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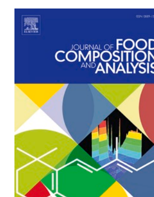

Original Research Article

The determination of intact β-casein in milk products by biosensor immunoassay

Harvey E. Indyk, Brendon D. Gill *, Jackie E. Wood, Sowmya Chetikam, Tadashi Kobayashi

Fonterra Co-operative Group Ltd, Cnr No. 1 Road & SH26, Waitoa, 3341, New Zealand

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ABSTRACT

A label-free optical biosensor immunoassay exploiting surface plasmon resonance detection for the estimation of the β-casein content in bovine milk and milk products is described. Samples were prepared by direct dilution with buffer and the protein was detected, under direct assay conditions, through binding with a commercially available anti-β-casein polyclonal antibody immobilised on the sensor surface. Assay conditions, selectivity and the potential for non-specific binding were defined. Analytical performance parameters included a method detection limit of 2.3 mg mL^{-1} for fluid milk, an intermediate precision RSD_{iR} of 10.7 % for a skim milk powder and a mean recovery of 101.7 %, with a single functionalised flow cell being stable for at least 200 cycles. The method was found to be rapid, sensitive, precise and accurate, and is reliable for a range of milk products containing intact β-CN, and provides a routine complement to alternative conventional immunoassay and separation-based methods.

1. Introduction

Caseins are the dominant group of heterogeneous proteins in bovine milk, are characterised by thermal tolerance and a pI of 4.6 and represent approximately 80 % *w/w* of the typical total protein content of about 3.5 % *w/v*. In raw milk, the four major casein fractions (α_{S1}-, α_{S2}-, β- and κ-) are present as micelles, of which approximately 30–35 % of the total protein is β-casein (β-CN), with a typical content of about 10 mg mL^{-1} (Dupont et al., 2013; Huppertz, 2013).

Bovine milk β-CN contains 209 amino acids, has a monomeric molecular mass of approximately 24 kDa and is the most hydrophobic of the amphiphilic caseins, with a high proline content that limits higher order structure. Although there are 12 known genetic variants, variants A1 and A2, which differ in a single residue, are the most prevalent in consumer milk sourced predominantly from *Bos taurus* herds, which contain both common variants in comparable amounts; this contrasts with *Bos indicus* milk, which is A2 dominant (Mishra et al., 2009). The hypothesis that the consumption of milk containing the A1 β-CN variant may represent a human health risk remains controversial (Küllenberg de Gaudry et al., 2019; Truswell, 2005). Endogenous enzymatic proteolysis of β-CN generates multiple products, including proteose-peptones, γ-caseins, β-casomorphins and other bioactive peptides that are implicated in diverse physiological roles (Capriotti et al., 2016; Szwajkowska et al., 2011). As an integral component of the casein fraction, β-CN is intimately involved in the physical, functional and nutritional properties of milk and in the manufacture of cheese. However, along with β-lactoglobulin, the intact caseins are most commonly implicated in IgE-mediated cow milk allergy that affects a small population of children (El-Agamy, 2007).

The ability to quantify intact β-CN content is important in evaluating the quality of bovine milk used for the manufacture of diverse dairy-based consumer products. Quantitative methods based on different analytical principles have been developed for the determination of the milk caseins, including separation-based techniques (e.g. gel electrophoresis, capillary electrophoresis and high performance liquid chromatography, exploiting various separation and detection modes) and biospecific immunoassay-based techniques (e.g. nephelometry, enzyme-linked immunosorbent assay, radial immunodiffusion and biosensors); the attributes and limitations of these strategies have been comprehensively reviewed (Dupont et al., 2013).

Optical biosensors utilising surface plasmon resonance (SPR) detection have become an important evanescent field technique for the label-free and real-time monitoring of biomolecular interactions at an interface. Although predominantly applied for evaluating kinetic and

* Corresponding author.
E-mail addresses: harvey.indyk@fonterra.com (H.E. Indyk), brendon.gill@fonterra.com (B.D. Gill), jackie.wood@fonterra.com (J.E. Wood), sowmya.chetikam@fonterra.com (S. Chetikam), tadashi.kobayashi@fonterra.com (T. Kobayashi).

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thermodynamic characteristics in drug discovery and life science environments, they have also been applied within the food sector to facilitate concentration analysis (Gauglitz, 2020). Given the mass sensitivity of SPR detection, immunoaffinity techniques may be utilised most simply in a direct or sandwich assay format for high molecular mass analytes such as proteins, whereby the primary antibody biorecognition ligand is immobilised on the sensor surface. Several quantitative methods based on this principle for the intact whey proteins of milk and for the evaluation of their vulnerability to denaturation have been reported (Billakanti et al., 2010; Dupont et al., 2013; Indyk et al., 2017). As caseins are generally less easily exploited as antigens than the globular whey proteins, there have been fewer quantitative SPR-based immunoassay applications for the milk caseins; studies have targeted milk species adulteration, raw milk quality, casein degradation during cheese manufacture, milk allergen detection, casein interactions and small molecule binding, and have required diverse assay formats (Ashley et al., 2017; Bahri et al., 2019; De Gobba et al., 2020; Hiep et al., 2007; Marchesseau et al., 2002; Muller-Renaud et al., 2005; Nehra et al., 2020; Yman et al., 2006).

