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Semi-continuous, Label-free Immunosensing Approach for Ca^{2+}-based Conformation Change of a Calcium Binding Protein

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RUNNING HEAD: Semi-continuous Immunosensing of Calcium Ions

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Abstract

A label-free immunosensing method based on the conformational change of calcium binding protein (CBP) depending on analyte concentration was explored for semi-continuous analysis of free Ca^{2+}. Glucose-galactose binding protein as a CBP and produced as a recombinant protein by *Escherichia coli* was used as the immunogen to produce monoclonal antibodies by hybridoma technology. We finally screened the 3-6F cell clone, which produced the desired antibody specific to a particular structural conformation of the protein that occurred only upon CBP-calcium complex formation. To construct an immunosensor, the antibody was immobilized via a secondary antibody on an Octet Red optical fiber-based label-free sensor. A calcium analysis was conducted on the sensor in combination with CBP previously added to the aqueous sample, which distinguished the sensor signal according to the analyte concentration. The immunosensor produced a signal in real time with a response time of approximately 15 min and could be reused for analyses of different samples in a semi-continuous manner. The minimum detection limit of the analyte under optimal conditions was 0.09 mM and the upper limit was about 5 mM (log-logit transformed standard curve linearity: R^2 > 98%). In sample tests with milk, analytical performance of the sensor was highly correlated (R^2 > 99%) with that of the reference system based on the $KMnO_4$ titration method (ISO 12081). Although the sensor showed cross-reactivity at high concentrations (> 1 mM) of cations including zinc, iron, manganese, and copper, these ionic components were not traceable (< 0.01 mM) in milk.

Key words: Glucose-galactose binding protein as a calcium binding protein, Calcium binding-induced conformation change, Protein conformation-specific antibody, Semi-continuous immunosensor, Real sample test for milk

Introduction

Many food components are critical to maintain a healthy body when included or excluded, and therefore need to be quantified through various analytical techniques.[1] One of these is calcium ions (Ca^{2+}), which is the most abundant mineral in the body, although only a relatively small amount circulates in the blood (<1% of total body calcium).[2] Serum Ca^{2+} is crucial in the control and regulation of physiological systems inside and outside of cells. Ca^{2+} supports muscle contraction, blood vessel expansion and contraction, secretion of hormones and enzymes, and transmission of impulses throughout the nervous system.[3] Furthermore, Ca^{2+} has been implicated in preventing colon cancer and osteoporosis.[4] An increasing number of people in aging societies are at risk for calcium deficiency, which must be supplied from the diet. Many foods are fortified with calcium.[5]

Although methods for determining calcium in food samples are well established,[1] the need for pre-treatment hinders automation of methods for routine analysis of dietary foods. To quantify calcium in food, chemical methods or atomic absorption spectroscopy has been used for specific mineral content only after sample pre-treatment for chelation and additional processing to detect the chelated complexes.[6] Ion-selective electrode (ISE) and enzyme-based biosensors have attracted interest because of potential simplification of analytical procedures and their inexpensive cost.[7] However, commercial ISEs suffer from interference caused by matrix effects and lack of day-to-day reproducibility, which may blunt the accuracy of the analysis.[8] A biosensor incorporating a calcium-requiring enzyme was proven too labile for reuse.[9]

Binding proteins can be used to bind an analyte to a protein for analyses of small molecular components or to dissociate the analyte from binding complexes, depending on the concentration in the sample.[10] Such binding reactions can be carried out without consuming reagents or producing additional chemical species, which would be a preferred automation analysis format.[11] Although a variety of binding proteins are known, typical examples for calcium analysis are calmodulin (CaM) and glucose-galactose binding protein (GGBP).[12] Interestingly, binding of a small substance to the designated site of a protein induces molecular motion for fitting, which alters the structural conformation of the protein. This

returns to the original molecular configuration in a reversible manner when the ligand dissociates from the complex due to a concentration decrease in the sample.[13]

