Talanta 109 (2013) 7–12

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

# Coupled solid phase extraction and microparticle-based stability and purity-indicating immunosensor for the determination of recombinant human myelin basic protein in transgenic milk

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ARTICLE INFO

*Article history:*
Received 2 February 2013
Received in revised form
14 March 2013
Accepted 16 March 2013
Available online 22 March 2013

*Keywords:*
Biopharmaceuticals
Optical immunosensors
Bio-Plex
Myelin basic protein
Solid phase extraction

ABSTRACT

An optical immunosensor was developed and validated on the surface of microparticles for the determination of a biopharmaceutical protein. The recombinant human myelin basic protein (rhMBP) was produced in milk of transgenic cows as a His-tagged fusion protein. Previous work indicated exclusive association of rhMBP with milk casein micelles that hindered direct determination of the protein in milk. In this work, a solid phase extraction using a cation exchange matrix was developed in order to liberate rhMBP from casein micelles. A sandwich-type immunoassay was then used for in-process monitoring of the full-length protein in the presence of major milk proteins. The assay was successfully employed for detection of ultra-traces of rhMBP (LOD = 6.04 ng $mL^{-1}$ ≈ 0.3 nmol $L^{-1}$) and for quantitative determination over a wide concentration range (10.00–10,000.00 ng $mL^{-1}$). The assay was able also to detect the rhMBP in the presence of its human counterpart that lacks the His-tag. The high sensitivity along with the ability of the assay to determine the full length protein enabled monitoring of the stability of rhMBP. The testing protocol is particularly useful for intrinsically unstructured proteins that are extremely sensitive to proteolysis and lack a traceable enzymatic activity. This immunosensor provides a specific, ultrasensitive high throughput tool for in-process monitoring in biopharmaceutical industry.



## 1. Introduction

Production of recombinant biopharmaceuticals is a complex process and a tremendous effort and cost is involved in the development of every new product. A well-defined analysis strategy that addresses the key features of the recombinant product has to be developed and validated [1–3]. A rapidly growing list of intrinsically unstructured proteins (IUP) has recently gained much attention as new therapeutic modalities. It is believed that the inherent flexibility and the highly adaptive structure of these proteins are crucial to their functions. They are able to associate with one or more binding partner according to the surrounding environment. IUP lack traceable enzymatic activity and are extremely sensitive to proteolysis. From a biopharmaceutical industry point of view, the production of IUP, with all their unique characteristics is challenging especially when it comes to monitoring the quality attributes of such products such as activity, stability and purity [4,5].

Human myelin basic protein (hMBP) is a typical IUP that forms about 35% of the protein fraction of the myelin sheath. The adaptive structure of hMBP along with its highly basic character ($pI > 10.5$) allows an efficient interaction between hMBP and the negatively charged phospholipids in the myelin sheath. Thus, insulate nerve fibers for efficient transmission of impulses [6]. The hMBP is generally considered as the autoantigen in multiple sclerosis (MS), an autoimmune disease characterized by active degradation of the myelin sheath. Recent research suggested that administration of neuroantigens including hMBP to MS patients can tolerize the autoimmune response [7,8].

In previous work, recombinant human myelin basic protein (rhMBP) was produced in the milk of transgenic cows in order to develop a therapeutic vaccine for multiple sclerosis. The recombinant protein was detected in milk as a multiple charge isoforms. This was attributed to different patterns of posttranslational modifications (PTM) [9]. The basic nature of rhMBP along with its intrinsically unstructured conformation resulted in exclusive association of rhMBP into milk micellar phase. This association

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http://dx.doi.org/10.1016/j.talanta.2013.03.043

was explained on the basis of an interaction between the highly basic rhMBP and milk caseins; a group of acidic, IUP present in milk [10]. Lack of a traceable enzymatic activity complicated the monitoring of the recombinant protein during its downstream purification [9].