The objective of this study was to develop and single-laboratory validate a simple, direct SPR-based method utilising a single fluidic biosensor instrument and a commercially available polyclonal antibody, for the routine quantitation of physiologically active intact β-CN in milk and milk products, which may be effective in indicating the presence of bovine milk where such control may be necessary.

2. Materials and methods

2.1. Instrumentation and reagents

The Biacore® Q optical biosensor, its instrument operation and data processing software (v3.0.5) and CM5 sensor chips were from GE Healthcare (Uppsala, Sweden). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDC, 0.4 mol L^{-1}), N-hydroxysuccinimide (NHS, 0.1 mol L^{-1}), ethanolamine-HCl (1 mol L^{-1}, pH 8.5), sodium acetate buffer (10 mmol L^{-1}, pH 4.0, 4.5, 5.0 and 5.5) and HBS-EP running buffer (10 mmol L^{-1} HEPES, 150 mmol L^{-1} NaCl, 3.4 mmol L^{-1} EDTA, 0.005 % surfactant P20, pH 7.4) were obtained from GE Healthcare. The regeneration solution was sodium hydroxide (10 mM) and water was of > 18 MΩ resistivity.

Rabbit polyclonal anti-bovine β-CN antibody (1 mg mL^{-1}), isolated by specific affinity fractionation over β-CN antigen, with purity demonstrated by Western Blot, was obtained from Biosensis (R-1794, Thebarton, South Australia, Australia). The stock antibody solution was sub-aliquoted and was stable for up to 12 months at −18 °C. Two other commercially available polyclonal anti-bovine β-CN antibodies were also obtained for screening evaluation (Antibodies-online, PA, USA; Abbiotec, CA, USA). β-CN was sourced from Sigma-Aldrich (St. Louis, MO, USA). β-CN was also isolated in-house from acid-precipitated casein from bovine skim milk, by fractionation using cation-exchange chromatography (S-Sepharose), extensive dialysis and freeze drying. The bovine milk proteins α-casein, κ-casein, α-lactalbumin, β-lactoglobulin, bovine serum albumin and immunoglobulin G were obtained from Sigma-Aldrich, bovine lactoferrin was isolated from skim milk as described previously (Indyk et al., 2007) and proteose peptone 5 (PP5) was prepared from plasmin-hydrolysed β-CN and was isolated by anion-exchange chromatography.

2.2. Biosensor surface preparation

During method development, the anti-bovine β-CN antibody and β-CN were immobilised on separately assigned flow cells of a CM5 sensor chip by standard amine coupling at 25 °C. Briefly, each surface was activated with a mixture of EDC and NHS (1:1 *v/v*, 10 μL min^{-1}, 7 min) followed by either antibody (35 μg mL^{-1} in 10 mmol L^{-1} sodium acetate, pH 5.0, 10 μL min^{-1}, 7 min) or β-CN (50 μg mL^{-1} in sodium acetate buffer, pH 4.0, 10 μL min^{-1}, 7 min). Following ligand immobilisation, unreacted ester functionalities were deactivated with ethanolamine (10 μL min^{-1}, 7 min). A reference flow cell surface was prepared similarly by omitting the ligand immobilisation step. Between analyses, functionalised chips were stored in a sealed container over silica gel desiccant at 4 °C.

2.3. Standards

Bovine β-CN was dissolved in water to approximately 1 mg mL^{-1} and was filtered twice (0.2 μm cellulose acetate membrane). The concentration of this clarified stock solution was determined spectrophotometrically (E1%: 280 nm = 4.6) and the solution was stored frozen. An intermediate standard was prepared at 100 μg mL^{-1} in HBS-EP buffer and sub-aliquots were stored at −18 °C. β-CN working standards (0–5500 ng mL^{-1}) were prepared daily by sequential dilution from the intermediate standard in HBS-EP buffer and were used to generate a calibration curve for the estimation of the β-CN content in samples.

Stock solutions of individual milk proteins were separately prepared to approximately 10 mg mL^{-1} in water and were stored frozen; working dilutions (1–10 μg mL^{-1}) of these stock solutions were prepared in HBS-EP buffer for the evaluation of antibody cross-reactivity.

