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Direct Optical Biosensor Analysis of Folate-Binding Protein in Milk

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An automated, rapid, sensitive, and label-free biosensor-based assay for folate-binding protein (FBP) in bovine milk utilizing surface plasmon resonance optical detection is described. The active concentration of FBP is estimated from its specific interaction with a pteroyl-L-glutamic (folic) acid derivative immobilized on the sensor surface in a direct binding assay format. Milk, colostrum, and milk powders are prepared for analysis by dilution into buffer. Analysis conditions, including ligand immobilization, flow rate, contact time, and regeneration, have been defined, and nonspecific binding considerations were evaluated. Performance parameters include a working range for FBP in buffer of 0−200ng/mL, a method detection limit of 0.13 μg/mL in fluid milk, overall instrument response RSD_R of 0.64%, a mean interassay RSD_R of 7.3% for skim milk powder, and surface stability over ca. 200 samples. The technique was applied to the measurement of active FBP content of consumer milks, the heat classification of skim milk powders manufactured under a wide range of thermal processing protocols, and change during early bovine lactation.

KEYWORDS: Folate-binding protein; optical biosensor; surface plasmon resonance; milk; heat treatment

INTRODUCTION

FBPs have been demonstrated to regulate the assimilation, distribution, and retention of folates in mammalian cells, extracellular fluids, and tissues. The high affinity binding of folates to FBP has been reported to increase bioavailability of the vitamin in animal studies (*1*). Both membrane-bound and soluble forms of FBP have been identified, with the former functioning as a cellular folate receptor, while the soluble form is predominant in milk, where it acts primarily to sequester and stabilize folate from maternal circulation for the neonate (*2*−*4*).

Soluble FBP is a minor component of the whey fraction of mammalian milk and has been isolated from several species by a range of affinity chromatographic techniques generally based on capture by immobilized folic acid. Bovine FBP has been sequenced and is a single polypeptide of ca. 30 kDa containing variable glycosylated moieties (*3*, *5*). It binds specifically to folates in a 1:1 molar ratio above pH 5 and exists in milk in the monomeric state but is vulnerable to polymerization under certain conditions (*5*−*7*).

FBP has been estimated with use of a two site sandwich ELISA assay (*8*) to investigate the effect of thermal processing on the retention of FBP in bovine milk and milk products (*9*, *10*). Recent developments in real-time, label-free biosensor techniques based on SPR applied to milk proteins have included quantitative detection of plant protein adulterants in dairy products, IgG in milk and colostrum, and FBP in milk (*11*−*13*), as well as a study of milk casein interactions (*14*). The technique has also been recently applied to the interaction between FBP and multivalent, folic acid-coupled nanoparticles, in view of their potential therapeutic role in clinical drug delivery (*15*). SPR-based immunoassays have been shown to be versatile, robust, and capable of producing rapid and reliable data for the quantitative analysis of components in complex food matrixes with minimal sample preparation. The present study describes an automated SPR biosensor assay for bioactive FBP with respect to its capacity to bind folate in milk and colostrum. It has been applied to an examination of consumer milks, temporal change in FBP levels during early bovine lactation, and a survey of the influence of heat treatment on active FBP content during industrial processing of milk powder.

MATERIALS AND METHODS

Instrumentation. An optical biosensor (Biacore Q) was obtained from Biacore AB (Uppsala, Sweden). Instrument operation and data processing were performed with Biacore Q control software 3.01.

Chemicals and Reagents. Amine coupling reagents EDC-HCl (0.4 M), NHS (0.1 M), and ethanolamine-HCl (1 M, pH 8.5), folic acid derivative, sensor chip CM5, and HBS-EP running buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20, pH 7.4) were all obtained from Biacore.

Commercially available bovine FBP (F0504) was obtained from Sigma-Aldrich (MO). Bovine FBP, isolated by ion exchange and affinity chromatography of whey, were kind gifts donated by Drs. Neill Haggarty (Fonterra Research Centre, Palmerston North, New Zealand),

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FBP Calibrant. Stock standards were prepared at 1.0 mg/mL in HBS-EP and aliquoted for storage at −18 °C. Intermediate standards (10 μg/mL) were prepared in HBS-EP, and a top-level working calibrant (200 ng/mL) was prepared daily by further dilution in HBS-EP.