The high specificity and sensitivity of immunoassays account for their indispensable role in quality control protocols. Enzyme linked immunosorbant assay (ELISA) has been the standard for quantitative protein immunoassays. ELISA is specific and reproducible yet time consuming, sample volume demanding and cannot determine more than one analyte per sample simultaneously. On the other hand, advances in biosensor technology provided valuable tools for the development of semi-automated, high throughput immunoassays [11,12]. The inherent recognition properties of antibodies impart biosensors with high sensitivity and selectivity [13,14].

Advances in particle encoding technologies enabled the development of semi-automated, multiplexed immunoassays on the surface of color coded micro beads. Each of these bead types can be used to probe an analyte on its surface using the sandwich assembly characteristic for ELISA. Coupling of the capture antibodies to the surface of the beads is carried out in a separate step which resulted in a significant reduction of the analysis time when compared to that required for ELISA [11,15–17]. Several applications covering a wide variety of analytes in different matrices have been reported in the literature [18–24]. However, the applicability of this technique for the analysis of milk components or the analysis of biopharmaceuticals produced in the milk of transgenic animals appears not explored yet.

In this work, a sandwich type, optical immunosensor on the surface of micro beads was developed. The applicability of solid phase extraction (SPE) for the reduction of milk sample complexity and the removal of matrix interference were investigated. The ability of the developed testing protocol to monitor the quality attributes of rhMBP in milk and along the downstream purification process was explored.

## 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 day sample from transgenic cows (TGmilk) and wild type control cows (WTmilk) of the same genotype, except for the transgene insertions. All milk samples used in this study were prepared from defatted, freeze-dried milk powder by dissolving suitable amounts in MilliQ water to 10% w/v concentration. A standard hMBP (1.0 mg $mL^{-1}$) was purchased from Research Diagnostics (USA) for comparative purposes (cat no. RDI-TRK8M79). A mouse monoclonal anti-hMBP antibody (100.0 μg $mL^{-1}$—cat no. sc-71547) which recognizes the amino acid sequence 130–136 of hMBP was purchased from Santa-Cruz Biotechnology (USA). A biotinylated rat anti-mouse (500.0 μg $mL^{-1}$—cat no. 553388) and a biotinylated anti-His tag monoclonal antibodies (200.0 μg $mL^{-1}$—cat no. 34440) were purchased from BD Biosciences (USA) and QIAGEN (Germany) respectively. Coupling reagents used for the preparation of the sensor were purchased from Bio-Rad, USA (cat no. 171-406001). For western and dot blotting, a rat anti-hMBP monoclonal antibody (cat no. ab7349) which recognizes amino acids sequence 82–87 and a horse radish peroxidase-labeled anti-rat monoclonal antibody (cat no. A5795) were obtained from Abcam (UK) and Sigma (USA) respectively. Nitrocellulose membranes were obtained from Bio-Rad (USA). All other chemicals were of analytical grade and were obtained from Sigma (USA).

### 2.2. Instruments

A Bio-Plex Suspension Array System controlled by Bio-Plex Manager software was used (Bio-Rad, USA). Surface plasmon resonance experiments were carried out using a Biacore X100 system at 25 °C. The Biacore control and Biacore evaluation modules were used to control the instrument and analyze the data respectively. Non-linear calibration models were generated using SPSS statistical package. The SPE experiments were carried out using a vacuum manifold and Bond Elut SPE cartridges, 3 mL with two Frits (Agilent Technologies, Germany) packed manually with SP Sepharose HP, 34 μm, CV=1 mL (GE Healthcare, USA).