2.4. Samples

Liquid consumer milks, milk powders, whey protein concentrates, whey protein isolates and infant formulae were obtained from milk processing facilities and commercial sources. Samples were prepared for analysis by adding HBS-EP buffer (1:20 *v/v*) to liquid milk or by adding water (1:20 *w/v*) to powdered samples, with vortex mixing, followed by ultra-sonication (15 min). Further serial dilutions were accomplished with HBS-EP buffer to a final optimised dilution level that depended on the expected β-CN content (liquid milk, 1:10,000 *v/v*; powdered milk, infant formula and protein products, 1:100,000 *w*/v).

2.5. Biosensor assay

The optimised direct assay for routine quantitative application utilised the tethered rabbit polyclonal anti-bovine β-CN antibody surface. β-CN calibration standards (in duplicate) and sample extracts (100 μL) were dispensed into a 96-well microtitre plate and were sequentially injected (5 min at 10 μL min^{-1}) with HBS-EP running buffer at 25 °C. Binding responses were measured 30 s after the initiation of the dissociation phase relative to the baseline, and were used to generate a calibration curve using a four-parameter logistic regression [$y = R_{hi} - ((R_{hi} - R_{lo})/1 + (Conc/A_1)^{A2})$], where R_{hi}= response at infinite concentration, R_{lo}= response at zero concentration, A_1= B_{50}, A_2= slope factor and Conc = concentration of β-CN (ng mL^{-1}). The β-CN concentration of unknown samples was determined by interpolation from the calibration curve. Following each cycle, the sensor surface was regenerated by a 10 μL injection of 10 mmol L^{-1} sodium hydroxide at 50 μL min^{-1}.

The maximum binding capacity (R_{max}) was estimated by performing multiple injections of β-CN (100 μg mL^{-1}) without surface regeneration. Additional validation trials incorporated a sandwich assay format, whereby the primary polyclonal anti-β-CN antibody (R-1794, Biosensis) was injected over the captured β-CN, prior to surface regeneration, in order to qualitatively confirm the integrity of the captured casein.

3. Results and discussion

3.1. Sensor surface

Subsequent to pH screening, performed without surface activation to select optimal conditions, sensor surface functionalisation with either antibody or β-CN was accomplished by exploiting standard amine-

coupling covalent chemistry, yielding a randomly orientated protein ligand. This protocol generated high ligand density surfaces of approximately 10 kRU and 3 kRU respectively (1 RU is a change in refractive index of 10^{-6}, equivalent to 1 pg mm^{-2} protein), which, for the antibody, represents about 65 fmol of tethered protein. The reproducibility relative standard deviation (RSD) of multiple ligand immobilisations was 11.1 % ($n = 13$), as estimated over three lots of antibody and five different sensor chips. A high affinity ligand is favoured for concentration immunoassay, and the anti-β-CN antibody was selected from a panel of three commercially available rabbit polyclonal anti-CN antibodies by initially evaluating uninhibited binding characteristics to a β-CN-immobilised surface. The selected antibody (R-1794, Biosensis) yielded a binding response of appropriate magnitude and was specific, as revealed by the absence of non-specific binding to a reference non-functionalised surface. In contrast, of the two alternative antibodies screened, one was resistant to regeneration and one gave low binding response. This latter observation of the poor performance of a high proportion of commercially available antibodies has been reviewed (Baker, 2015; Perkel, 2014). The selected antibody was then further evaluated under direct assay conditions, whereby β-CN was injected over the immobilised antibody, confirming both acceptable binding response and specificity, as demonstrated by the absence of non-specific binding of β-CN to the non-functionalised surface. The described immobilisation chemistry is suitable for a concentration assay despite the non-orientated configuration of surface tethered antibody, although an orientated affinity capture technique over Protein A has been reported (Hiep et al., 2007).

The R_{max} of the active antibody ligand surface was estimated to be approximately 0.25 of theoretical under direct assay conditions, and was considered to be suitable for the quantitation of intact β-CN. Following injection of either β-CN or sample extract, regeneration of the antibody-functionalised surface was achieved with sodium hydroxide, in preference to a low pH reagent, which would potentially result in casein precipitation within the flow cell.

3.2. Method development and validation

Specificity was evaluated by quantifying the extent of binding inhibition by antibody under competitive direct assay conditions. Thus, when β-CN was equilibrated in solution with excess anti-β-CN antibody (12–65 M excess), its subsequent binding to the antibody-functionalised surface was inhibited by > 98 %. The selectivity of the tethered polyclonal antibody was further assessed under direct assay conditions by estimating its cross-reactivity, relative to β-CN, for individual milk proteins and the principal β-CN proteolytic PP5 peptide. Compared to β-CN, negligible binding was observed for the major whey proteins (β-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulin G) as illustrated in Fig. 1, with PP5 exhibiting minor cross-reactivity (approximately 2%); however, at high concentrations, lactoferrin was found to bind non-selectively to the sensor surface due to its high pI. When corrected for molecular mass, a minor, but significant binding cross-reactivity of about 5% was demonstrated for α- and κ-casein isoforms, both of which are present in milk at levels comparable with that of β-CN. In the absence of an available monoclonal antibody exclusively specific for β-CN, the selectivity of the polyclonal antibody was considered to be acceptable.

