

Determination of Minor Proteins of Bovine Milk and Colostrum by Optical Biosensor Analysis

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Automated, rapid, sensitive, and label-free biosensor-based immunoassays for immunoglobulin G (IgG), folate binding protein, lactoferrin, and lactoperoxidase in bovine milk using surface plasmon resonance optical detection with direct binding assay format are described. Samples are prepared for analysis by direct dilution into buffer. Analysis conditions, including ligand immobilization, flow rate, contact time, and regeneration are defined and nonspecific binding considerations evaluated. The technique has been applied to the measurement of these proteins in consumer milks, colostrum, milk products, and infant formulas, and their temporal change during early bovine lactation followed.

Milk contains numerous minor proteins with physiological properties targeted at providing immunoprotective, growth, and antimicrobial factors to the neonate, as distinct from the nutritionally more significant major proteins. Many of these minor bioactive proteins are found in the whey fraction of mammalian milks and are generally present at elevated levels in colostrum, reflecting their importance to early neonatal health (1, 2).

In contrast to human neonates, calves are agammaglobulinemic at birth, and their passive immunity is dependent entirely on the absorption of maternal colostrum immunoglobulin antibodies (Ig) during the early neonatal period. The principal Ig glycoprotein in bovine colostrum and milk is IgG (>80%), of which serum IgG₁ dominates. The relatively high levels of IgG in early bovine colostrum thus provide an essential source of this nutrient to the calf immediately following parturition and until it is able to establish immunosufficiency. Several factors, including individual variation, breed, parity, and lactation, influence the concentration of IgG in bovine colostrum, although pathologies may also have a profound effect. Although the

commercial milk supply should be colostrum-free, there has been significant interest in colostrum-supplemented products based on the alleged prophylactic and immunotherapeutic benefits of absorbed IgG. Thus, colostrum milk from both immunized and nonimmunized herds have been processed into hyperimmune products designed for pharmaceutical and nutraceutical usage.

Folate binding proteins (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. 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. 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 about 30 kDa containing variable glycosylated moieties. 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.

Lactoferrin (Lf), ca 80 kDa, is a basic iron-binding, bilobal secretory transport sialylated glycoprotein, and is a member of the transferrin family characterized by bicarbonate anion-dependent, high affinity, and reversible binding of 2 Fe³⁺ per molecule. Although present in milk as a result of *in situ* synthesis within the mammary gland, it is also present in several other exocrine fluids. In addition to its antimicrobial activity, Lf may function in intestinal iron uptake and regulation, immune response, and antioxidant activity. Lf content is significantly higher in human milk and colostrum than the bovine equivalent, and this has stimulated an increasing trend of supplementation of bovine milk-based infant formulas with fractionated bovine Lf, despite both a 69% primary sequence homology with human Lf and a poorly characterized understanding of its absorption in the infant gastrointestinal tract.

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Lactoperoxidase (LPO) is a basic endogenous enzyme in milk with an isoelectric point of 9.6 and a typical natural concentration of ca 30 g/mL in bovine milk. The protein has antimicrobial qualities when thiocyanate and peroxide are present, and functions as a preservative in raw milk. LPO is a single-stranded polypeptide of 612 amino acids (MW: 78 kDa) and exists in 2 isoenzyme forms, is highly glycosylated, and relatively thermostable. The structure of LPO is unusual in that the prosthetic heme group is covalently linked to the protein backbone by ester linkages to carboxylic acids in the active site of the enzyme.

The content of these minor proteins is known to be species-dependent. The increasing commercial interest in exploiting their therapeutic value has stimulated the need for reliable concentration assays for their determination at endogenous levels in milk and colostrum, at supplemental levels in infant formulas, and at pharmaceutical levels in milk protein isolates and nutraceuticals.

Various chromatographic modes for bovine whey proteins have been reviewed, while a recent review of the different immunological techniques available concludes that immunodiffusion techniques have inherently low sensitivity and have generally been superseded by the more sensitive enzyme-linked immunosorbent assay (ELISA) techniques. More recently, nephelometric immunoassays have also been developed.

Recent developments in real-time, label-free optical biosensor techniques based on surface plasmon resonance (SPR) as applied to milk proteins have included the quantitative detection of plant protein adulterants, IgG, FBP, Lf, insulin-like growth factor (IGF-1), α -casein, and the kinetic study of milk casein interactions (3–10). Ferritin, a cellular iron-binding storage protein, has recently been quantitated in serum by this technique (11). SPR-based biosensor immunoassays exploit the specific and reversible interaction between ligand and target protein and are versatile, robust, and capable of producing rapid and reliable data for the quantitative analysis of components in complex food matrixes with minimal sample preparation. This article summarizes development of automated SPR-biosensor assays for the quantitation of IgG, FBP, Lf, and LPO in milk, colostrum, and infant formula.