This conformational change in CaM has been utilized to quantitatively detect Ca^{2+} by employing distinct signal generation technologies.[14] As the motion change of the protein may alter the local environment of the molecule surrounding the amino acid, residues emitting fluorescence, such as tryptophan, are altered in relation to the ligand concentration in a sample.[15] The signal can also be modulated by the distance change between two arms tagged with a fluorescent donor and acceptor, respectively. The distance is far enough in the absence of the analyte to emit the excited light energy from the donor into the bulk solution, whereas it contracts upon binding the analyte to transfer the energy to the accepter for quenching. However, such sensing principles require a label, such as a chemical or biological fluorophore, to produce a signal. Thus, the analytical performance would be dependent on several other factors related to label stability, and interference with signal transmission of the detector.[16]

In this study, a label-free sensing approach was investigated to directly measure calcium binding to a calcium binding protein (CBP) designated GGBP, as an alternative method for automated food analysis. As GGBP also shows a structural conformation change upon binding to calcium,[17] the interaction was semi-continuously monitored on a label-free sensor via immunological signal amplification. The calcium binder was produced by genetic recombination and used as an immunogen to produce a monoclonal antibody specific to a particular structural conformation of GGBP that occurs only upon protein-calcium complex formation. The complex was finally quantified using the Octet Red optical fiber-based sensor[18] with the antibody immobilized on the sensor surfaces, which can be reused in a semi-continuous manner. GGBP has a single calcium-binding site per molecule, unlike other CBPs,[17] facilitating an estimate of the specific region that the antibody reacts with, and subsequent clarification of the proposed analytical concept. CaM contains four identical subunits, each of which binds a metallic ion, and shows different protein conformations according to the degree of complex formation.[19] Label-free sensing of calcium in a real sample (milk) demonstrated that the same sensor can be repetitively used for analysis of ionic ingredients in food with minimal sample handling.

Materials and Methods

Materials

Potassium phosphate, magnesium sulfate heptahydrate, zinc chloride, iron chloride hexahydrate, maganese chloride, cupric chloride, aluminum hydroxide, hypoxanthine-aminopterin-thymidine (HAT) medium supplement, goat anti-mouse IgG antibody (specific to whole IgG) conjugated with horseradish peroxidase (HRP), 3,3',5,5'-tetramethylbenzidine (TMB), casein (sodium salt type, extracted from milk), and polyoxyethylene sorbitan monolaurate (Tween 20) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The Ni-NTA Purification System containing Ni-NTA agarose, guanidinium lysis buffer, and imidazole were supplied by Qiagen (Venlo, the Netherlands). Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Welgene (Daegu, Korea) and PAA Laboratory (Etobicoke, Ontario, Canada), respectively. The Octet Red anti-mouse capture (AMC) sensor tip was supplied by ForteBio (San Francisco, CA, USA). Calcium chloride dihydrate and potassium chloride were provided by Daejung (Siheung, Korea) and Junsei (Tokyo, Japan), respectively. Other reagents used were of analytical grade.

Production of the Mutated GGBP Molecule

Gene manipulation. Residues Asp14 and Gln26 on the GGBP molecule were mutated to provide binding specificity to glucose and to introduce a sulfhydryl group, respectively.[20] For site-directed mutagenesis of the residues, the construction was carried out to change the GGBP Asp14 to glutamate and Gln26 to cysteine on the GGBP expression vector, pET30(c)/mglB, by the Quik Change method (Strategene, La Jolla, CA, USA) using mutagenic primer pairs described previously.[12] The sequences of the created mutants were confirmed.

Microbial cell culture. *Escherichia coli* BL21(DE3) cells harboring the pET30(c)/mglB plasmid were cultured initially in a flask, and the protein was then induced in the medium containing isopropyl β-D-1-thiogalactopyranoside (IPTG). A single colony from a selective antibiotic LB agar plate was inoculated in 10 mL of LB medium containing ampicillin (final

concentration, 50 μg/mL) and grown at 37ºC for 16 h. This culture was transferred to 100 mL of the same medium and further cultured with vigorous shaking at 37ºC for 3 to 4 h until an optical density of 0.6 at 600 nm was reached. The protein was expressed by adding IPTG (final concentration, 1 mM) and maintaining the culture for additional 4 h. The cells were harvested by centrifugation at 4,000 rpm for 20 min, and the cell pellets were stored frozen at −20ºC. The expressed protein was analyzed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[21]