HBS-EP buffer containing EDTA, used to prepare both calibration standards and sample solutions, has previously been demonstrated to be effective in disrupting casein micelles and releasing monomeric forms for quantitative analysis (Muller-Renaud et al., 2005). The use of authentic β-CN in this study for method calibration was preferred over the use of a reference milk powder for the quantitation of the β-CN content in milk-based samples, as described in a previous study (Muller-Renaud et al., 2005). Prior to the spectrophotometric determination of the β-CN calibrant concentration, it was necessary to methodically filter the stock standard in order to clarify the turbid solution, yielding a purity of < 100 %, as compared with the certificate of analysis. Once corrected for purity, both commercial and in-house sources of β-CN were determined to yield equivalent binding responses to immobilised anti-β-CN antibody.

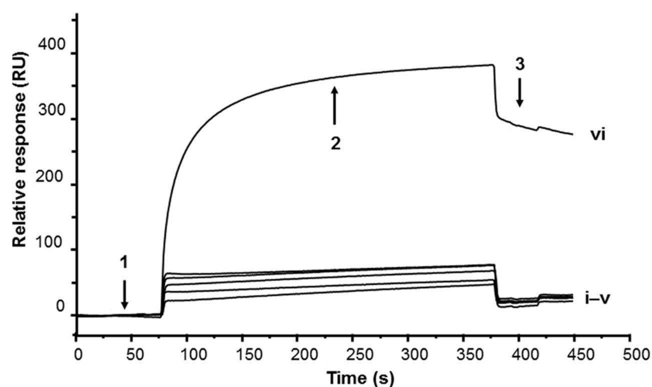

Fig. 1. Superimposed sensorgrams for protein standards (5000 ng mL^{-1}) [(i) HBS-EP blank, (ii) β-lactoglobulin, (iii) α-lactalbumin, (iv) bovine serum albumin, (v) immunoglobulin G, (vi) β-casein] over an anti-β-casein antibody immobilised surface under direct assay conditions (regeneration phase of each cycle removed for clarity): 1, baseline; 2, association; 3, binding level.

Immunoassays, irrespective of platform, are potentially vulnerable to non-specific binding, which, if present, will compromise method reliability. Therefore, the extent of non-specific binding of the selected antibody, authentic β-CN and a range of dairy sample extracts, including whey proteins, was assessed over a reference, ligand-free carboxymethyldextran surface; in all cases, it was confirmed to be negligible ($<$ 2 RU), thereby facilitating a direct sample dilution in HBS buffer to within the assay calibration range, without the requirement for prior lipid removal or additional sample clean-up. Analysis of liquid skim milk, low-fat milk and whole milk samples containing 0.1–3.3 % milkfat confirmed that lipid removal was unnecessary during sample preparation. As the binding response of β-CN to the immobilised antibody was found to be independent of sodium chloride concentration from 0.15 to 0.50 M, the ionic strength of the sample diluent was optimal at 0.15 M.

As bovine milk that is devoid of β-CN is unavailable, potential unidentified matrix binding was further evaluated by analysis of serial dilutions of liquid milk, skim milk powder and infant formula powders under the described direct assay conditions, with the β-CN concentration determined to be within $\pm$ 10 % of the mean value, thereby confirming both an absence of significant matrix non-specific binding and optimum sample dilution levels for routine application.

For a high molecular mass analyte such as β-CN, a simple direct assay over immobilised antibody is the most facile, with a single reagent format, minimal requirement of antibody and where response is directly related to analyte concentration. However, the alternative inhibition assay format over immobilised target protein was investigated during method development, and although the expected inverse dose–response calibration was observed, self-association of β-CN precluded this assay format for routine use. This observation confirms previous reports of the binding interaction of solution and immobilised β-CN as determined by SPR-based techniques (Bahri et al., 2019; De Gobba et al., 2020; Marchesseau et al., 2002).