Experimental

Instrumentation

The Biacore® Q optical biosensor was obtained from Biacore AB (Uppsala, Sweden). Instrument operation and data processing were performed with Biacore Q control software 3.0.1.

Reagents

Amine coupling reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDC, 0.4 M), *N*-hydroxysuccinimide (NHS; 0.1 M), and ethanolamine-HCl (1 M, pH 8.5), sodium acetate buffer (10 mM, pH 4.5–5.5), Sensor Chip CM5, and HBS-EP running buffer (10 mM HEPES, 150 mM NaCl,

3.4 mM EDTA, 0.005% surfactant P20, pH 7.4) were all obtained from Biacore.

Biosensor Surface Preparation

Surfaces were prepared with affinity-purified goat anti-bovine IgG, folic acid derivative, goat anti-bovine Lf, or rabbit anti-LPO on a CM5 sensor chip via amine coupling under instrument control. Briefly, the designated flow cell was activated with a mixture of EDC and NHS (1:1, v/v), followed by ligand in appropriate buffer. Finally, unreacted ester functionalities were deactivated with ethanolamine. 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 ligand.

Samples

Raw early lactation bovine milk and a range of retail liquid consumer milks, commercial milk powders, colostrum powders, and infant formulas were all prepared for analysis by dilution to between 1:1000 and 1:50 000 in HBS-EP, depending on analyte and level.

SPR Biosensor Assay

Calibration curves were established by serial dilutions of 10 000 ng/mL bovine IgG, 200 ng/mL FBP, 1000 ng/mL Lf, or 10 000 ng/mL LPO standard in HBS-EP buffer. Calibrants and sample extracts were dispensed into 96-well microtiter plates and were injected for 3 min at 20 L/min (IgG), 8 min at 20 L/min (FBP), or 5 min at 10 L/min (Lf and LPO) with HBS-EP running buffer. Binding response units (RUs) 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. Regression analysis was routinely performed under software control by a 4-parameter polynomial fit. The surface was regenerated by injection of 25 mM phosphoric acid (IgG), 75 mM sodium hydroxide (FBP), 10 mM glycine-HCl, pH 1.75 (Lf), or 10 mM glycine-HCl, pH 1.50 (LPO).

Results and Discussion

Selection of Ligand

A typical protein immobilization procedure is illustrated in Figure 1 for the generation of a goat anti-bovine IgG specific surface. While ligand levels for anti-IgG, anti-Lf, and anti-LPO are directly visualized during the immobilization procedure, comparable information for low molecular weight ligands such as folic acid must be inferred based on subsequent binding capacity measurements. Such immobilization protocols typically aim to produce a high ligand binding capacity surface that is generally beneficial in concentration assays, because the relative binding response is directly related to the level of immobilized ligand.

Selection of Calibrant

Bovine IgG and LPO were obtained from Sigma (St. Louis, MO), purified FBP and Lf were isolated from skim

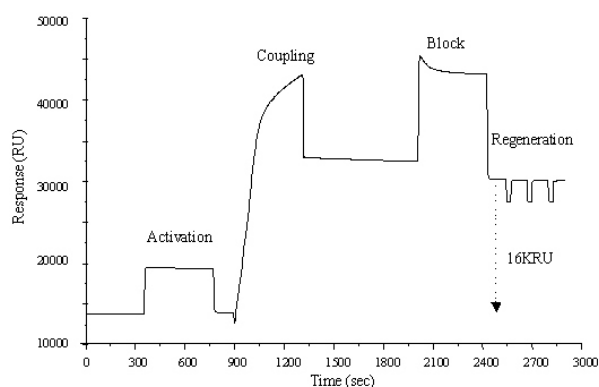


Figure 1. Sensorgram of goat anti-bovine IgG immobilization over a CM5 sensor chip. Amount immobilized, ca 16 ng/mm².

milk whey following affinity chromatography, ion-exchange liquid chromatography, and gel filtration.

Optical Biosensor Analysis

Figure 2 displays typical dose-response calibration curves obtained under assay conditions for IgG, FBP, Lf, and LPO.

Method Validation

Specificity.—It is imperative for any immunoassay format that nonspecific interferences be evaluated. The extent of potential nonspecific binding from both target analyte and sample matrix components to the dextran support was therefore assessed over the reference ligand-free surface and determined to be negligible under the described assay conditions. For IgG and FBP, a diluent ionic strength of 0.15 M sodium chloride was adequate, while for the significantly more basic Lf and LPO, ionic strength was increased to 0.5 M.