### 2.3. Preparation of rhMBP reference standard and control samples

An in-house reference standard of the rhMBP was prepared from TGmilk samples as previously described [9]. Briefly, direct capture of the rhMBP from the TGmilk was achieved using cation exchange chromatography (SP Sepharose BB, GE Healthcare, USA). The rhMBP was eluted from the column using 50 mmol $L^{-1}$ HEPES (pH 7.0) containing 0.5 mol $L^{-1}$ NaCl "SPBB fractions". Fractions containing the rhMBP were further purified using immobilized metal affinity chromatography (IMAC, $Ni^{2+}$ Sepharose FF, GE Healthcare, USA). A gradient elution was employed (50–500 mmol $L^{-1}$ imidazole) and fractions containing the rhMBP were pooled together "IMAC fractions". Concentration and buffer exchange to 1.0 mg $mL^{-1}$ were then carried out using 50 mmol $L^{-1}$ HEPES (pH 7.0). The identity, purity and integrity of the rhMBP in the final preparation were confirmed using SDS-PAGE followed by western blotting detection with anti-hMBP and anti-His tag antibodies. The total amount of rhMBP was determined by the dot blotting assay using anti-hMBP antibody. The freeze and thaw stability, short-term temperature stability as well as the stock solution stability of the rhMBP preparation was evaluated and results were documented in accordance to the FDA guidelines [25]. The same procedure was repeated using the WTmilk for preparation of a negative control sample (WTcontrol). The rhMBP standard (1.0 mg $mL^{-1}$) and the WTcontrol were stored in aliquots (−80 °C) and were used for studying matrix effects and validation of the immunoassays.

### 2.4. Coupling of anti-hMBP antibody to the fluorescent micro beads

Coupling of the anti-hMBP antibody to the surface of the fluorescent polystyrene micro beads (diameter 6.5 ± 0.2 μm) was carried out employing an amine coupling protocol according to the manufacturer's guidelines and a published protocol [26]. The coupling reaction was carried out using three different amounts of the mouse anti-rhMBP antibody (1.2, 1.8 and 2.4 μg). The efficiency of the surface coverage in each case was evaluated employing a biotinylated anti-mouse antibody and streptavidin coupled *R*-phycoerythrin (SA–PE) and the median fluorescence intensity (MFI) obtained in each case was compared. Preparation of a larger number of beads was then carried out and the coupled beads were stored away from light at 4 °C.

### 2.5. Optimization of the analysis conditions

In order to determine the optimum concentration of the detection antibody (anti-His tag antibody), three aliquots of the rhMBP reference standard over a wide concentration range were prepared in 50 mmol $L^{-1}$ HEPES (pH 7.0). A similar procedure was followed to prepare an equivalent serial dilution of the WTcontrol sample. Aliquots of the coupled bead suspension (≈10,000 beads) were transferred to each well of a 96-well filter plate pre-wetted with the HEPES buffer then washed with the same buffer. Aliquots of 50 μL of the test and control samples were transferred to the

filter plate (in duplicate) and were incubated with the coupled beads for 30 min, followed by another incubation step for 30 min with different amounts of the biotin-labeled anti-His tag antibody (25 μL of 1:250, 1:500 and 1:1000). Detection was carried out using the Bio-Plex system after an incubation step with 50 μL SA–PE (1:1000) for 10 min and the optimal dilution factor of the anti-His tag antibody was determined. All incubation steps were carried out at RT, away from light and on the surface of a plate shaker (300 rpm). Washing steps (3 × 100 μL of HEPES buffer) were carried out after each incubation step and excess reagents/washing buffers were removed from the filter plate by vacuum.

### 2.6. Calibration and validation

A serial dilution of the rhMBP standard covering a wide range of concentration, blank and WTcontrol samples were prepared using the HEPES buffer in a 96-well microtiter plate. Aliquots of 50 μL of the samples were transferred to the corresponding wells in the 96-well filter plate. Samples were incubated with the coupled beads, the biotin-labeled anti-His tag antibody and the SA–PE as described above. A calibration curve was obtained by plotting the MFI vs. the rhMBP concentration. The same protocol was used to analyze all validation samples.

### 2.7. Sample pre-processing and application

Matrix interference was investigated through spiking of known concentrations of rhMBP standard into different sample matrices derived from WTmilk as described above. For determination of rhMBP in neat milk, an offline casein micelle disruption was carried out. A suitable volume of dilute milk with MilliQ water was passed through a 3 mL Bond Elut SPE cartridge packed with SP Sepharose HP (34 μm, CV = 1 mL) using a mild vacuum. The cartridge was equilibrated and washed with 3 mL 50 mmol $L^{-1}$ HEPES (pH 7.0) and elution was carried out using 3 mL 50 mmol $L^{-1}$ HEPES (pH 7.0) containing 1.0 mol $L^{-1}$ NaCl. Samples of chromatographic fractions were measured without pre-processing after dilution with 50 mmol $L^{-1}$ HEPES (pH 7.0).