Optimised contact time (5 min), flow rate (10 μL min^{-1}) and ligand regeneration (sodium hydroxide, 10 mM at 50 μL min^{-1}) conditions were defined for the direct-binding assay used for routine application, yielding acceptable binding response for the highest level β-CN calibration standard (> 200 RU), stable baseline response of immobilised ligand over multiple cycles, with a repeatability relative standard deviation (RSD_r) of 0.10 % ($n = 15$) demonstrating stability of the immobilised antibody during multiple regeneration cycles. Each of the four addressable functionalised flow cell surfaces performed

consistently for approximately 200 injections across multiple storage cycles at 4 °C. In practice, 1–2 injection cycles are initially required to establish a stable relative binding response for both calibration standards and sample extracts. Sensorgrams acquired for a single set of β-CN calibration standards and a derived dose-response curve are presented in Fig. 2.

It is apparent from the non-linearity during both the association phase and calibration curve, and confirmed by the variance of the initial binding rate with increasing flow rate (10–75 μL min^{-1}), that the interaction between immobilised ligand and β-CN is partially mass-transport limited over the entire association phase, with the described end-point binding level measurement therefore found to be optimal for the reliable quantitation of β-CN.

Over the calibration interval, a four-parameter logistic regression effectively described the dose–response relationship, with an intermediate precision (RSD_{iR}) of 13.8 % ($n = 13$) for the estimated B_{50} value over multiple assays and immobilised surfaces. The instrumental limit of detection (response + 3 × SD of blank) was estimated to be 0.1 ng mL^{-1} ($n = 5$), and the method detection limit (SD × $t_{n-1,\ 0.01}$) determined by replicate analysis of a fluid milk sample ($n = 7$) was 2.3 mg mL^{-1} (Su, 1998), suitable for the estimation of β-CN content in milk products. Repeatability (RSD_r) was estimated for the binding response of the top level β-CN calibration standard to be 4.9 % ($n = 15$). The RSD_r for a fluid skim milk was estimated to be 5.30 % ($n = 7$), and the RSD_{iR} was 10.1 % for replicate independent fluid skim milk samples ($n = 26$) and 10.7 % for a skim milk powder ($n = 12$). In the absence of an international reference method for the measurement of βCN, a certified reference material for β-CN, or any proficiency testing scheme, method accuracy was evaluated by estimating recovery subsequent to dosing a skim milk sample with β-CN at 50, 100 and 150 % of the endogenous content, with the mean recovery estimated to be 101.7 % (88.2–116.7 %) over two independent immobilised surfaces.

The sandwich immunoassay format was evaluated for its potential to both verify antibody specificity and enhance assay sensitivity, and was accomplished by employing the same primary antibody as the enhancement reagent. Despite the relatively low concentration of enhancement antibody (20 μg mL^{-1}) due to its limited supply, the secondary signal was, as expected, amplified because of the higher molecular mass of immunoglobulin G relative to that of surface-captured β-CN, as illustrated in Fig. 3. Although not employed for the routine quantitation of β-CN due to increased analysis time, a high antibody consumption, a relatively high dissociation rate and procedural complexity, this technique provides qualitative information regarding the integrity of β-CN, a protein that is vulnerable to proteolytic cleavage within processed milk products.

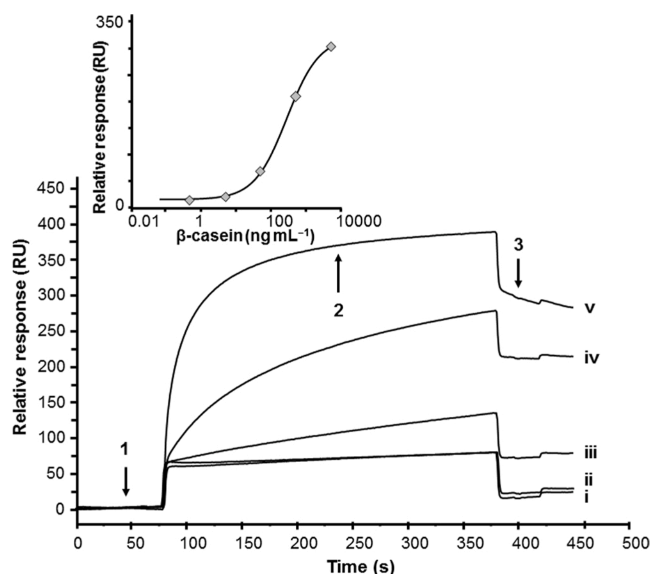

Fig. 2. Superimposed sensorgrams for β-casein calibration standards [(i) 0 ng mL^{-1}, (ii) 5.5 ng mL^{-1}, (iii) 55 ng mL^{-1}, (iv) 550 ng mL^{-1}, (v) 5500 ng mL^{-1}], in singlicate, over an anti-β-casein antibody immobilised surface under direct assay conditions (regeneration phase of each cycle removed for clarity): 1, baseline; 2, association; 3, binding level. Inset: derived dose–response calibration curve.

3.3. Application to bovine milk products

The specificity and sensitivity of the SPR-based biosensor immunoassay facilitated application to bovine milk products, subsequent to a simple sample preparation involving direct dilution in buffer.