Samples. Raw bovine milk was collected from a single 4 year old Jersey (second calving) between days −1 prepartum and +14 postpartum. Samples were diluted 1:50 in HBS-EP buffer, divided into 1.0 mL aliquots, and frozen (−18 °C) until analyzed by SPR biosensor assay. In preparation for analysis, aliquots were thawed at 37 °C and further diluted to a final 1:1000 (<day 1 postpartum), 1:500 (day 1−2), or 1:200 (>day 2) with HBS-EP buffer. A range of liquid consumer milks was retail purchased and diluted 1:50 in HBS-EP buffer. A range of skim milk powders (ultralow heat to high heat) was obtained from New Zealand milk processing facilities and prepared for analysis at 1:500 w/v final dilution in HBS-EP.

Biosensor Surface Preparation. Folic acid was immobilized on sensor chip CM5 via amine coupling under instrument control. Briefly, the designated flow cell was activated with a mixture of EDC and NHS (1:1 v/v) (10 μL/min, 7 min) followed by a 5 mM solution of the folic acid derivative (Biacore) (in 10 mM borate buffer containing 1 M NaCl at pH 8.5) (10 μL/min, 7 min). Finally, unreacted ester functionalities were deactivated with ethanolamine (1 M, pH 8.5, 10 μL/min, 7 min). Following immobilization, the chip was stored between analyses over desiccant at 4 °C in a sealed container. A reference flow cell was prepared similarly but without folic acid ligand.

SPR Biosensor Assay. A multilevel calibration curve was established by serial dilution of a 200 ng/mL FBP standard in HBS-EP. Standards and sample extracts (in duplicate) were dispensed (200 μL) into 96 well microtiter plates and were injected for 8 min at 20 μL/min with HBS-EP running buffer at a temperature of 25 °C. Binding responses (RU) acquired 30 s after the end of the injection were measured relative to the initial baseline and used for generation of calibration curve and interpolation of unknown samples. The surface ligand was regenerated by injection of 80 μL of 75 mM sodium hydroxide at 50 μL/min.

RESULTS AND DISCUSSION

Biosensor Surface Preparation. The binding site of FBP involves the folate pteridine-amine functionality, and it has previously been demonstrated that for both affinity purification of FBP (*16*) and its determination by biosensor assay (*13*), folic acid must be immobilized via its glutamate-carboxyl moiety. Immobilization of the carboxyl-modified derivative via conventional amine coupling yielded an FBP surface binding capacity of ca. 15 000 RU (1 RU = 1 pg/mm^2). On the basis of a 1:1 binding stoichiometry, this value of R_{max} is equivalent to ca. 200 pg of immobilized ligand. While the relative proportion of folic acid immobilized via pteridine or glutamyl moieties is unknown, the population of glutamyl-orientated ligand provides a high density FBP specific surface suitable for concentration analysis.

FBP Calibrant. FBP isolates obtained from four different sources were evaluated as potential assay calibrants, by estimating binding responses of nominally equimolar dilutions over the folic acid immobilized biosensor surface under identical assay conditions. Commercially available FBP (Sigma) gave significantly lower responses as compared to the three noncommercial isolates acquired, probably due to the significant levels of serum albumin found present in this material. All four sources have been independently confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis analysis to contain at least three glycosylated forms of FBP.

Of the remaining three FBP isolates available, one was selected as calibrant in the described routine SPR biosensor assay based on its specific binding response to immobilized folic acid, absence of nonspecific binding to the reference surface, and its characterization as >95% pure by conventional techniques used for estimating protein purity (*17*).

Method Evaluation. It is imperative for any immuno- or binding assay format that the extent of potential nonspecific interferences from complex biological fluids be evaluated, especially for label-free detection systems (*18*). The ability to perform comparative binding experiments with the SPR biosensor system described provides the capability of both assessing and minimizing nonspecific binding. The extent of potential nonspecific binding to the dextran support was therefore evaluated over the reference ligand-free surface and for FBP standards was essentially absent (<2 RU), while a range of individual consumer milks ($n = 26$) of varying fat content (0.5−3.3%) at analytical dilution levels yielded responses <30 RU. For milk samples, the extent of such predominantly electrostatic nonspecific binding to the hydrophilic and negatively charged carboxymethyl dextran surface was considered acceptably low in comparison to the specific response (<5%). Although preliminary experiments confirmed the benefits of increased diluent ionic strength (150−800 mM NaCl) for reducing such nonspecific response, this was at the expense of the specific association, which was also suppressed, albeit to a lesser extent. The potential for nonspecific binding to the ligand was also evaluated by analysis of serial dilutions of milk (1:50−1:200) over the active folic acid immobilized surface. Equivalent levels of measured FBP at these dilutions confirmed the absence of any significant matrix interference.

