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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.

2.3. Reducing SDS-PAGE, western blotting and dot blotting

Reducing sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Comassie Blue (CB) detection was carried out according to the method of Laemmli [37]. Immunodetection was carried out on the surface of NC membranes (0.2 μm) using either rat anti-hMBP monoclonal antibody followed by a secondary HRP-coupled anti-rat monoclonal antibody or HRP-coupled anti-His tag monoclonal antibodies. Chemiluminescence detection of HRP-coupled antibodies was carried out according to published protocols [38]. Optimization of electro-blotting and development protocols was carried out in-house [39]. Anti-hMBP monoclonal antibody was used in all immunodetection experiments unless otherwise specified. A semi-quantitative immunoassay was developed using dot blot technique in order to estimate the concentration of the rhMBP in chromatography fractions. A serial dilution of each test sample was compared to an equivalent serial dilution of hMBP standard. Comparisons were carried out by blotting 1 μl of each solution on the surface of a NC membrane. Detection was carried out in a similar way to the membranes obtained by electro-blotting. The signal intensity which was obtained from the rhMBP samples was compared densitometrically to that obtained from the standard hMBP in order to estimate the rhMBP concentration relative to that of hMBP concentration.

2.4. Preliminary investigations

Several approaches employing casein micelle disruption were trialed: (i) isoelectric point precipitation of caseins using 1.0 M acetic acid to pH 4.6 and solubilization of the casein pellet using 8.0 M urea, (ii) disruption of the casein micelles by addition of either urea or arginine to the milk samples to a final concentration of 8.0 and 2.0 M respectively, and (iii) selective precipitation of caseins using CAP nanoparticles as previously described [26]. The rhMBP was then recovered from the clarified fractions obtained in each case using Ni^{2+} Sepharose FF followed by SP Sepharose FF in the presence of 8.0 M urea (PD-10 disposable columns, cv 5 ml each). The employed denaturants were then removed using Vivaspin centrifugal filters (500 Da molecular weight cut off) [39].

2.5. Milk fractionation

Two fractionation approaches were trialed in order to locate the recombinant protein in various milk fractions. Isoelectric point precipitation of caseins followed by centrifugation ($8000 \times g$ for 10 min) was carried out using 1.0 M acetic acid to pH 4.6. The casein fraction (CNF) was prepared by dissolving the pellet obtained in 8.0 M urea while the supernatant was considered as the whey fraction (WF). The second fractionation approach was based on the direct pelleting of casein micelles by centrifugation of a milk sample at $8000 \times g$ for 1 h at room temperature. The pellet obtained was washed twice with MilliQ water then re-suspended in MilliQ water and was considered to contain intact casein micelles (CNM). The supernatant was re-centrifugated again ($8000 \times g$ for 1 h at room temperature), the pellet was discarded and the supernatant was considered as the serum fraction (SF). The described protocols were applied on TGmilk and WTmilk samples. All fractions were subjected to SDS-PAGE and western blotting analysis.

2.6. Development of the purification protocol

2.6.1. Media screening

A disposable column (PD-10) was packed with 5 ml SPBB resin and used to investigate the usefulness of the direct chromatographic recovery of the rhMBP from TGmilk. A loading buffer of 50 mM HEPES (pH 7.0) was used for column equilibration and washing. Milk samples (10 ml) were loaded onto the column by gravity and elution was carried out using 10 cv of the same buffer in the presence of 1.0 M NaCl. The collected fractions and the supernatant obtained after boiling a sample of the SPBB resin with SDS-PAGE sample buffer were analyzed using SDS-PAGE followed by western blotting analysis. Fractions containing the rhMBP were used to investigate the applicability of the IMAC technique using HisGravi columns (Ni^{2+} Sepharose 6 FF - cv 1 ml). A loading buffer of 50 mM HEPES buffer (pH 7.0) containing 0.5 M NaCl and (5–50) mM imidazole was used for column equilibration and washing steps. Elution was carried out using the same buffer but in the presence of 500 mM imidazole. The flow-through fractions and the eluted fractions were analyzed using SDS-PAGE followed by western blotting analysis.

2.6.2. Column format

The suggested modifications to the chromatography system were carried out in order to bypass the line of detectors during the sample loading step and column wash as described above. A Tricorn 10/100 column was packed with 8 ml of SPBB resin and used for optimization of the purification protocol. A loading buffer of 50 mM HEPES (pH 7.0) was used for column equilibration and washing while elution was carried out using a buffer of similar composition but in the presence of 1.0 M NaCl. The TGmilk samples (30 ml) were loaded on to the column using the sample pump at a flow rate of 1.0 ml/min followed by a column washing step (50 mM HEPES pH 7.0 - 6 cv). The flow was diverted through the bypass line to the fraction collector and the flow-through and 3 cv of the washing buffer were collected before returning the flow to the detectors line again. The conductivity of the flow-through was recorded during the sample loading step using an offline conductometer connected to the bypass line. Elution of the retained proteins from the column was then carried out using 50 mM HEPES (pH 7.0) in the presence of 1.0 M NaCl either as a gradient or as a step elution over 10 cv from 0.0 to 1.0 M NaCl. The flow-through fractions and salt-eluted fractions were analyzed using SDS-PAGE followed by western blotting or dot blotting techniques, as required in each experiment. The fractions containing the rhMBP were pooled together and were further purified using a HisTrap HP column (cv 5 ml). A loading buffer of 50 mM HEPES (pH 7.0)–0.5 M NaCl–50 mM imidazole and an elution buffer of the same composition and pH but using 0.5 M imidazole were used. A suitable amount of imidazole was dissolved in the pooled fraction containing the rhMBP up to 50 mM concentration in order to bring the sample composition to the loading buffer composition. Samples were loaded onto the column at 2.5 ml/min followed by 3 cv washing step using the loading buffer. A gradient elution was carried out at 5.0 ml/min over 2 cv followed by 5 cv of the elution buffer. The column effluent was collected in fractions of 2 cv each and fractions containing rhMBP isoforms were pooled together.