Whey protein, whey protein concentrate and whey protein isolate powders manufactured from either rennet whey or acid whey, with various protein contents, were analysed by the described method. Whey protein and whey protein concentrate, although predominantly containing the major whey proteins, can also contain low levels of soluble casein-derived peptides such as glycomacropeptide and PP5, which, along with trace residual β-CN, were observed to bind to a minor extent with low cross-reactivity to the immobilised anti-β-CN antibody. Because of its higher whey protein content and more exhaustive manufacturing protocol, whey protein isolate is essentially free of potential casein-derived peptides and hence was observed to generate negligible binding to the antibody. These observations with whey protein products provide further confidence in the selectivity of the method for β-CN.

The β-CN content of retail consumer milks was estimated over several months and ranged between 9.2 and 13.7 mg mL^{-1} with a mean of 11.6 mg mL^{-1} ($n = 26$). These values are consistent with the range of 8.0–14.8 mg mL^{-1} that has generally been reported in other studies utilising a wide variety of both separation-based and immunoassay-based analytical techniques (Anema, 2009; Bär et al., 2019; Dupont et al., 2013; Huppertz, 2013; Muller-Renaud et al., 2004; Zhou et al., 2013), indicating both method accuracy and, given the minor binding response of PP5, that negligible endogenous plasmin proteolysis of β-CN had occurred in the fresh retail milks tested. The concentrations of the major milk proteins, including β-CN, are typically reported as a range because of their dependence on many factors including lactation, breed, season, udder health and feed, although analytical variability will undoubtedly also be a factor.

A skim milk powder was analysed as a quality control sample across multiple analytical runs ($n = 12$) and yielded a mean β-CN content of 94.8 mg g^{-1} (range 81.2–113.0 mg g^{-1}). This value is consistent with the expected value of 103.0 mg g^{-1}, based on measured total protein (33.0 %) and values reported for the proportion of total protein that is β-CN (31.3 %). The method was also applied to a whey-based infant formula

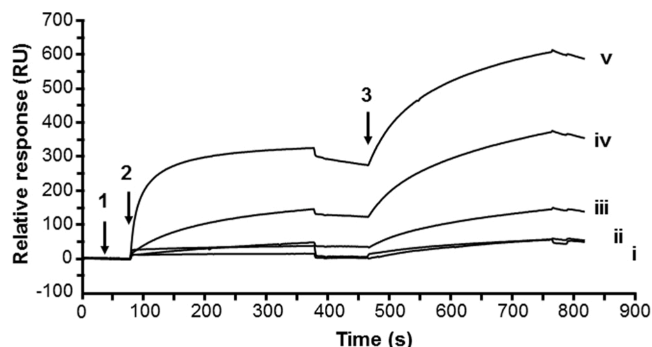

Fig. 3. Superimposed sensorgrams for β-casein calibration standards [(i) 0 ng mL^{-1}, (ii) 5.5 ng mL^{-1}, (iii) 55 ng mL^{-1}, (iv) 550 ng mL^{-1}, (v) 5500 ng mL^{-1}] over an anti-β-casein antibody immobilised surface under sandwich assay conditions (regeneration phase of each cycle removed for clarity): 1, baseline; 2, β-casein injection; 3, secondary antibody injection.

powder and the β-CN content was estimated to be 22.4 mg g^{-1} ($n = 4$), a value that is consistent with a calculated β-CN content of 21.0 mg g^{-1} based on a measured total protein content of 15.6 % and a whey protein: casein ratio of 65:35 *w/w*.

The caseins, and in particular β-CN, are vulnerable to cleavage by endogenous proteolytic milk enzymes, a process that commonly occurs as part of cheese manufacture; the remaining intact casein constitutes the final cheese matrix, and the casein proteolytic products are eliminated as part of the soluble whey fraction. Two studies have previously described SPR-based biosensor immunoassays for intact α-CN and β-CN; they were configured in a two-step sandwich mode and employed separate monoclonal antibodies produced in-house that were demonstrated to be specific for the N- and C-terminal peptides of each casein (Muller-Renaud et al., 2004, 2005). The present study has demonstrated a similar strategy for interrogating the qualitative integrity of β-CN, although its specificity for the intact protein is lower due to the use of the same polyclonal antibody as both primary and secondary reagent. Nonetheless, the described method does not require the use of monoclonal reagents that are commercially unavailable, and the demonstrated utility of the commercially available polyclonal antibody facilitates method implementation in a routine product release environment. However, further work will investigate the utility of complementary antibodies, in order to increase the specificity for intact β-CN by discriminating peptides originating from the parent protein.