Sample preparation was accomplished in this study by direct dilution in buffer, in contrast to the preliminary rennet-facilitated removal of caseins as described recently (*13*). The benefits of a direct dilution protocol prior to analysis include operational simplicity, enhanced analytical precision, and the avoidance of losses of FBP due to potential coprecipitation with caseins. In this context, while spike recoveries were not reported previously (*13*), losses of other minor proteins in milk have been observed where prior sample manipulation incorporated casein precipitation. However, analysis of milk-derived whey was appropriate where speciation of FBP (free and folic acid-bound) was targeted (*13*).

It is common practice to generate calibration curves in analyte-free matrix as a further strategy for minimizing the impact of matrix interference, and several recently reported SPR-based concentration assays for incurred contaminants in milk have adopted this technique (*19*−*21*). This approach was evaluated in the present study, since endogenous milk FBP is efficiently destroyed by heat thereby providing a potential blank milk matrix. However, the concomitant release of native bound folate compromised the assay through inhibition of spiked FBP calibrant.

A sandwich immunoassay format was investigated, primarily in order to confirm assay specificity for FBP but also to achieve potential enhancement of analytical response. **Figure 1** illustrates use of a polyclonal anti-bovine FBP antibody (Statens Serum Institute, Copenhagen, Denmark) (pAb) as secondary reagent to confirm specificity of surface-bound FBP. Signal enhancement is predominantly due to association of pAb to surface-bound FBP (ca. 1500 RU), confirmed by the minimal response following its direct injection over immobilized folic acid (ca. 40 RU), as shown in the inset. The sandwich assay protocol was, however, considered unacceptable for routine use, since affinity-purified pAb is not commercially available.

Competition experiments were also performed to confirm specificity of immobilized folate for FBP, based on inhibition by free folate in solution. Following establishment of solution

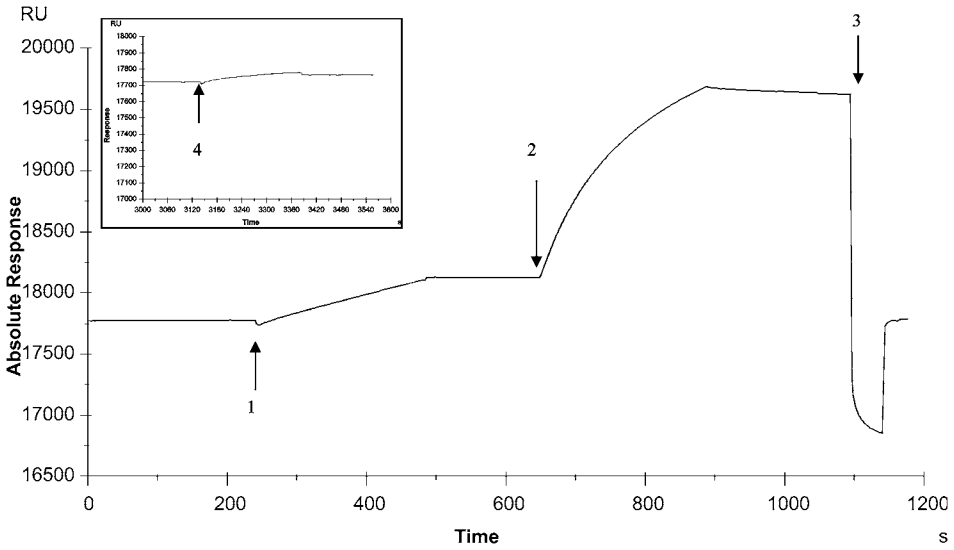

Figure 1. Sensorgram obtained after injection of FBP (500 ng/mL) for 4 min at 20 μL/min (1) followed by polyclonal anti-bovine FBP (10 μg/mL) for 4 min at 20 μL/min (2) prior to regeneration (3). Insert: direct injection (4) of polyclonal anti-bovine FBP (10 μg/mL) for 4 min at 20 μL/min over folic acid immobilized surface.