2.6.3. Factors affecting the dynamic capacity of the cation exchange step

The described method (above) was used as a template to study various factors affecting the dynamic capacity of the column such as the effects of: (i) sample loading flow rate, (ii) sample dilution, (iii) endogenous milk components and (iv) loading the sample in aliquots with a column washing step after each aliquot.

2.7. Downstream purification of rhMBP from TGmilk

The optimized method for the direct capture of the rhMBP from TGmilk using the Tricorn 10/100 (cv 8 ml) was scaled-up by a factor of 2.5. A new column (XK 16/20) was packed to 10 cm height (cv 20 ml) and was used for isolation of the rhMBP from TGmilk. A loading buffer of 50 mM HEPES (pH 7.0) was used for column equilibration and washing. Milk samples were loaded at 2.0 ml/min in aliquots of 0.64 cv each followed by 2 cv of loading buffer at

5.0 ml/min. Elution was carried out using the same buffer in the presence of 1.0 M NaCl at 10.0 ml/min in two steps (0.5 M and 1.0 M NaCl – 5 cv each). Column effluent was collected in fractions (2.5 cv each). The fractions containing the rhMBP were pooled together and were further purified using a HisTrap HP column (cv 5 ml) as described above. The collected fractions were analyzed using SDS-PAGE/western blotting. Fractions containing the rhMBP were pooled together and the amount of the rhMBP was estimated using the dot blotting assay.

2.8. Stability study

A preliminary qualitative stability study was carried out in order to evaluate the stability of the rhMBP throughout the time and conditions employed for product recovery. The short-term stability of the rhMBP in milk, SPBB fractions, IMAC fractions and desalted concentrated fractions (rhMBP stock) was studied over 24 h in order to evaluate the matrix effect on stability of rhMBP. TGmilk samples were prepared and processed according to the developed purification protocol. A sample of the pooled IMAC fractions was desalted using 50 mM HEPES (pH 7.0) and concentrated (10×) using Vivaspin centrifugal filters (500 Da molecular weight cut off). The final preparation was considered as "rhMBP stock". Aliquots of each of the four sample types were incubated at room temperature, 4–8 °C (purification conditions) and at the specified time (0, 1, 2, 4, 8, 12 and 24 h) aliquots were taken and immediately frozen at −80 °C. Samples obtained at t_0 were considered as controls. On the other hand samples incubated for 24 h at room temperature were considered as forced degradation in order to obtain a qualitative estimate for the degradation (if any) for each set of matrix/conditions. Samples were analyzed using SDS-PAGE followed by western blotting analysis as previously described.

3. Results and discussion

A recombinant protein expressed in the milk of Tg animals could be associated to some extent with any of the three phases of the milk system. However, it has been reported that most recombinant proteins produced in milk partition into the aqueous phase and thus downstream purification usually begins with lipid and casein removal [13,14,19]. Initial western blotting analysis confirmed expression of the rhMBP and revealed that it was exclusively associated with skim milk and not found in milk fat fraction (data not shown). Therefore, skim milk was used in all subsequent investigations.

3.1. Understanding the starting material

The rhMBP was expressed as one major isoform that could be detected by either the anti-hMBP or anti-His tag monoclonal antibody. The apparent molecular weight of the rhMBP major isoform was estimated from SDS-PAGE/western blotting analysis to be 21 kDa which is in agreement to the calculated one (predicted from the gene construct employed). Dot blot assay using anti-hMBP antibody showed an expression level of 2–3 mg/ml in skim TGmilk. At first, various approaches were trialed in order to reduce the complexity of milk samples before chromatographic purification such as isoelectric point precipitation of caseins, the inclusion of additives (urea or arginine) to disrupt casein micelles and the selective precipitation of caseins using CAP nanoparticles as described above.

The use of isoelectric point precipitation of caseins or denaturants in the clarification of milk samples resulted in a poorly soluble product after the buffer exchange step. The selective precipitation of caseins using CAP particles was more successful than using the more aggressive micelle disruption procedures using either acid or denaturants regarding the solubility of the rhMBP in the final preparation. However, the rhMBP was detected also with the precipitated caseins (in the CAP particles fractions) which could explain the low yields (results not shown).

In order to design a robust and scaleable purification approach, we first investigated the localization of the rhMBP in the TGmilk system in more detail. Two milk fractionation procedures were trialed. Isoelectric point precipitation was employed as an example for milk fractionation via micelle disruption while centrifugation was considered as a good method to fractionate milk without disruption of the micelles [40]. In the latter case, the possibility of entrapment of serum milk proteins with the micelle fraction is minimal.

The resulting fractions were analyzed using SDS-PAGE and western blotting techniques. Comassie Blue-stained gels showed no difference between TGmilk and WTmilk samples (Fig. 1). The

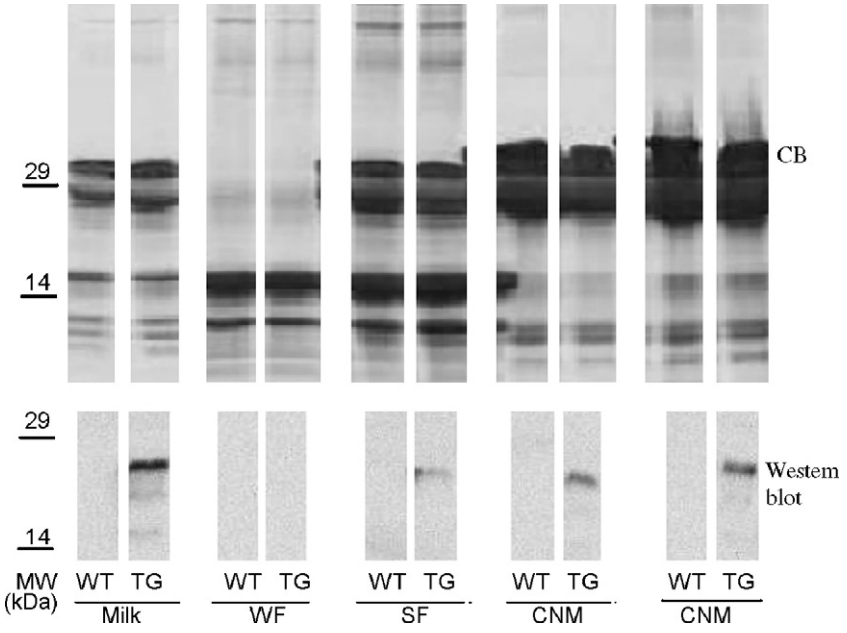

Fig. 1. CB-stained gels (top) and western blots using anti-hMBP antibody (bottom) of WTmilk, of TGmilk samples and fractions obtained from each type of milk upon centrifugation and isoelectric point precipitation. WF, whey fraction; SF, serum fraction; CNF, casein fraction and CNM, casein micelles fraction.