Apart from its utility as a routine technique for dairy product quality control, the described biosensor immunoassay technique for quantifying intact bovine milk β-CN may have, despite its moderate sensitivity under the described conditions, further applications in allergy control strategies, both for foods potentially containing bovine milk ingredients, and confirming the absence of milk caseins in manufacturing environments where carry-over on a common packing line is involved (Alves et al., 2016; Ashley et al., 2017; Nehra et al., 2020; Yman et al., 2006).

4. Conclusions

A direct, single-antibody SPR-based biosensor immunoassay for the routine quantitation of β-CN in bovine milk and milk products is described. The assay exploits an immobilised, commercially available polyclonal antibody ligand that was demonstrated to be stable to multiple regeneration cycles, and avoids the requirement for a more complex sandwich immunoassay format strategy utilising non-commercially accessible monoclonal antibody reagents. Assay variables that influence analytical performance were optimised and the method was found to be rapid, sensitive, precise and accurate, and is reliable for a range of milk products containing intact β-CN.

In common with alternative immunoassay methods, it is a single protein quantitation technique, which is a limitation by comparison with separation-based methods that facilitate multiplex protein determination. Nonetheless, because of its inherent antibody-based biospecific detection strategy, the method allows for an estimation of the physiologically active concentration, incorporates a sample preparation of minimal complexity involving direct dilution in buffer, and may therefore be considered to complement alternative techniques.

Author statement

Harvey E. Indyk: Conceptualization; Methodology; Roles/Writing - original draft.

Brendon D. Gill: Writing - review & editing.

Jackie E. Wood: Writing - review & editing.

Sowmya Chetikam: Conceptualization.

Tadashi Kobayashi: Conceptualization.

Declaration of Competing Interest

The authors report no declarations of interest.

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References

Alves, R.C., Barroso, M.F., Gonzáles-García, M.B., Oliveira, M.B.P.P., Delerue-Matos, M. C., 2016. New trends in food allergens detection: towards biosensing strategies. Crit. Rev. Food Sci. Nutr. 56, 2304–2319. https://doi.org/10.1080/10408398.2013.831026.

Anema, S.G., 2009. The use of "lab-on-a-chip" microfluidic SDS electrophoresis technology for the separation and quantification of milk proteins. Int. Dairy J. 19, 198–204. https://doi.org/10.1016/j.idairyj.2008.11.001.

Ashley, J., Piekarska, M., Segers, C., Trinh, L., Rodgers, T., Willey, R., Tothill, I.E., 2017. An SPR based sensor for allergens detection. Biosens. Bioelectron. 88, 109–113. https://doi.org/10.1016/j.bios.2016.07.101.

Bahri, A., Henriquet, C., Pugnière, M., Marchesseau, S., Chevalier-Lucia, D., 2019. Binding analysis between monomeric β-casein and hydrophobic bioactive compounds investigated by surface plasmon resonance and fluorescence spectroscopy. Food Chem. 286, 289–296. https://doi.org/10.1016/j.foodchem.2019.01.176.

Baker, M., 2015. Blame it on the antibodies. Nature 521, 274–276. https://doi.org/10.1038/521274a.

Bär, C., Mathis, D., Neuhaus, P., Dürr, D., Bisig, W., Egger, L., Portmann, R., 2019. Protein profile of dairy products: simultaneous quantification of twenty bovine milk proteins. Int. Dairy J. 97, 167–175. https://doi.org/10.1016/j.idairyj.2019.01.001.

Billakanti, J.M., Fee, C.J., Lane, F.R., Kash, A.S., Fredericks, R., 2010. Simultaneous, quantitative detection of five whey proteins in multiple samples by surface plasmon resonance. Int. Dairy J. 20, 96–105. https://doi.org/10.1016/j.idairyj.2009.08.008.

Capriotti, A.L., Cavaliere, C., Piovesana, S., Samperi, R., Laganà, A., 2016. Recent trends in the analysis of bioactive peptides in milk and dairy products. Anal. Bioanal. Chem. 408, 2677–2685. https://doi.org/10.1007/s00216-016-9303-9308.

De Gobba, C., Møller, M.S., Rauh, V., Svensson, B., Lund, M.N., 2020. Casein–casein interactions in the presence of dairy associated carbohydrates analysed using surface plasmon resonance. Int. Dairy J. 105, 104686 https://doi.org/10.1016/j.idairyj.2020.104686.

Dupont, D., Crouennec, T., Brodkorb, A., Kouaouci, R., 2013. Quantitation of proteins in milk and milk products. In: McSweeney, P.L.H., Fox, P.F. (Eds.), Advanced Dairy Chemistry, Vol 1A: Proteins: Basic Aspects, 4th ed. Springer Science, New York, NY, USA, pp. 87–134.

El-Agamy, E.I., 2007. The challenge of cow milk protein allergy. Small Rumin. Res. 68, 64–72. https://doi.org/10.1016/j.smallrumres.2006.09.016.