## 3. Results and discussion

### 3.1. Preparation of rhMBP reference standard and control samples

While developing the downstream purification protocol [9], the commercially available CNS-derived hMBP standard was used for comparative purposes in western and dot blotting applications using anti-hMBP antibodies. The hMBP is the product of differential splicing of a single mRNA transcript that results in four molecular weight isoforms (21.5, 20.2, 18.5 and 17.2 kDa), with the 18.5 kDa representing the major isoform. Further microheterogeneity exists due to a myriad number of PTM resulting in a variety of charge isoforms [27,28]. A standard of only one molecular weight isoform is available from recombinant *E. coli* but lacks the characteristic PTM of mammalian proteins.

The studied rhMBP was produced in milk of transgenic cows as N-terminal His tagged fusion protein. The recombinant protein was detected in TGmilk by western blotting as one major isoform (≈21 kDa) and several charge isoforms (18–20 kDa) that were attributed to PTM [9]. Development and validation of analytical methods requires the availability of a reference standard of identical nature to the analyte and of known purity and concentration [29,30]. Thus, an in-house rhMBP reference standard was prepared, characterized against the commercially available hMBP standard and was used for the immunosensor development and validation. Western blotting confirmed the identity and stability of the rhMBP standard over three freeze/thaw cycles, at room temperature for 4 h and 4–8 °C for 6 h [31]. A dot blot immunoassay was used to estimate the concentration of the rhMBP in the final preparation (1.0 mg $mL^{-1}$). The rhMBP concentration, determined using the dot blot assay, was compared to the total protein concentration, determined using a capillary electrophoresis method [32], and the purity in the final preparation was estimated to be ≈90%. The same procedure was followed using the WTmilk in order to prepare a set of negative control samples for assay validation.

### 3.2. Assay development and optimization

In this study, an anti-hMBP antibody, which detects an epitope very close to the C-terminus of the rhMBP was immobilized to the surface of the fluorescent beads. The detection of the captured rhMBP was achieved using a biotin conjugated anti-His tag antibody. The detection antibody was shown before in our western blotting studies to have a similar detection profile of the rhMBP when compared to the anti-hMBP antibody. The well characterized interaction between the biotin label and the fluorescent marker SA–PE was used to detect the antibody sandwich using the Bio-Plex system. Taking into consideration that this matched antibody pair detects two epitopes at the opposite ends of the rhMBP molecule, it could be concluded that only full length His tagged rhMBP molecules will be detected regardless of the PTM pattern. The sensor was employed for the development of in-process stability and activity indicating assay for the rhMBP since the immunogenic activity of rhMBP is the only activity to trace.

Three aliquots of the mouse anti-hMBP antibodies were coupled to three aliquots of an equal number of beads. The amount of anti-hMBP antibody coupled to the bead surface in each case was determined using a biotin-labeled anti-mouse antibody. An amount of 1.8 μg anti-hMBP antibody/20 μL beads was found to be the optimal amount for the coupling reaction. The stability of the coupled beads was evaluated over a period of 6 months (results not shown). The functionality of the coupled beads was investigated via the analysis of a set of the rhMBP standard samples. Blank samples and an equivalent set of WTcontrol samples were included for comparative purposes. Different concentrations of the detection antibody were trialed in order to optimize the detection step. Results indicated that the selected antibody pair was able to probe the rhMBP in the samples. The control samples gave a MFI comparable to that of the blank samples which indicated that any impurities remaining in the standard does not interfere with the assay. A low background signal suggested that the washing protocol employed had successfully removed excess reagents. An anti-His tag antibody dilution of 1:1000 was selected since the MFI obtained at this dilution was comparable to that obtained from the more concentrated antibody preparations (results not shown).