recombinant protein was detected by western blotting technique in CNF and CNM, traces were found in SF and no rhMBP was detected in WF (Fig. 1). These results were a clear evidence for the association of the rhMBP with the casein micellar phase. The faint response for rhMBP which was detected in SF could be attributed to the rhMBP associated with small casein micelles which remained in solution at the centrifugation speed employed. Acid disruption of the casein micelles resulted in complete precipitation of the rhMBP in the casein pellet. A large excess of urea was necessary to disrupt this interaction and dissolve the rhMBP.

3.2. Design of the purification strategy

3.2.1. Direct capture of rhMBP from milk

The chromatographic recovery of recombinant proteins from crude feed stock is a known concept and a few examples have been reported in literature [41,42]. The applicability of the direct capture of endogenous milk basic proteins using cation exchanger resins has been reported also [22,27,28]. However, this technique has hardly ever been used for downstream purification of recombinant proteins expressed in milk and clarification of milk samples prior to chromatographic processing has been a standard treatment as discussed earlier.

The theoretically calculated p*I* value for the rhMBP based on the amino acid sequence results in an isoelectric point of 10.5. It could be expected that the recombinant protein will carry a net positive charge at the pH of milk (pH ≈ 6.7). Cation exchange chromatography using SPBB could be then an ideal approach for the capture of the recombinant protein from milk. Taking into consideration that milk contains minimal amounts of endogenous basic proteins (when compared to the total amounts of proteins in milk), a high purification factor could be expected upon using cation exchange chromatography.

The zwitterionic organic buffer (HEPES) was selected for testing the usefulness of SPBB for isolation of the rhMBP from TGmilk. It has a buffer region (pH 6.8–8.2) which spans that of milk and it lacks interaction with calcium. Therefore the HEPES buffer was expected to have a minimal effect on casein micelle integrity. Moreover, the low conductivity properties of HEPES made it an ideal choice for screening of biological samples using electrophoretic techniques without extensive sample pre-treatment [43–45].

Preliminary experiments employing TGmilk loaded directly on to a small disposable column (PD-10 - cv 5 ml) confirmed excellent retention of rhMBP on the SPBB resin and showed that elution with 1.0 M NaCl was sufficient to elute any specifically or non-specifically bound proteins from the column as described above (results not shown). Once the usefulness of the direct capture approach was established, modifications to the chromatographic system were carried out. A post-column bypass line was installed in order to avoid problems associated with milk flowing through the flow cells of the detectors. Suitable software solutions were utilized via modification of the built-in strategies in order to maintain the automated capabilities of the chromatographic system.

A small column (Tricorn 10/100 – cv 8 ml) was packed with SPBB resin according to the manufacturer's guidelines. Milk samples were loaded directly to the column and elution was carried out using an increasing NaCl concentration (0.0–1.0 M). The rhMBP was detected in fractions obtained over salt concentrations ranging from 0.15 to 0.45 M using western blotting analysis (data not shown). These results suggested that a linear gradient was unable to resolve the rhMBP from other endogenous milk proteins eluting below 0.5 M salt. A step elution was considered in order to obtain rhMBP in one fraction. Direct capture of rhMBP from TGmilk was carried out using two steps of the elution buffer containing 0.5 and 1.0 M NaCl (5 cv each) as shown in Fig. 2A. Collected fractions were analyzed using SDS-PAGE and western blotting (Fig. 2B). No difference was noted in CB-stained gels; however, multiple isoforms of the recombinant protein were detected in 0.5 M salt fractions (as one major isoform and several small molecular weight minor isoforms). The 1.0 M fractions were found to contain lactoferrin (LF) which is one of the milk endogenous basic proteins, as will be discussed later. Contrary to what was expected from a highly basic