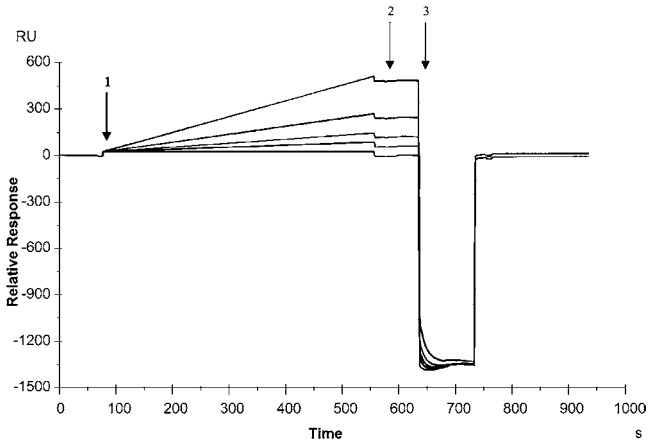

Figure 2. Superimposed sensorgrams of duplicate FBP calibrants (200, 100, 50, 25, and 0 ng/mL in HBS-EP) injected for 8 min at 20 μL/min (1) followed by regeneration (3). Response relative to initial baseline (2) used to establish dose–response curve.

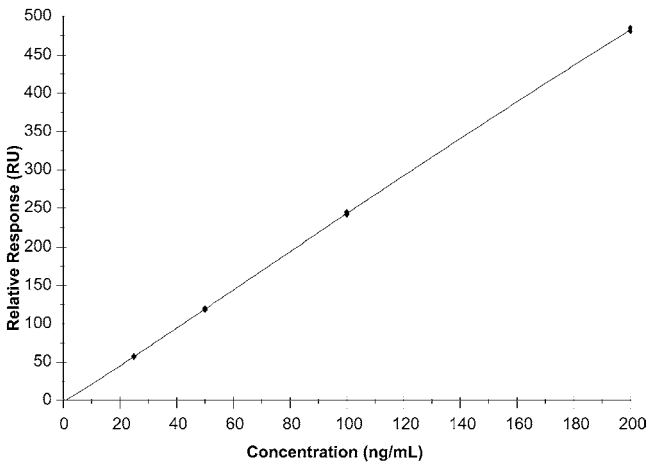

Figure 3. Dose–response curve established from relative responses (duplicates) acquired in **Figure 2**.

equilibrium of either FBP calibrant or diluted milk (12.5 pmol/mL) in the presence of excess folic acid (1250 pmol/mL), binding inhibition was estimated at >95%.

Method Performance. Duplicate sensorgrams of FBP standards over immobilized folic acid are illustrated in **Figure 2**, and a typical calibration curve is illustrated in **Figure 3**.

Over the optimized working range (0−200 ng/mL), a linear regression adequately described the dose−response relationship, while the four parameter curve fit employed routinely yielded equivalent interpolation. The instrumental limit of detection (response + 3 SD of blank calibrant) for FBP over several independent runs was estimated as 0.7 ng/mL (equivalent to 0.11 ng of FBP injected). The method detection limit (SD × $t_{n-1,0.01}$) was estimated from replicate analysis of a low FBP fluid milk sample processed under medium heat conditions and measured at 0.13 μg/mL ($n = 5$).

Instrument precision was estimated from replicate analysis of the active FBP concentration of a single level calibrant (100 ng/mL) over several runs and measured 0.64% ($n = 10$) RSD. Intra-assay repeatability RSD_r for a fluid milk sample measured 0.47%, while inter-assay reproducibility RSD_R over several immobilized surfaces, for independently sampled fluid low fat milks and a control skim milk powder, were estimated to be 11.5 and 7.3%, respectively. Recovery efficiency was determined following replicate spiking of fluid low fat milk with FBP at 1× and 2× endogenous levels and measured 92.6−109.0%.

Performance over sequential analyses illustrates the stability of both ligand and surface analyte binding capacity, as well the effectiveness of the regeneration protocol, as shown in **Figure 4** for a typical replicate analysis. Furthermore, it was found that the folic acid immobilized surfaces were durable over at least 200 injections with minimal baseline drift under the described assay conditions.