Further, binding specificity of the immobilized ligand was formally evaluated through measuring cross-reactivity for individual milk proteins (—casein, —casein, —casein, —lactalbumin, —lactoglobulin, IgG, bovine serum albumin, Lf, and LPO) at levels expected in milk. In the absence of analyte-free milk, the potential for binding interferences originating from nonanalyte sample components was also evaluated by analysis of serial dilutions of milk over the active immobilized surface and nonspecific matrix interferences confirmed as negligible.

Competitive inhibition experiments were performed to further evaluate specificity of the immobilized ligand and confirmed both ligand specificity and minimal nonspecific binding.

Sandwich immunoassays were also investigated primarily as an additional confirmation of assay specificity. Despite signal enhancement and confirmation of specificity, they were not adopted for routine analysis because of inherent requirements of increased analysis time and complexity.

Performance

Performance figures-of-merit for the 3 fully validated immunoassays are summarized and presented in Table 1. Indicative data are included for the LPO assay currently subject to single-laboratory validation. Performance over sequential analyses illustrates the stability of the surface ligand and its analyte binding capacity as well as the effectiveness of the regeneration protocol, as shown in Figure 3 for replicate analysis of an FBP calibrant.

In the absence of a reference milk or colostrum containing certified levels of these minor proteins, analytical accuracy may be assessed based on recovery and method comparison studies. The described SPR biosensor assays for IgG, FBP, and Lf were evaluated against alternative liquid chromatography (LC), radial immunodiffusion (RID), and ELISA techniques for milk-based samples representative of type. Statistical analysis confirmed reasonable between-method equivalence, suggesting an essentially unbiased estimate of analyte content, despite both the fundamentally different analytical principles and nonreference status of the alternative methods used.

Method Applications

The described biosensor-based direct immunoassays, using an immobilized protein-specific ligand, have been applied to a range of cow's milk products following a sample preparation scheme involving simple dissolution in buffer to high dilution levels, a protocol facilitated by the inherent sensitivity of SPR detection. Such minimal manipulation

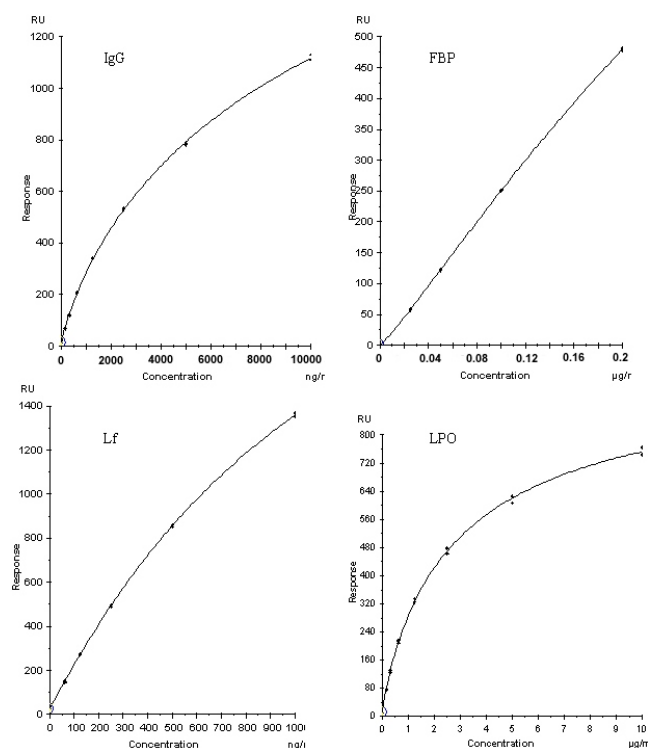


Figure 2. Calibration curves for IgG, FBP, Lf, and LPO.

Table 1. Method performance

	Range, ng/mL	LOD, ng/mL ^a	MDL, g/mL ^b	RSD _r , % ^c	RSD _R , % ^d	HorRat ^e	Recovery, % ^f
IgG	150–10000	16.8	80	2.6	10.5	1.8	>96.0
FBP	5–200	0.7	0.13	0.5	9.4	0.8	92.6–109.0
Lf	10–1000	1.1	19.9	3.6	10.8	1.3	101.7–106.8
LPO	150–10000	75	ND ^g	3.5	ND	ND	ND

^a LOD = Limit of detection; response + 3 SD blank.^b MDL = Method detection limit.^c Within-run precision.^d Between-run precision.^e Measured RSD_R:predicted RSD_R (2^[1-0.5logC]).^f Estimated by standard addition.^g ND = Not yet determined.

avoids the potential for recovery losses associated with casein removal, filtration, and centrifugation protocols commonly associated with alternative analytical techniques.