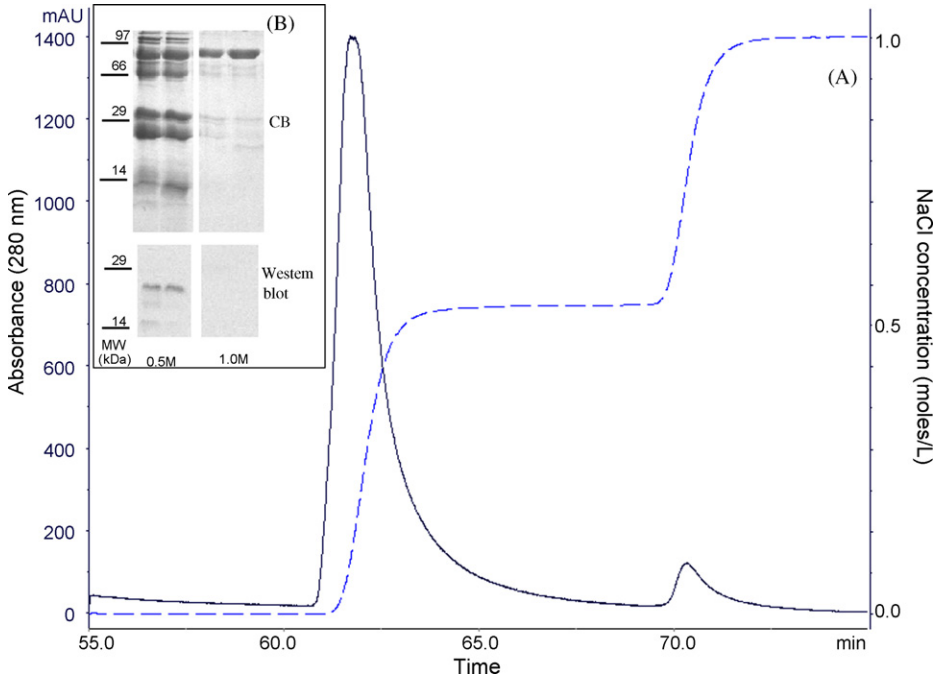

Fig. 2. (A) Chromatogram showing the elution pattern of milk basic proteins from the cation exchanger column using a two-step elution (0.5 and 1.0 M NaCl). Solid line: UV trace at 280 nm (mAU) and dashed line: NaCl concentration in (M). (B): CB-stained gels (top) and western blots (bottom) of the fractions collected from the column at 0.5 and 1.0 M steps showing the elution of the rhMBP as multiple isoforms at 0.5 M NaCl. Column: Tricorn 10/100 – cv 8 ml, sample: 30 ml TGmilk loaded at 1.0 ml/min, elution flow rate: 5.0 ml/min, loading buffer: 50 mM HEPES (pH 7.0) and elution buffer: 50 mM HEPES (pH 7.0)–1.0 M NaCl.

protein with no ordered structure such as hMBP, the recombinant protein was found to elute from the cation exchanger at low salt concentration (<0.5 M). This elution pattern along with the presence of multiple isoforms of smaller apparent molecular weight (18–20 kDa) suggested that the rhMBP is post-translationally modified. A similar banding pattern was detected when the anti-His tag antibody was used in western blotting detection which indicated that the smaller molecular weight bands are not due to proteolysis. Investigation of the nature of these isoforms is beyond the scope of this study.

3.2.2. Affinity purification of rhMBP

Taking advantage of the N-terminal His tag of rhMBP, we utilized IMAC using Ni^{2+} Sepharose resins for further purification. This method has the additional advantage that it does not add any animal-derived impurities to the final protein preparation such as the antibody-based affinity chromatography techniques. Again, a preliminary study using HisGravi columns (cv 1 ml) was carried out to optimize the IMAC step using the pooled SPBB fractions containing the rhMBP as described above (2.6.1). A loading buffer of 50 mM HEPES buffer (pH 7.0) containing 0.5 M NaCl and 50 mM imidazole was found to provide good rhMBP retention and to minimize the non-specific binding of milk proteins whereas the increase of the imidazole concentration to 0.5 M resulted in the complete elution of the bound rhMBP (data not shown).

A HisTrap HP column (cv 5 ml) was used in order to develop a column format purification step. Fractions collected from the optimized cation exchange step (as described below) were adjusted to the loading buffer specification by adding imidazole to a final concentration of 50 mM. A gradient elution over 10 cv (50 mM–500 mM imidazole) was employed and the rhMBP was detected in fractions collected at ≈0.4–0.5 M imidazole (results not shown). A sharp gradient elution over 2 cv followed by 5 cv of elution buffer was used in order to obtain the rhMBP in concentrated fractions. The IMAC purification step resulted in elution of the recombinant product in high purity as shown from the CB-stained gel in Fig. 3. It resulted also in enrichment of the minor isoforms of the recombinant protein in one fraction as shown by the western blotting detection in Fig. 3.

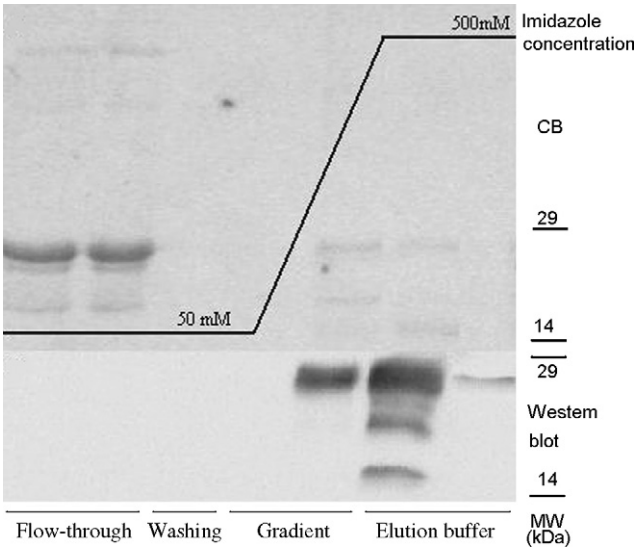

Fig. 3. Fractions collected from an IMAC run analyzed by SDS-PAGE (top) and western blotting using anti-hMBP antibody (bottom) showing the elution pattern of rhMBP with imidazole concentration in various fractions collected during the run. Column: HisTrap HP – cv 5 ml, sample: 50 ml pooled SPBB fractions loaded at 5.0 ml/min, elution flow rate: 5.0 ml/min, loading buffer: 50 mM HEPES–0.5 M NaCl–50 mM imidazole (pH 7.0) and elution buffer: 50 mM HEPES–0.5 M NaCl–500 mM imidazole (pH 7.0).

3.2.3. Short-term stability of rhMBP

Several proteolytic enzymes are present in milk and proteolysis has been a key event that determines the textural characteristics of milk products in dairy industry. However, in order to obtain high yields of a recombinant protein expressed in milk, chances for proteolysis should be minimized [13,20]. In this study, a two-step purification protocol for downstream purification of rhMBP from milk was described. However, the stability of the product has to be ensured throughout the time and conditions employed before investing in optimization of the product recovery. Short-term stability of the rhMBP in milk, SPBB fractions, IMAC fractions and rhMBP stock was studied over 24 h as described above. Unexpectedly, the rhMBP was found to be highly stable in milk even at room temperature. In contrast, rhMBP was no longer detectable in SPBB fractions following 24 h incubation at room temperature indicating complete degradation under such conditions. The rhMBP showed a good stability in IMAC fractions at 4–8 °C. However, the rhMBP stock showed intermediate stability at room temperature with degradation becoming detectable after 12 h.