### 3.3. Calibration and validation

The hMBP lacks the His tag located at the N terminus of of rhMBP. The ability of the immunosensor to differentiate between the recombinant protein and its human counterpart was utilized as a measure of its specificity. This was demonstrated via the analysis of a set of the hMBP standards equivalent to the rhMBP standard series. Lack of any specific response from the hMBP standard series provided good evidence that the assay is specific to full-length rhMBP (Fig. 1) and degraded protein molecules will not interfere. Since all rhMBP isoforms are His-tagged [9], the developed method was considered as a total activity and stability-indicating assay for the rhMBP.

Serial dilution of the rhMBP standard, WTcontrol samples and blank samples were prepared and analyzed. The MFI was plotted

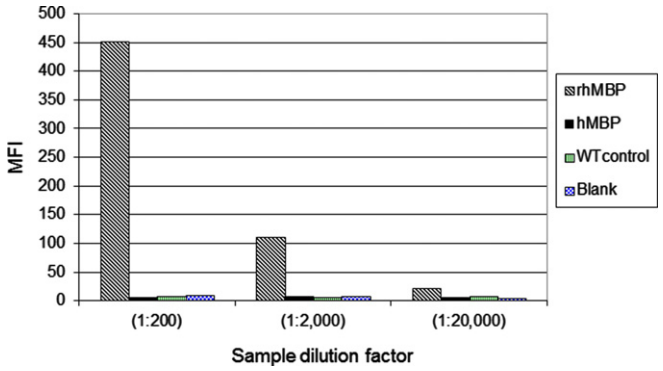


**Fig. 1.** A plot of the MFI obtained by analyzing diluted samples (1:200, 1:2000 and 1:20,000) of the rhMBP standard (1.0 mg $mL^{-1}$), hMBP standard (1.0 mg $mL^{-1}$), WTcontrol and blank samples using the SPE-immunosensor method showing the specificity of the method to the rhMBP and lack of matrix interference.

against the log of the rhMBP concentration (Fig. 2). The 4-PL nonlinear regression model [29,33] was found to be the best fitting model ($R^2$ 0.998). The fit was found not to improve upon using the 5 PL model which indicated that the response was symmetric across the inflection point of the calibration curve. The resulting regression equation was used to back predict the concentrations of the reference standards used to generate the model. The average recovery percent at P 0.05 (observed/expected × 100) was found to be 101.53 ± 3.01 The LOD was calculated from the standard deviation of the background MFI (blank response $n=12$) and was found to be (6.04 ng $mL^{-1}$). The LLOQ and the ULOQ were obtained so that the calculated percent relative error (%RE) meets an intra-assay precision of ≤15% and inter-assay precision of ≤20%. The assay range was found to be (10.00–10,000.00 ng $mL^{-1}$). The predicted concentrations of the standards were compared to the actual ones using ANOVA (P 0.10). No significant differences indicated good accuracy of the model.

A series of the rhMBP standards covering the assay range was analyzed in triplicate. The predicted concentrations were compared to the actual concentrations. Intra-assay variability (within the same day) was carried out by comparing the MFI obtained at each concentration level. The same procedure was repeated over two different days and the inter-assay variability was investigated in a similar manner. Statistical comparisons were carried out using ANOVA (P 0.10) and no significant difference indicated good repeatability and reproducibility of the method.

### 3.4. Application

#### 3.4.1. Determination of rhMBP in milk and chromatography fractions

A strong interaction between the rhMBP and other milk proteins has been demonstrated and confirmed [9,10]. Thorough investigation of possible matrix effects was essential in order to evaluate the applicability of the method for determination of the rhMBP concentration in various milk fractions. A sample of the WTmilk was processed using the optimized downstream purification protocol. Samples of the WTmilk and of each of the chromatography fractions (WT-SPBB and WT-IMAC) were obtained and equal amounts of the rhMBP were spiked in each sample at three different levels. The MFI of each set was compared to that obtained from an equivalent set of the rhMBP standard prepared in the assay buffer. The response obtained from spiked WTmilk was much lower than expected which confirmed the severe matrix interference (Fig. 3). Such interference was eliminated upon application of the SPE pre-processing step. Upon application of milk to the SPE cartridge, micelle disruption took place as indicated by the transparent flow through. Micelle disruption was attributed to binding of milk calcium that is crucial for micelle integrity to the active sites of