Recovery and purification. The periplasmic fraction was prepared by sonication of the bacterial medium, and the supernatant was collected after centrifugation, as described previously.[12] Briefly, the cell pellets were resuspended in lysis buffer containing lysozyme and benzonase nuclease on ice for 30 min. The cell debris was centrifuged, and the lysate was dialyzed against 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl (Buffer A) and 10 mM imidazole. The samples were subsequently loaded onto a Ni-NTA agarose column (2 mL gel volume) and fractionated according to the manufacturer's protocol. After washing with three column volumes of Buffer A containing 20 mM imidazole, the mutant GGBP was eluted with Buffer A containing 250 mM imidazole. The purification and expression levels of GGBP were determined by SDS-PAGE, and the protein concentration was determined by the Bradford method.[22]

Production of a Monoclonal Antibody Specific to GGBP

Mice immunization. Monoclonal antibodies specific to the mutated GGBP were produced using a modified standard protocol.[23] Four 6-week old Balb/c female mice were injected intraperitoneally with purified GGBP (40 μg/mouse), which had been emulsified with complete Freund's adjuvant (150 μL) combined with the same volume of sterile phosphate-buffered saline (PBS), pH 7.4. The mice were subsequently injected with the identical immunogen twice except for the use of incomplete Freund's adjuvant at a 2-week interval. The serum samples from each mouse were collected on day zero for the control and 7 days after each injection. Serum activity was tested via enzyme-linked immunosorbent assay (ELISA) with GGBP immobilized in wells of a microtiter plate as described below.

Cell fusion and screening. After immunization, the mouse splenocytes were fused with myeloma (sp2/0 Ag14) using a standard protocol.[23] The fused hybridoma cells were then screened based on HAT selection, and the activity of the produced antibodies was tested using ELISA with immobilized antigen as described below. Hybridoma clones showing various activities were pre-screened and were cultivated into a single cell line by limiting dilution.

Selection of antibodies responding to calcium. Among the clones, antibodies with activity towards a particular GGBP conformation under differential binding conditions, with and without calcium, were selected. Microwells were coated with the mutated GGBP (1 μg/mL; 100 μL each) diluted in 10 mM Tris containing 140 mM NaCl, pH 7.4 (Tris buffer). The plate was placed in a sealed box, maintained at 100% humidity, and incubated at 37ºC for 1 h. It was then washed three times with deionized water. These conditions were also employed after each step below unless stated otherwise. After blocking the remaining surfaces with Tris buffer containing 0.5% casein (200 μL; Tris-Casein), the antibodies produced from the cell culture were diluted 1:10 with 1 mM $CaCl_2$ in Tris-Casein containing 0.1% Tween 20 (Tris-Casein-TW) and then incubated in each well. After washing with 1 mM $CaCl_2$ in Tris buffer ($CaCl_2$-Tris), goat anti-mouse IgG antibody conjugated with HRP was reacted in the calcium-containing Tris-Casein-TW. After washing with $CaCl_2$-Tris, the substrate solution (100 μL) containing TMB for HRP was finally reacted at room temperature for 15 min, and color development was stopped by adding 2 M sulfuric acid (50 μL).[24] The color densities were measured at an absorbance of 450 nm using a Versa Max microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA). To discriminate a negative response upon removal of calcium, the identical procedure above was carried out using the same aqueous medium without calcium.