These results were of extreme value since milk is the sample required to withstand the longest processing time. The good stability of the rhMBP in milk minimizes chances for product loss during the early in-farm processing steps. The relative instability of rhMBP in SPBB fractions when compared to IMAC fractions had a direct impact on the downstream purification strategy. Fractions obtained from SPBB have to be either processed to the next step as soon as they were collected or stored frozen until the next step. A possible explanation for the instability of rhMBP in SPBB fractions could be attributed to the non-specific retention of some of the proteolytic enzymes on the SPBB resin. On the other hand, the noted stability of the rhMBP in milk even at room temperature could be explained on the basis of association of the recombinant protein with the micellar phase. In order to minimize any chances for degradation during the buffer exchange and concentration steps, the use of a refrigerated centrifuge was recommended for future applications. The good stability of the rhMBP stock under different temperatures was crucial for the product analysis steps.

In this experiment, western blotting analysis was used successfully to investigate the stability of the rhMBP in milk fractions throughout the conditions encountered during its purification. However, to obtain a quantitative evaluation of the stability of the rhMBP, a more reliable analytical technique which is capable of tracing minor changes in intact rhMBP amounts has to be employed. The development and optimization of such testing protocol for rhMBP forms part of an ongoing work.

3.3. Optimization of the direct capture of rhMBP from milk

3.3.1. Premature breakthrough

The downstream purification protocol was considered successful with respect to the following: direct capture of intact rhMBP from milk with no denaturing additives, good purity (≈90%) and solubility of the product in the final preparation, and the ability to monitor the rhMBP throughout the purification steps. However, when milk samples were loaded to the cation exchanger column, a premature breakthrough was noted in the flow-through fractions.

Association of the rhMBP with milk caseins has been suggested after studying the results obtained by centrifugation and isoelectric point precipitation of TGmilk samples. The relative stability of rhMBP in TGmilk samples supported this hypothesis. The amount of the rhMBP retained on the SPBB column, from the same volume of milk, was found to improve upon reducing the sample loading flow rate which indicated a slow kinetics which controls the release

of the rhMBP into serum (results not shown). Investigation of the type of the molecular interaction between the rhMBP and other milk proteins is beyond the scope of this study and forms part of an ongoing work.

3.3.2. Effect of endogenous milk basic proteins

The elution pattern of the rhMBP suggested that it does not bind strongly to the resin as discussed earlier. The weak binding of the rhMBP to the resin could be also contributing to the early breakthrough noted since milk contains other endogenous basic proteins which compete with the rhMBP for the binding sites. Lactoferrin is one of the endogenous milk basic proteins which has a molecular weight of 77 kDa [20]. Extraction of LF from milk has been reported before using SPBB and it was found to elute in a pure form at high salt strength (0.9–1.0 M NaCl) [22,27,28]. In this study, LF was detected in the 1.0 M fraction using SDS-PAGE by its molecular weight (Fig. 2B) and was considered as a marker for a strongly bound endogenous milk basic protein. In this experiment, the integrated peak area was employed as an estimate of the total amount of basic proteins retained by the column and eluting with 0.5 and 1.0 M NaCl. A two-fold increase in the volume of loaded TGmilk sample to the cation exchanger column (under the same experimental conditions) resulted in increase in integrated peak areas of 82% and 255% for 0.5 and 1.0 M fractions respectively (Fig. 4A). The band corresponding to LF in the latter case (CB-stained gel) was found to be more intense while that corresponding to the rhMBP (dot blotting) was found to be less intense upon increasing the milk volume loaded (Fig. 4B). These results suggested that the amounts retained of the strongly bound endogenous milk basic proteins such as LF was enriched on the expense of the weakly bound rhMBP. The competition between the rhMBP and endogenous milk basic proteins for the active sites of the resin does not favor rhMBP retention.

3.3.3. Effect of milk calcium

In a systematic study for evaluation of the influence of major components in milk on the chromatographic recovery of a target protein from milk, the effect of calcium was found to be significant. Milk calcium was found to compete for the adsorption sites of the resin leading to a significant reduction in mass transfer process. The effect of calcium was found of higher significance than that of either casein micelles or lipid [23]. Milk contains approximately 30 mM of calcium with about 68% associated with caseins. Micellar calcium plays a key role in the maintenance of the structure and stability of casein micelles and it has been reported that it is in equilibrium with the soluble calcium fraction [46,47].

The effect of reducing the milk sample ionic strength by dilution with either MilliQ water or loading buffer on the retention behavior of rhMBP was studied. A four-fold dilution was found sufficient to bring the conductivity of the milk samples close to that of the loading buffer. The amounts of rhMBP retained on the column were determined using the developed dot blot assay for rhMBP (results not shown). There was no apparent difference in using water or loading buffer as the diluting agent. Dilution resulted in an increase in the amount of rhMBP that was retained on the column. However, the effect of dilution was found comparable to reducing the sample loading flow rate to the extent that kept the residence time of a sample in the column constant.

It could be concluded from these results that the early breakthrough is mainly due to the association of the rhMBP with the casein micelles. Alkaline earth metals in milk reduce the dynamic capacity of the cation exchanger column as discussed above. Under such reduced capacity, increasing the milk volume loaded results in enrichment of the more strongly bound milk proteins over the weakly bound ones including our target protein. The effects of either reducing the sample loading flow rate or reducing the sample ionic strength are comparable. However, dilution does not release the rhMBP from the micelles and reducing the sample flow rate does not help reduce the effect of milk calcium. It could be suggested also that in order to enhance the recovery of the rhMBP from milk samples, gentle disruption of the casein micelles and reducing the effect of calcium in milk, in a more efficient way than dilution, are the key parameters.