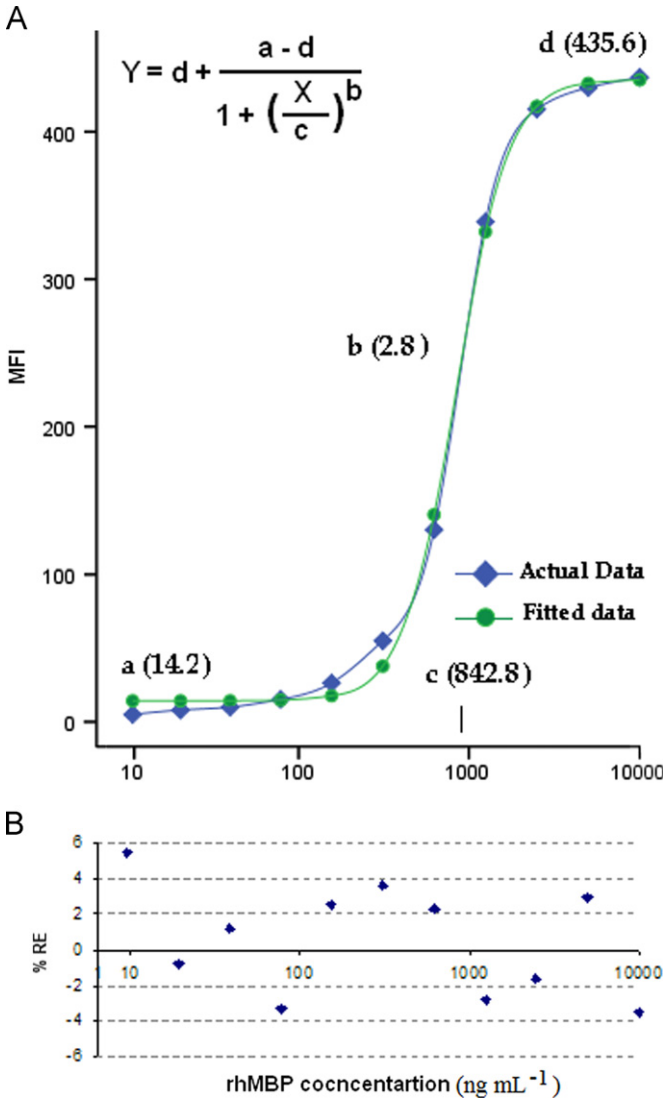


**Fig. 2.** A: Calibration curve (MFI vs. rhMBP concentration) for the SPE-immunosensor assay with the actual and fitted data using the 4-PL regression model. The equation representing the model and the estimated values of the parameter were overlaid. B: A plot of the %RE vs. rhMBP concentration calculated for a set of validation samples. RE: relative error and 4-PL: 4-parameter logistic.

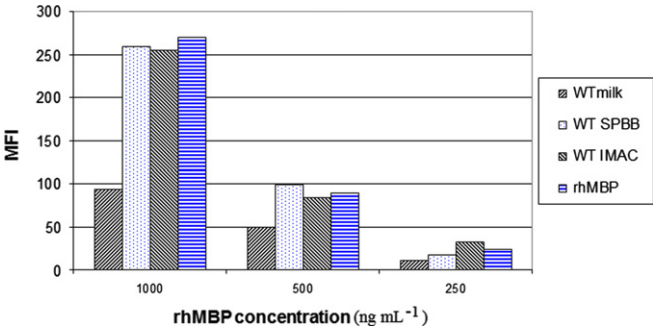


**Fig. 3.** A plot showing the MFI obtained by spiking of three different concentrations of rhMBP (1000.00, 500.00 and 250.00 ng $mL^{-1}$) into various control milk matrices (WTmilk, WT-SPBB and WT-IMAC) compared to that obtained from an equivalent rhMBP standard solutions.

the cation exchanger resin. No significant difference (ANOVA at P 0.10) was noted between the results of spiked WTmilk, WT-SPBB and WT-IMAC as summarized in Table 1. This indicated that it was necessary to disrupt the casein micelles and remove a large extent

of the endogenous milk proteins before analysis as revealed by SDS-PAGE (results not shown). Coupling of SPE to the immunoassay enabled fingerprinting of the expression level of the rhMBP in milk from each genetically modified animal, at different stages of the lactation cycle and over different lactation cycles with minimal effort and time.

