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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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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.

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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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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

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