3.3.4. On-line casein micelles disruption

While developing this capture protocol using the PD-10 disposable columns (as described), it was noted that the first few flow-through fractions always had an unusually transparent appearance. The total volume of these fractions was found to be larger than the void volume of the column. Casein micelles disrup-

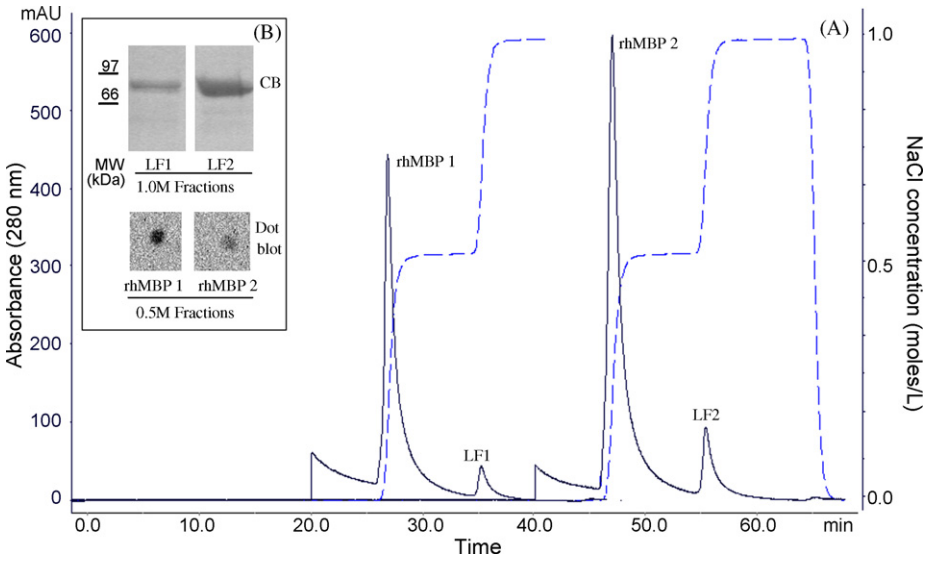

Fig. 4. (A) Chromatograms showing the relative increase in peak height and peak area upon increasing the volume of milk sample loaded to the cation exchanger column from 10 to 20 ml respectively. Solid line: UV trace at 280 nm (mAU) and dashed line: NaCl concentration in (M). (B) CB-stained gel (top) showing the increase in intensity of LF band and dot blots using anti-hMBP antibody (bottom) showing the decrease in intensity of the rhMBP bands upon increasing the volume of milk loaded to the cation exchanger column. Column: Tricorn 10/100 – cv 8 ml, sample: 10 and 20 ml TGmilk loaded at 1.0 ml/min, elution flow rate: 5.0 ml/min, loading buffer: 50 mM HEPES (pH 7.0) and elution buffer: 50 mM HEPES (pH 7.0)–1.0 M NaCl.

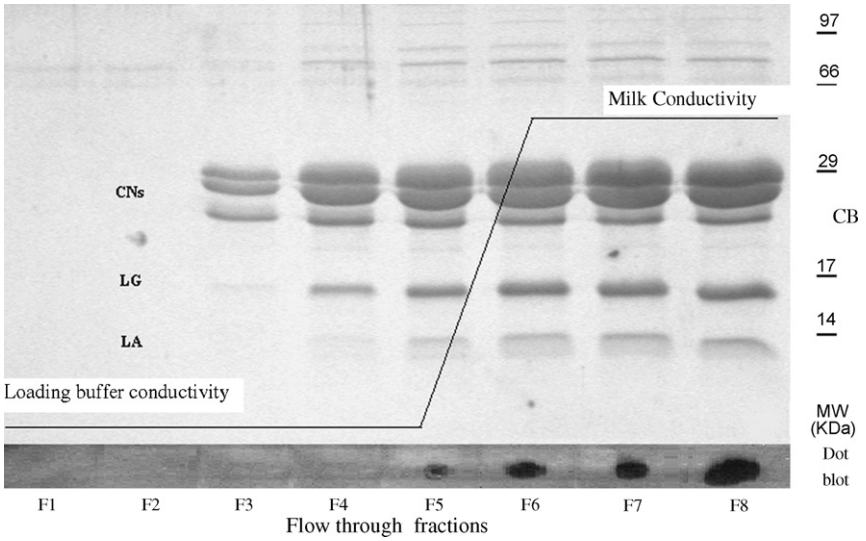

Fig. 5. CB-stained gel (top) of the flow-through fractions collected upon loading TGmilk to the cation exchanger column and dot blot analysis using anti-hMBP (bottom) for the same fractions showing that the rhMBP is detectable in the flow-through fractions F5–F8. A diagrammatic representation of the change in ionic strength of the flow-through fractions is overlaid. Column: Tricorn 10/100 – cv 8 ml, sample: 25 ml TGmilk loaded at 1.0 ml/min, elution flow rate: 5.0 ml/min, loading buffer: 50 mM HEPES (pH 7.0) and elution buffer: 50 mM HEPES (pH 7.0)–1.0 M NaCl.

tion during the loading step was then considered as the main reason for a transparent flow-through. Additional investigations were carried out in order to evaluate the significance of this phenomenon on the retention capacity of SPBB resin towards the rhMBP.

A sample of TGmilk was loaded to the column (Tricorn 10/100 – cv 8 ml) at 1.0 ml/min and the flow-through fractions were collected (2 ml each) and analyzed using SDS-PAGE and dot blot analyses. Fig. 5 shows that the early flow-through fractions (F1–F2) contain high molecular weight milk proteins only which indicated that the column dead volume has been exceeded. Caseins were detected in the following flow-through fractions (F3–F4) while the flow-through appearance was still transparent. This observation clearly showed that the casein micelles disruption is in progress during this stage. On-line monitoring of the conductivity of the flow-through indicated that the conductivity of the transparent flow-through fractions was similar to that of the starting buffer. The presence of the caseins in the flow-through without a milky appearance or an increase in the conductivity of the flow-through strongly suggested that disruption of the casein micelles was due to adsorption of the calcium to the active sites of the resin. Dot blot screening of the transparent fractions showed no detectable rhMBP specific signal. When the flow-through started to look like milk, the conductivity of the flow-through was found to increase progressively and the rhMBP was detected in the flow-through (F5–F8). These results suggested that the appearance of the rhMBP in the flow-through is directly linked to the presence of intact casein micelles in the flow-through fractions.