### 3.4.2. Monitoring of the elution pattern of rhMBP in chromatography fractions

In this experiment, a transgenic milk sample was processed using the optimized purification protocol and the immunosensor was used to trace the rhMBP in the chromatography fractions collected from the second chromatography step (IMAC). Aliquots of the loaded sample (SPBB fractions), the flow through fractions and the eluted fractions (IMAC fractions) were analyzed. The responses recorded indicated a similar elution pattern of the recombinant protein (Fig. 4) to that obtained previously by western blotting analysis [9]. These results confirmed that the immunosensor is suitable for in-process qualitative and quantitative monitoring of the rhMBP throughout the downstream purification protocol. The amount of the rhMBP and the total amount of protein were calculated employing the immunoassay and the CZE assay respectively. Results are summarized in Table 2.

### 3.4.3. Monitoring of the stability of the rhMBP

In this experiment, we focused on studying the short-term temperature stability at RT as well as the stock solution stability at 4–8 °C of the rhMBP in the final preparation. Suitably diluted aliquots of the rhMBP reference standard were incubated at room temperature and 4–8 °C. Samples were taken after 0, 1, 4, 8 and 24 h and immediately frozen (−80 °C) until analyzed. The concentration of the rhMBP in each sample was determined from the calibration curve and was plotted vs. the incubation time (Fig. 5). The results showed good stability of the rhMBP reference standard at 4–8 °C for up 24 h and at room temperature for up to 4 h which was in agreement to the previously reported results using western blotting analysis [9]. Results confirmed the ability of the immunosensor to detect small changes in the rhMBP concentration.

**Table 1**
Recovery results of spiked control samples over three concentration levels using the SPE technique for pre-processing of neat milk samples.

| Level of spiked samples (ng mL$^{-1}$) | Recovery (%) (mean ± SD, $n$=9) | | |
|---|---|---|---|
| | WTMilk | WT-SPBB | WT-IMAC |
| 250.00 | 92.45 ± 12.84 | 96.18 ± 7.31 | 97.34 ± 4.65 |
| 500.00 | 105.27 ± 11.52 | 97.34 ± 5.50 | 101.50 ± 5.18 |
| 1000.00 | 103.84 ± 9.82 | 104.71 ± 6.26 | 99.22 ± 3.81 |

**Table 2**
Summary of the purification results for the rhMBP from a TGmilk sample.

| Sample | Volume (mL) | rhMBP[a] (mg) | Recovery[b] (%) | Total protein[c] (mg) | Purity[d] (%) |
|---|---|---|---|---|---|
| **Defatted TGmilk** | 25.6 | 2.60 | 100 | 896.25 | 0.29 |
| **Cation exchange chromatography** | 50.0 | 2.05 | 78.85 | 2.56 | 80.08 |
| **Affinity chromatography** | 20.0 | 2.00 | 76.92 | 2.18 | 91.74 |

[a] Determined using the SPE-immunosensor assay.
[b] Cumulative recovery mg/mg % relative to rhMBP amount in TGmilk.
[c] Determined using the CZE assay [30].
[d] rhMBP (mg)/total protein (mg) × 100.