Method Applications. It has been reported that native folate is bound to FBP in unprocessed milk, while heat treatment of milk during processing releases protein-bound folates. On the basis of the accepted 1:1 molar binding stoichiometry at neutral pH, the described biosensor assay will estimate the active, free FBP level of bovine milk. As compared to raw milk, the reduction in FBP content was estimated as ca. 10−15% from commercial pasteurization and >80% as a consequence of UHT treatment, both values consistent with previously reported data (*9*, *10*). A selection of retail-pasteurized milks containing variable fat was analyzed. Low fat (0.5% v/v) milk contained

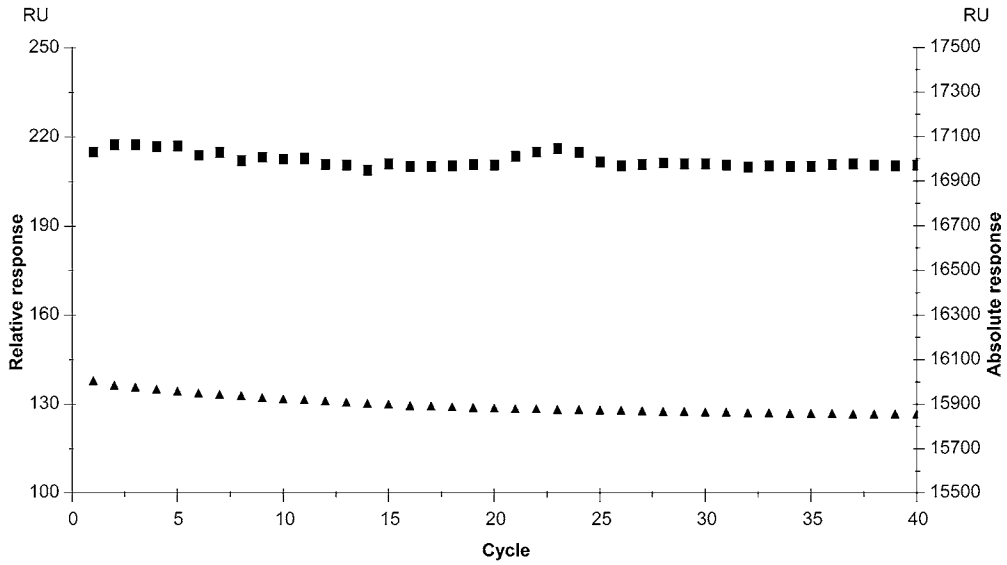

Figure 4. Baseline (▲) and relative response (■) of FBP (100 ng/mL) over folic acid immobilized surface for 40 sequential analyses.

a mean of 6.7 μg/mL (range, 5.1−7.9 μg/mL; $n = 17$; RSD_R, 14.5%), "reduced fat" (1.5% v/v) milk contained a mean of 7.4 μg/mL (range, 7.2−7.6 μg/mL; $n = 3$; RSD_R, 2.8%), and full fat (3.3% v/v) milk contained a mean of 6.6 μg/mL (range, 4.6−8.0 μg/mL; $n = 9$; RSD_R, 15.9%). Overall, this survey of retail milks found an active FBP content between 4.6 and 8.0 μg/mL (mean, 6.7 μg/mL; $n = 29$; RSD_R, 14.1%), a range comparable to reported values of 4.5−10.0 μg/mL (*1*, *9*, *10*, *13*, *16*). Despite the somewhat higher levels of active FBP detected in milk as compared to a recent SPR-based study (*13*), the general equivalence of FBP content between different methods is encouraging in view of both the radically divergent analytical schemes utilized and the absence of an accepted FBP reference material. In this respect, the ability of the antibody-based ELISA to detect total FBP has previously been promoted (*13*).

The biological properties of proteins reside primarily in their native three-dimensional polypeptide conformation. The thermal conditions under which fluid milk is processed will inevitably correlate with progressive denaturation of FBP, and this has been confirmed in both this and previous studies, when milk is exposed to pasteurization and UHT conditions (*9*, *10*). To date, the kinetics of FBP denaturation have not been rigorously established. However, differential scanning calorimetry has been utilized to investigate the stability thermodynamics of bovine FBP and apo-FBP between 20 and 75 °C at varying pH, indicating both reversibility of protein unfolding and increased stability when bound to folate (*22*). Because the described SPR biosensor technique responds only to biologically active FBP with respect to its capacity to bind folate, it has therefore been used to monitor the denaturation of both isolated FBP and native FBP in milk at 100 °C. The observed binding response over immobilized ligand will plausibly be specific for undenatured, and therefore active protein, as shown in **Figure 5**.