Based on the above results and taking into consideration the key role of calcium in maintaining casein micelle integrity it could be concluded that micelle disruption was a direct impact of calcium binding to the adsorption sites of the resin. Micelle disruption during milk loading could provide a novel approach for the recovery of recombinant proteins expressed in milk and exclusively associated with the micellar phase. In order to investigate this hypothesis, two TGmilk samples (25 ml) were loaded to the column (Tricorn 10/100 – cv 8 ml) either as one aliquot (Fig. 6A) followed by a wash step or in five aliquots (Fig. 6B) with column washing in between. A wash step of 2–3 cv of the loading buffer was enough to reduce the conductivity of the flow-through to that of the loading buffer. A software modification to the Unicorn platform controlling the chromatography system was carried out in-house in order to maintain the whole process automated and to integrate this function with the bypass function developed before. The flow-through fractions were collected and elution was carried out in one step using 1.0 M NaCl. The total amount of protein retained on the column was compared using the values of integrated peak area (Fig. 6, peak 2) obtained in each case. The amounts of non-specifically retained milk proteins (Fig. 6, peak 1) on the column was compared. Trans-column pressure build-up was monitored during the sample loading steps (sample pump) and throughout the run (main system pump). The dot blot assay was used to estimate the concentration of the rhMBP in eluted fractions in both cases.

The chromatograms obtained were compared with respect to integrated peak area, width and height. The rhMBP concentration in the fractions collected at 1.0 M NaCl was compared. All results were expressed relative to those obtained by loading the milk sample in one aliquot and summarized in Table 1.

A relative increase in the integrated peak area (29.10%) and the peak height (31.53%) accompanied by a decrease in the peak width at half height (14.78%) were noted when the sequential sample loading method was employed. These results demonstrated that the total amount of protein retained on the column was significantly higher using this method. Narrower peak width indicated the improvement in column dynamics (Fig. 6) by loading the milk sample in aliquots with column washing steps after each aliquot. Moreover, the dot blot assay revealed a 25% increase in the amount of eluted rhMBP when the sequential loading approach was employed. A decrease of 88.16% in the amount of non-specifically retained proteins (peak 1) was also noted. A profound decrease in the trans-column pressure during the sample loading, wash and elution steps was recorded which indicated an improvement in the hydrodynamic properties of the column when the sample was loaded in aliquots. This reduction in the trans-column pressure could be attributed to the decrease in the amount of casein micelles physically blocking the pores of the resin.

The flow-through was found to remain transparent over a much larger volume which indicated that the casein micelle disruption phase was extended when the sample was loaded in aliquots (Fig. 6). This observation suggested that the calcium occupying the active sites of the resin was washed off the column by the loading buffer regenerating free adsorption sites available for binding of additional rhMBP during the next loading step. Once most of

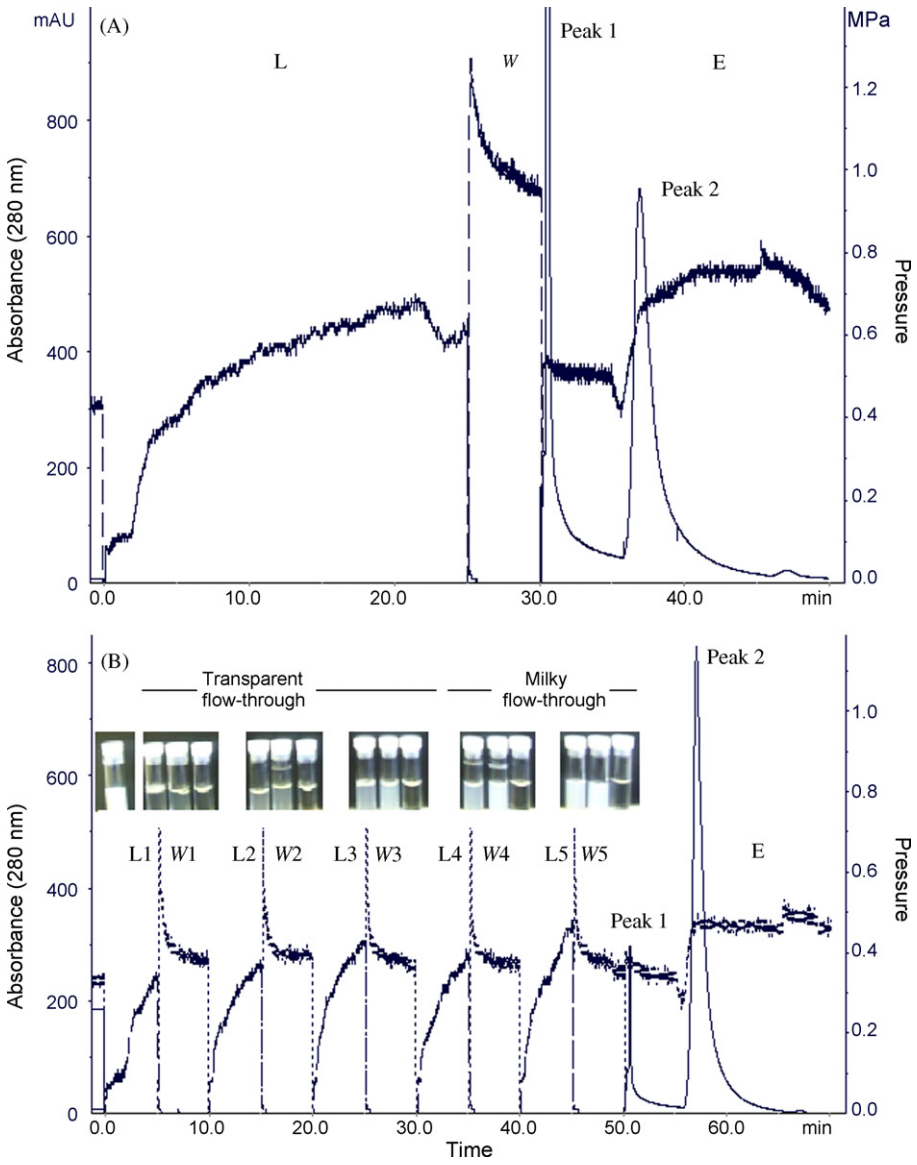

Fig. 6. A comparison between the conventional sample loading approach (A) and the sequential loading approach (B) showing pictures for the flow-through fractions collected at the latter case. The UV trace (left *Y*-axis vs time) shows the elution pattern from the column with "Peak 1" representing the non-specifically retained proteins eluting during the wash step and "Peak 2" representing elution of all selectively retained proteins from the column using a step elution (1.0 M salt). The trans-column pressure (right *Y*-axis vs time) recorded at different parts of the run was overlaid; L, loading; W, wash and E, elution. Column: Tricorn 10/100 – cv 8 ml, sample: 25 ml TGmilk loaded at 1.0 ml/min, elution flow rate 5.0 ml/min, loading buffer: 50 mM HEPES (pH 7.0) and elution buffer: 50 mM HEPES (pH 7.0)–1.0 M NaCl.