Label-free Calcium Biosensing

Analytical protocol. Using the Octet Red label-free sensor system (ForteBio, Menlo Park, CA, USA), a biosensor sensitive to calcium was constructed by combining an antibody (clone 3-6F) immobilized on the sensor as the capture binder with the mutated GGBP as CBP in the liquid phase. To prepare the biosensor, the AMC sensor was dipped into a well containing

PBS buffer (200 μL) for 600 sec, and transferred to Tris-Casein to block residual surfaces for a further 900 sec. The sensor was then placed within a well containing the antibody harvested from the culture medium and diluted 1:2 in Tris-Casein-TW. After a 30ºC incubation for 900 sec, the sensor was transferred to Tris-Casein-TW to remove the excess reagent from the sensor surfaces for a further 900 sec. The sensor was then moved into wells containing the calcium sample, which was diluted in Tris-Casein-TW including CBP at a final concentration of 10 μg/mL. The reactions were allowed to continue for 900 sec. The sensor tip was subsequently moved into the calcium-free medium for the same period. This cyclic response was repeated to analyze the next sample under the same conditions. The identical experiments except for the absence of CBP were also carried out as control. The kinetic data were collected and recorded using the Data analysis 6.3 program provided by the manufacturer.

Dose responses. Dose responses of the sensor were obtained with standard samples containing different concentrations (0.05–5 mM) of calcium in Tris-Casein-TW. The overall analytical procedure was identical to that described above in the presence of a constant CBP concentration (10 μg/mL). The control was also run in the absence of CBP. The sensor signal for each measurement was determined at the end of the time response and corrected by subtracting the original signal from the control. Each analysis was carried out in triplicate, and the mean value was plotted against the analyte concentration to prepare the standard curve. The detection limit was determined by multiplying the standard deviation of the blank value by three. The curve was linearized through log-logit transformation, which was used to determine the dynamic range, and a regression line was obtained using the least-squares method.[25]

Real sample tests. The analytical procedure was applied to the tests of actual samples (milk products in this study) and the results were compared with those determined by the reference system based on a $KMnO_4$ titration method (ISO 12081).[26] Three milk products (low fat milk; Maeil, Seoul, or Namyang) purchased from the market were serially diluted 1/25 to 1/200 in Tris-Casein-TW. The prepared samples were analyzed in triplicate according to the label-free immunosensing protocol described earlier. The signal value obtained for each sample was converted to calcium concentration using the linearized standard curve in the log-logit scale. The same samples were also simultaneously analyzed using the $KMnO_4$ titration

method.[27] Briefly, the milk sample was first treated with trichloroacetic acid to precipitate the milk proteins. After filtration, calcium in the filtrate was precipitated using ammonium oxalate, and the resulting calcium oxalate was sequentially isolated by centrifugation. The precipitates were washed with ammonia solution and centrifuged again, which was repeated twice more. They were then completely dissolved in sulfuric acid at a warm temperature (<60ºC) and titrated with $KMnO_4$ until the first persistent pink color appeared. The amount of $KMnO_4$ added was corrected by subtraction from that obtained for deionized water as a control under the same conditions, which was finally used to calculate calcium content. The two values for each milk sample were plotted on the respective axes of a graph to examine the correlation between the performances of the two systems, and the coefficient (R^2) was determined as a measure of the proportion of variability.[26]

Selectivity tests. Because CBP could also be reactive with ions other than calcium, analytical selectivity was tested for the ionic ingredients contained in milk. In preliminary tests, we selected eight different ions in addition to calcium, which were present in relatively high concentrations in raw cow milk[25]: phosphorus, magnesium, potassium, zinc, iron, manganese, copper, and aluminum. Standard samples for each ion were prepared in a range of 0.01 to 5 mM in Tris-Casein-TW medium and then analyzed in the same manner as that for the selection of the aforementioned antibodies except for the inclusion of each test ionic species in the respective medium. Based on the preliminary test results, four multivalent cations (zinc, iron, manganese, and copper) showed cross-reactivity and were further examined with the label-free immunosensor. In this experiment, standard samples containing each ion (30 μM for zinc, 2 μM for iron, 0.2 μM for manganese, and 0.3 μM for copper) were prepared in 10 times higher doses than those in milk. Duplicate measurements for each sample were carried out by following the procedure described above.

