Experimental

2.1. Chemicals and samples

The entire milk from consecutive afternoon and morning milking was collected and pooled to form a representative one daily milk sample from transgenic cows (TGmilk) and wild type control cows (WTmilk) with, except for the transgene insertions, the same genotype. The milk samples were skimmed and freeze-dried for storage. All milk samples used in this study were prepared from milk powder by dissolving suitable amounts in MilliQ water to 10% concentration. A rat anti-hMBP monoclonal antibody (cat no. ab7349) which recognizes amino acids sequence 82–87, horse radish peroxidase (HRP)-labeled anti-rat monoclonal antibody (cat no. A5795) and HRP-labeled anti-His tag monoclonal antibody (cat no. 15165) were obtained from Abcam (UK), Sigma (USA) and Pierce (USA) respectively. Nitrocellulose membranes (NC) were obtained from Bio-Rad (USA). A standard hMBP (1.0 mg/ml) was purchased from Research Diagnostics (USA) for comparative purposes (cat no. RDI-TRK8M79). The CAP nanoparticles were purchased from BioSante Pharmaceuticals (USA). All other chemicals were of analytical grade and were obtained from Sigma (USA). Vivaspin centrifugal filters (500 Da molecular weight cut off) were purchased from Vivascience (Germany).

2.2. Instruments

All chromatographic separations were carried out at 4–8 °C using an AKTA Explorer 100-Air controlled by Unicorn 5.11 software (GE Healthcare, Sweden). Hardware and software modifications were carried out in the lab in order to bypass the detectors (UV spectrophotometer, conductometer and pH meter) during the sample loading step. These modifications helped avoid fouling of the detectors' flow cells and obviate the need for extensive clean-in-place protocols between runs. Modifications in the software were carried out in order to incorporate the bypass function as well as the suggested loading approach in the automated strategies of the Unicorn platform. An offline conductivity monitor was connected to the bypass line in order to monitor the conductivity of the flow-through. The scale of the conductivity monitor was calibrated to the loading buffer conductivity as 0% response and the milk conductivity as 100% response. An electronic module (LabPro Datalogger, Vernier, USA) was used to interface the signal obtained to the PC controlling the AKTA system. The cation exchanger resins SPBB and SP Sepharose FF^{TM}, empty columns of different sizes (Disposable PD-10, Tricorn 10/100 and XK 16/20) and immobilized metal affinity chromatography (IMAC) prepacked columns; HisGraviTrap – column volume (cv) 1 ml (Ni^{2+} Sepharose 6 FF^{TM}) and HisTrap HP – cv 5 ml (Ni^{2+} Sepharose HP^{TM}) were purchased from GE Healthcare, Sweden. A Bio-Rad gel imaging system (USA) and a Fujifilm intelligent dark box (Japan) were used for documentation of stained gels and NC membranes after western blotting respectively.