The mean contents of these proteins as determined by SPR immunoassay are summarized in Table 2 and are consistent with ranges reported by alternative techniques. In mammals, physiological expression of milk components is highly influenced by the stage of lactation, and the SPR-biosensor technique was therefore applied to IgG, FBP, and Lf expression during the progression from early bovine colostrum to mature milk. Figure 4 illustrates the temporal trend for an individual animal over the first 15 days following parturition. For these minor proteins, early colostrum was found to contain significantly elevated levels relative to mature milk, illustrating a significant physiological response to lactogenesis.

Bioactive minor proteins in bovine milk will be increasingly exploited in the expanding international trade of milk products, and such trade will be increasingly reliant on the traceability of internationally validated analytical methods. In the absence of any currently accepted reference methods, the described SPR biosensor binding assays may fulfill this need for IgG, FBP, Lf, and LPO in milk, colostrum,

and milk-based infant formula products. Although discrepancies between analytical methods will generally occur due to differences in detection principles, it is also apparent that there are factors that critically influence their quantitation irrespective of the endpoint measurement technique. It will therefore be expedient to identify internationally accepted primary reference materials in order to facilitate a more realistic assessment of the equivalence of available analytical methods.

Conclusions

The sensitivity, specificity, and quantitation range of biospecific methods are generally determined by ligand:analyte affinity, and an optimized SPR immunoassay therefore relies predominantly on appropriate ligand selection and immobilization chemistry, as well as buffer conditions, contact time, and regeneration protocol. The ligands used were found to provide excellent performance parameters, stable surfaces under multiple regeneration conditions, low detection limits, and minimal nonspecific binding at the high sample dilution levels facilitated by the inherent sensitivity of SPR detection. The described label-free, real-time, and automated biosensor immunoassays are rapid, sensitive, precise, and accurate, and provide analytical information comparable to available alternative methods.

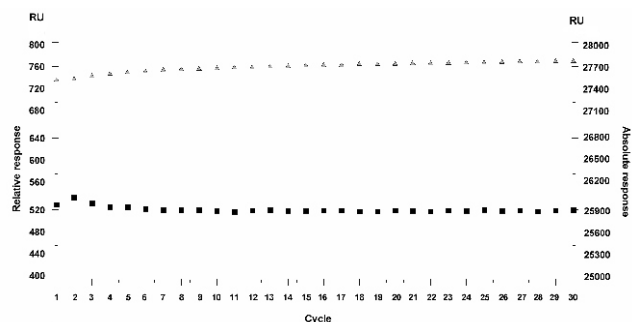


Figure 3. Stability of relative binding response of FBP (■) and absolute baseline (▲) over immobilized folic acid (30 replicate injections).

Table 2. Minor bovine milk protein content by SPR

	Milk	Milk powder	Colostrum (<1 day)
IgG, mg/g	0.4–0.6	2–3	50–60
FBP, g/g	4.6–8.0	10–60	80–100
Lf, g/g	50–200	ND ^a	2000–5000
LPO, g/g	23–34	150–210	ND

^a ND = Not determined.

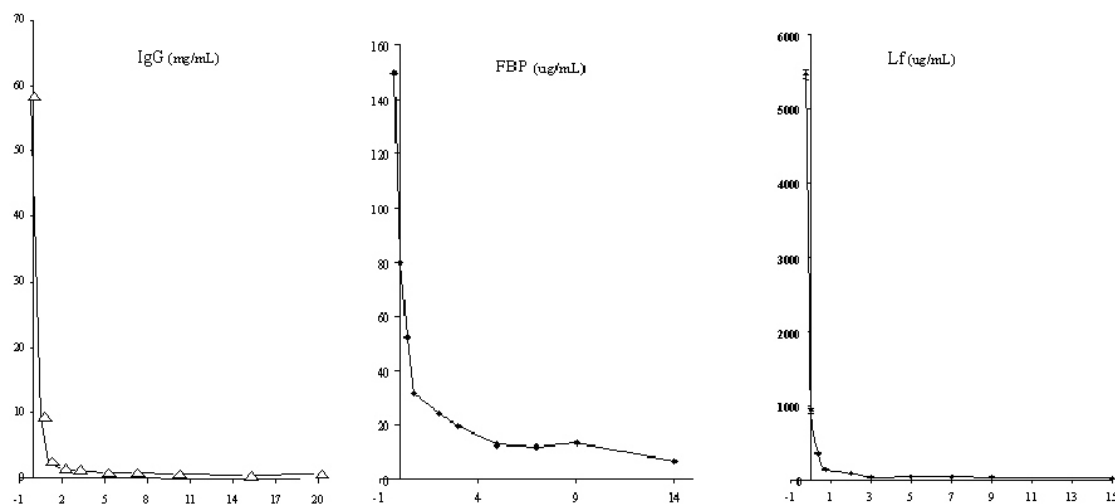


Figure 4. Minor protein expression during transition from colostrum to milk in a single animal. X-axis = days postpartum.

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