the adsorption sites were again occupied calcium could no longer bind, the micelles remained intact and the flow-through started to appear milky as shown in Fig. 6B. Two to three sequential loadings of ≈0.64 cv were found to be the maximum volume of the loaded milk sample. Loading larger volumes of milk samples resulted in enrichment of other strongly bound endogenous milk basic proteins (such as LF) and thus premature elution of the rhMBP as discussed previously.

A scale-up experiment (scaling factor 2.5) was carried out in order to evaluate the applicability of the modified loading approach at scale. A bigger column (XK 16/20 – cv 20 ml) was packed to a bed height of 10 cm using the same resin (SPBB). The performance of

Table 1
A comparison between the direct sample loading approach and the sequential loading approach showing the relative increase/decrease in the parameters studied as obtained from Fig. 6.

Parameter		Direct loading method	Sequential loading method
Peak 1	Integrated peak area (mAU*min)	677.99	80.30 (−88.16%)[a]
Peak 2	Peak height (mAU)	618.01	812.87 (+31.53%)[a]
	Peak width at half height (min)	1.15	0.98 (−14.78%)[a]
	Integrated peak area (mAU*min)	811.78	1047.90 (+29.10%)[a]
	rhMBP concentration[b] (μg/ml)	16.0	20.0 (+25%)[a]

[a] Relative to the corresponding values obtained using the direct loading method.
[b] Dot blot assay.

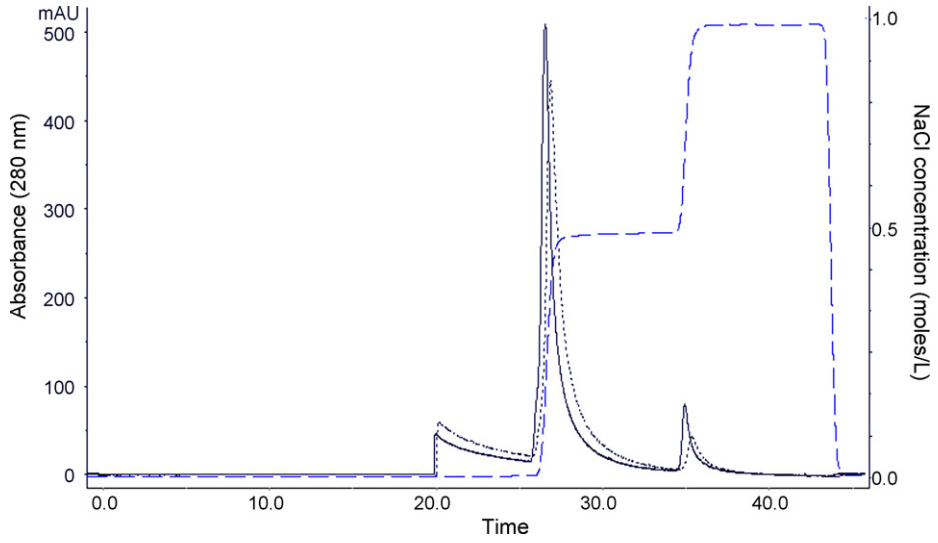

Fig. 7. Chromatograms showing the overlap in elution pattern obtained from the columns used in the scale-up experiment. Column 1: Tricorn 10/100 – cv 8 ml (------), sample volume: 2 × 5 ml TGmilk loaded at 1.0 ml/min, elution flow rate: 5.0 ml/min. Column 2: XK 16/20 – cv 20 ml (—), sample volume 2 × 12.8 ml TGmilk loaded at 2.6 ml/min, elution flow rate 12.8 ml/min. Loading buffer: 50 mM HEPES (pH 7.0) and elution buffer: 50 mM HEPES (pH 7.0)–1.0 M NaCl. Solid line: UV trace at 280 nm (mAU) and dashed line: NaCl concentration in (M).

the XK column was compared to that of the Tricorn 10/100 (cv 8 ml) which was used for method development. The loading and elution steps were carried out at the same linear velocity and using the same number of column volumes. The milk sample was loaded in two aliquots while maintaining the ratio of milk volume per aliquot to the bed volume at 0.64 cv. A good overlap in elution profile over the time scale of the run indicated a successful scale-up experiment (Fig. 7). The fractions containing the rhMBP were pooled together and further purification was carried out using the HisTrap HP column (cv 5 ml) as previously described. The concentration of the rhMBP in the starting TGmilk sample was compared to that of the pooled fractions after the SPBB and the IMAC steps employing the dot blotting assay and the recovery percentage was found to be 78%.

4. Conclusion

In this study, a novel automated approach for the direct chromatographic capture of a model transgenic protein was developed. The method proved to be useful for transgenic basic proteins which could be partially or completely associated with the milk micellar phase. The interaction between the micellar calcium and the adsorption sites of the cation exchanger resin was utilized in order to disrupt the micelles and liberate the target protein. Simultaneous product capture and on-line casein micelle disruption obviated the need for the use of denaturing additives. The new approach was found to improve not only the retention properties of the target protein but also the hydrodynamic properties of the chromatographic matrix. Characterization of different rhMBP isoforms as well as understanding the nature of the interaction between the model transgenic protein and milk caseins on the biomolecular level forms a part of an ongoing study.

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