The SPR biosensor assay has also been applied to survey the FBP content of spray-dried skim milk powders manufactured under a wide range of heat processing conditions. The variable heat treatment protocols, applied predominantly during the preheating stage, can significantly affect the nutritional and sensory properties of the final milk powder and define the intended use of the product. The progressive denaturation of milk serum protein has most commonly provided the basis for heat classification methods, involving either direct measurement of residual undenatured or indirect measurement of denatured

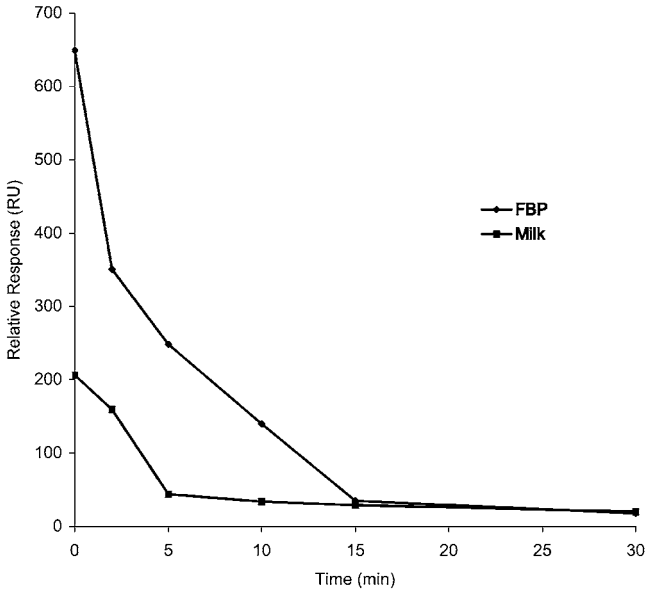

Figure 5. Effect of exposure of authentic FBP (◆) and native FBP in milk (■) to heat treatment at 100 °C on binding response to immobilized folic acid sensor surface.

whey protein. Such methods include turbidimetric, dye-binding spectrophotometric, and high-performance liquid chromatography-based techniques yielding a numerical classification system for ultralow to high heat milk powder (*23*−*25*). In the present study, levels of biologically active undenatured FBP were found to both correlate with the conventional dye-binding method (*24*) and provide a significantly enhanced sensitivity to the severity of preheat conditions, as shown in **Table 1**.

The variable retention of FBP in skim milk powders, all of which are exposed to similar high temperatures during the final spray-drying process, concurs with previously reported data (*10*) and confirms the significance of liquid preheating with respect to the content of FBP in the final product. Furthermore, the determination of biologically active FBP by the described SPR biosensor assay may be a suitable indicator for the heat classification of such products, based on the direct measurement of a specific undenatured whey protein.

Lactating cells of the mammary epithelium produce substantial quantities of membrane-bound FBP, and the conversion to