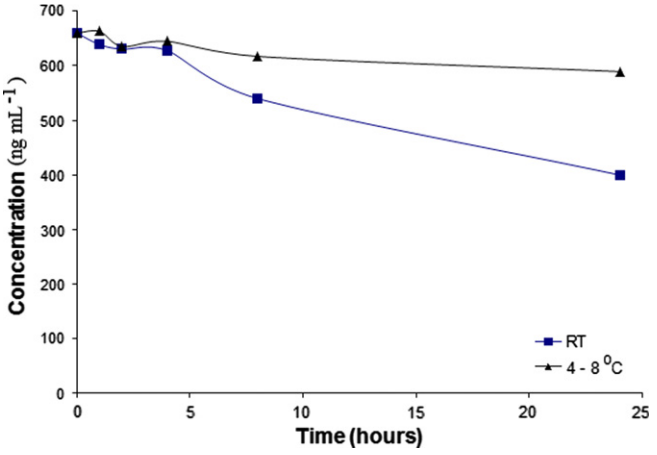


**Fig. 5.** A plot representing the change in the concentration of two rhMBP standard samples incubated at 4–8 °C and room temperature over 24 h showing the ability of the SPE-immunosensor assay to determine the small changes in rhMBP concentration.

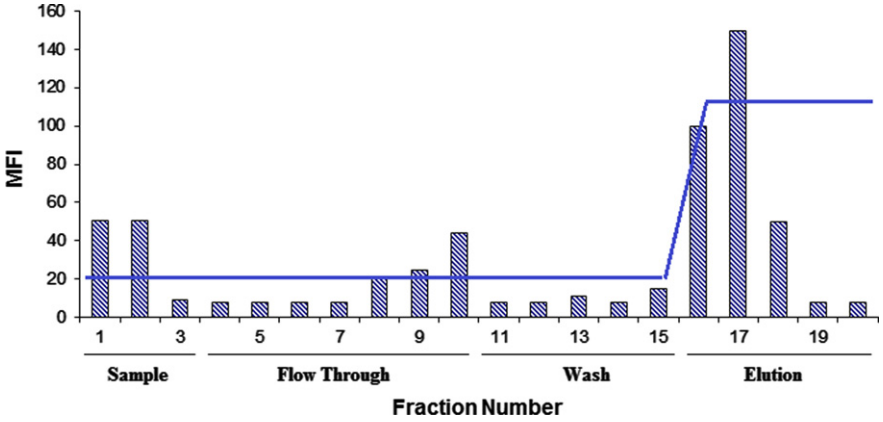


**Fig. 4.** A plot of the MFI obtained from fractions collected throughout a chromatography experiment for the purification of the rhMBP. The X-axis represents the fraction numbers collected throughout the run, 1–2: starting sample, 3–10: flow through, 11–14: column wash, 15–16: gradient elution (50.0–500.0 mmol L$^{-1}$ imidazole) and 17–19: eluted fractions (500.0 mmol L$^{-1}$ imidazole). Column: HisTrap HP—5 mL, sample: 50 mL pooled SPBB fractions loaded at 5 mL/min, elution flow rate: 5 mL/min, loading buffer: 50 mmol L$^{-1}$ HEPES—0.5 mol L$^{-1}$ NaCl—50 mmol L$^{-1}$ imidazole (pH 7.0) and elution buffer: 50 mmol L$^{-1}$ HEPES—0.5 mol L$^{-1}$ NaCl—500 mmol L$^{-1}$ imidazole (pH 7.0).

## 4. Conclusions

In this study, a sandwich type, immunosensor was developed for monitoring of the rhMBP in transgenic milk and milk fractions using the Bio-Plex system. The developed solid phase extraction step helped overcome interference caused by milk proteins. The assay protocol was considered as a total activity and stability indicating method that is applicable to His-tagged proteins. It can determine the total amount of intact rhMBP without interference from neither its native counterpart nor endogenous milk proteins. A large number of applications were achieved; monitoring of the stability of rhMBP, determination of the recombinant protein in chromatography fractions, fingerprinting of the expression level of the rhMBP in milk with minimal effort and time. Theoretically, the multiplexing capabilities of this immunosensor can help extend its value via simultaneous monitoring of process-related, product-related and host-related impurities and possibly monitoring of markers for the health condition of each animal using the same assay protocol.

## Acknowledgment

We thank Evelyn Spittle (Malaghan Institute of Medical Research, New Zealand) for technical assistance. This research was supported by Interdisciplinary Research Grants (IRG), Faculty of Pharmacy, Cairo University, Egypt and Foundation for Research, Science and Technology (FRST), New Zealand.

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