Gauglitz, G., 2020. Critical assessment of relevant methods in the field of biosensors with direct optical detection based on fibers and waveguides using plasmonic, resonance, and interference effects. Anal. Bioanal. Chem. 412, 3317–3349. https://doi.org/10.1007/s00216-020-02581-0.

Hiep, H.M., Endo, T., Kerman, K., Chikae, M., Kim, D.-K., Yamamura, S., Takamura, Y., Tamiya, E., 2007. A localized surface plasmon resonance based immunosensor for the detection of casein in milk. Sci. Technol. Adv. Mater. 8, 331–338. https://doi.org/10.1016/j.stam.2006.12.010.

Huppertz, T., 2013. Chemistry of the caseins. In: McSweeney, P.L.H., Fox, P.F. (Eds.), Advanced Dairy Chemistry, Vol 1A: Proteins: Basic Aspects, 4th ed. Springer Science., New York, NY, USA, pp. 135–160.

Indyk, H.E., McGrail, I.J., Watene, G.A., Filonzi, E.L., 2007. Optical biosensor analysis of the heat denaturation of bovine lactoferrin. Food Chem. 101, 838–844. https://doi.org/10.1016/j.foodchem.2005.12.025.

Indyk, H.E., Hart, S., Meerkerk, T., Gill, B.D., Woollard, D.C., 2017. The β-lactoglobulin content of bovine milk: development and application of a biosensor immunoassay. Int. Dairy J. 73, 68–73. https://doi.org/10.1016/j.idairyj.2017.05.010.

Küllenberg de Gaudry, D., Lohner, S., Schmucker, C., Kapp, P., Motschall, E., Hörrlein, S., Röger, C., Meerpohl, J.J., 2019. Milk A1 β-casein and health-related outcomes in humans: a systematic review. Nutr. Rev. 77, 278–306. https://doi.org/10.1093/nutrit/nuy063.

Marchesseau, S., Mani, J.-C., Martineau, P., Roquet, F., Cuq, J.-L., Pugnière, M., 2002. Casein interactions studied by the surface plasmon resonance technique. J. Dairy Sci. 85, 2711–2721. https://doi.org/10.3168/jds.S0022-0302(02)74358-0.

Mishra, B.P., Mukesh, M., Prakash, B., Sodhi, M., Kapila, R., Kishore, A., Kataria, R.R., Joshi, B.K., Bhasin, V., Rasool, T.J., Bujarbaruah, K.M., 2009. Status of milk protein β-casein variants among Indian milch animals. Indian J. Anim. Sci. 79, 722–725.

Muller-Renaud, S., Dupont, D., Dulieu, P., 2004. Quantification of β-casein in milk and cheese using an optical immunosensor. J. Agric. Food Chem. 52, 659–664. https://doi.org/10.1021/jf034723o.

Muller-Renaud, S., Dupont, D., Dulieu, P., 2005. Development of a biosensor-immunoassay for the quantification of α_{S1}-casein in milk. J. Dairy Res. 72, 57–64. https://doi.org/10.1017/S0022029904000664.

Nehra, M., Lettieri, M., Dilbaghi, N., Kumar, S., Marrazza, G., 2020. Nano-biosensing platforms for detection of cow's milk allergens: an overview. Sensors 20, 32. https://doi.org/10.3390/s20010032.

Perkel, J.M., 2014. The antibody challenge. BioTechniques 56, 111–114. https://doi.org/10.2144/000114143.

Su, G.C.C., 1998. A comparison of statistical and empirical detection limits. J. AOAC Int. 81, 105–110. https://doi.org/10.1093/jaoac/81.1.105.

Szwajkowska, M., Wolanciuk, A., Barłowska, J., Król, J., Litwińczuk, Z., 2011. Bovine milk proteins as the source of bioactive peptides influencing the consumers' immune system—a review. Anim. Sci. Pap. Rep. 29, 269–280.
Truswell, A.S., 2005. The A2 milk case: a critical review. Eur. J. Clin. Nutr. 59, 623–631. https://doi.org/10.1038/sj.ejcn.1602104.
Yman, I.M., Eriksson, A., Johansson, M.A., Hellenas, K.-E., 2006. Food allergen detection with biosensor immunoassays. J. AOAC Int. 89, 856–861.
Zhou, Y., Song, F., Li, Y.S., Liu, J.Q., Lu, S.Y., Ren, H.L., Liu, Z.S., Zhang, Y.Y., Yang, L., Li, Z.H., Zhang, J.H., Wang, X.R., 2013. Double-antibody based immunoassay for the detection of β-casein in bovine milk samples. Food Chem. 141, 167–173. https://doi.org/10.1016/j.foodchem.2013.03.010.