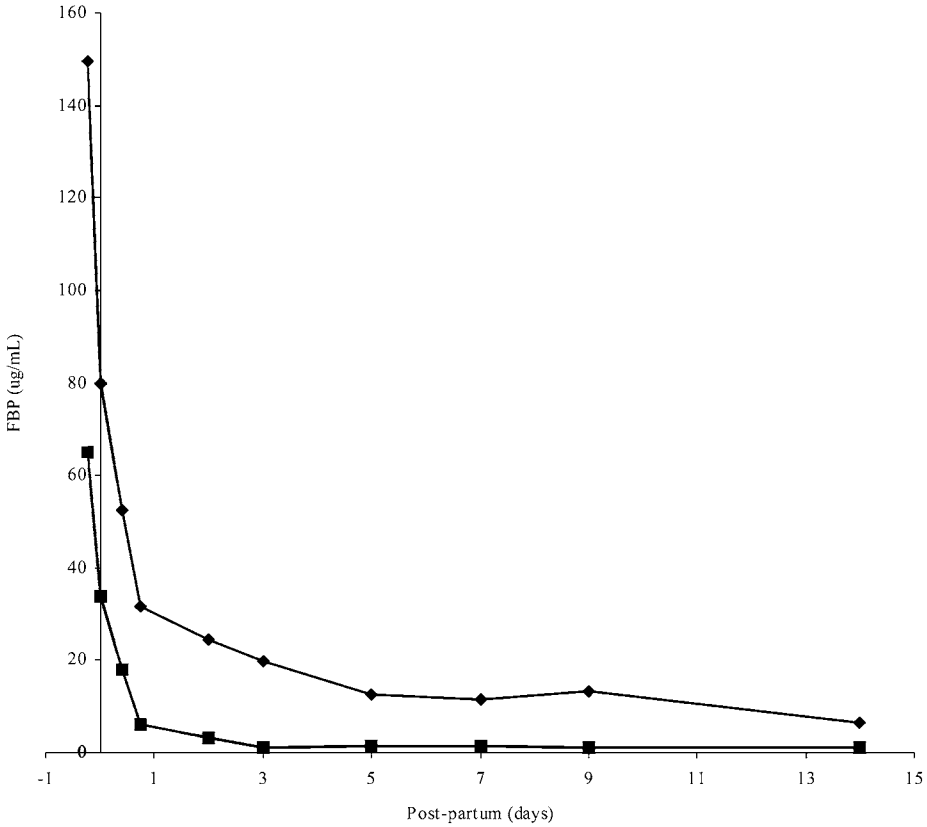

Figure 6. FBP content of early lactation milk acquired from the second calving of a 4 year old Jersey. Binding to folic acid immobilized surface (◆) and nonspecific binding to reference surface (■).

Table 1. Comparison of FBP Content (μg/g) and Whey Protein Nitrogen Index (WPNI) of Skim Milk Powders Manufactured under Varying Preheat Conditions

	FBP[a](μg/g)			
heat class[b]	1	2	3	WPNI[c]
low	50.1	59.8	48.5	7.2
low	33.9	42.1	31.5	5.9
low–medium	25.2	30.7	23.1	5.3
medium	13.2	15.5	12.3	4.5
medium	11.5	16.8	10.0	4.0
medium	9.9	11.8	10.1	2.9
medium–high	5.1	4.4	4.8	2.0
high	2.8	1.0	2.1	0.1

[a] FBP estimated by three independent replicate analyses using described SPR biosensor method. [b] Heat classification based on whey protein nitrogen index (*23*). [c] WPNI: whey protein nitrogen index (*24*).

soluble FBP facilitates the accumulation of folate in milk (*3*). In mammals, the expression of most milk components is highly influenced by the stage of lactation, and the SPR biosensor technique was therefore applied to investigate the variation in FBP content of early transitional bovine milk. **Figure 6** illustrates the temporal trend for an individual animal over the first 14 days following parturition.

As shown, the content of FBP in early colostrum was found to be ca. 10× higher than in mature milk expressed beyond day 5, an observation comparable to previous data based on a radioisotope-binding assay (*26*). The progression from early colostrum to mature milk coincided with a significant reduction in nonspecific interactions (as monitored over a reference surface) as indicated in **Figure 6**. The contribution of nonspecific binding in very early colostrum (<1 day) is likely to originate from the significantly higher levels of whey proteins as compared to mature milk. Because further strategies will be required to eliminate sources of early colostral nonspecific binding, the temporal trend in FBP content of colostrum reported in this study must be considered preliminary.

Nutritionally significant minor proteins in bovine milk will be increasingly exploited in the expanding international trade of milk products (*27*). Such trade will be increasingly reliant on the traceability of analytical methods, and in the absence of an internationally recognized reference method, the described SPR biosensor binding assay may fulfill this need for FBP. Indeed, this approach is only the second literature report of the potential for SPR-based biosensor analysis of FBP (*13*). Both studies share a similar overall analytical approach, although each differs in aspects of sample preparation, speciation, and application to industry issues. Thus, it is to be anticipated that content and distribution in milk and dairy products, vulnerability to heat processing conditions, and potential influence on folate availability will receive increased attention. It is axiomatic that discrepancies between analytical methods will generally occur due to differences in specific interaction chemistries. It is also apparent that there are several common factors that critically influence the analysis of FBP in milk and colostrum, irrespective of the end point measurement technique. Thus, it will be expedient to identify an internationally accepted FBP primary reference material, to facilitate a more realistic assessment of the equivalence of available analytical methods.

ABBREVIATIONS USED

SPR, surface plasmon resonance; FBP, folate-binding protein; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, N-hydroxysuccinimide; HBS, Hepes-buffered

saline; EDTA, ethylenediaminetetraacetic acid; RU, response units; RSD, relative standard deviation; UHT, ultrahigh temperature.

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