Results and Discussion

Analytical Approach Using Antibody Sensitive to CBP Conformation Change

Analytical concept. We devised a novel immuno-analytical approach for signal amplification of a surface-sensitive, label-free sensor for calcium. This was achieved by employing an antibody that specifically recognizes the structural conformation of CBP that forms when the analyte in the aqueous medium is bound to the calcium binder. In the proposed analytical concept, sample containing calcium was mixed with CBP to induce binding (complex 1) and a conformational change in the protein consequently occurs (Figure 1, Reaction 1). As this mixture is added to the antibody immobilized on the surfaces of the label-free sensor (1A), complex 1 sequentially binds to the antibody to form the second complex (Reaction 2, complex 2). As such complex formation increases surface layer thickness, the sensor may produce a signal proportional to the analyte concentration. However, upon depletion of calcium, complex 1 returns to the original conformation of CBP in Reaction 1, causing the calcium binder to dissociate from the antibody in Reaction 2 (1B). The sensor signal consequently decreases back to the background level in the absence of calcium in the sample.

A commercial product, Octet Red, was used as the label-free sensor, which is sensitive to the thickness change due to the binding complex formation on the interfacial area (refer to details below). Because the protein is much larger than a calcium ion, its presence or absence on the sensor surfaces significantly varied signal intensity in an amplified fashion. This effect enables quantitative monitoring of the calcium concentration in continuous mode if a constant CBP can be confined within the sensor compartment by using, for example, a semi-permeable membrane. Therefore, the analytical concept lends itself to automated food analysis.

As shown in the sections below, we first raised a monoclonal antibody specific to the CBP conformation, constructed a semi-continuous immunosensor by reusing the antibody-immobilized sensor, and finally demonstrated the concept through real sample testing.

Production of antibody discriminating different CBP conformations. A genetically modified GGBP as a CBP was first produced from *E. coli* by recombination and purified on a Ni-NTA column (Figure 2A). The bacterial culture was carried out initially in LB medium and then in the same medium with the addition of IPTG. The cells were harvested after culture and were lysed, after which the protein was finally recovered by separating it on a Ni-NTA chromatography column. The fractionated GGBP had a molecular weight of 34 kDa when analyzed by SDS-PAGE, as reported in a previous study.[28]

Animals were immunized with the recombinant GGBP to raise antibody recognizing the protein conformation formed upon calcium binding. By employing a standard monoclonal antibody production technology protocol,[24] such binders were selected at the time of screening hybridoma clones and showing positive antibody production against target (Figure 2B). We applied additional differential binding conditions to the conventional screening procedure. Each culture was diluted with aqueous medium with or without calcium, which was added to different wells containing pre-immobilized GGBP. The antibody bound in each well was traced by employing a secondary antibody labeled with an enzyme, as per the previous ELISA method description. We then screened for an antibody (clone 3-6F) sensitive to the CBP conformation depending on presence of the ligand (see 'Antibody clone desired' indicated in the figure).

Because the antibody clone displayed binding selectivity to a calcium-dependent conformation of the CBP, we applied the binder to construct an immunosensor for the analyte. In this case, a label-free optical fiber sensor system was applied for detection.

CBP Conformation-dependent Label-free Immunosensing for Calcium

Immunosensor construction. Octet Red was used as the label-free sensor device to monitor calcium. When molecular interactions between the binder and ligand occur on the sensor surfaces, formation of the binding complex causes a change in the interference pattern of white light reflected from the immobilized layer, which is relative to that from an internal reference layer.[24] This results in a wavelength shift, the intensity of which is proportional to the change in thickness of the sensor layer. According to the manufacturer's guide, the antibody (clone 3-6F) was immobilized on the sensor surfaces and the interaction with CBP in the liquid phase was used to monitor calcium (Figure 3). The AMC-type optical fiber sensor was first wet with buffer and was immersed in an antibody solution to fix the protein on the sensor tip via a secondary antibody, which changed the thickness of the molecular layer formed on the sensor tip surfaces (Step 1). In the next step, blocking of the residual surfaces was carried out by dipping each sensor in a casein solution (Step 2).

The sensor was used to detect calcium in samples initially containing a constant amount of CBP. As the sensor was transferred to the calcium (1 mM)-containing well for 15 min, the signal increased (Analyte; Figure 3, Step 3). When the sensor sequentially moved to the next well containing medium without calcium, the signal decreased (Step 4). The binding curves for the association and dissociation of CBP were clearly revealed, and the response time for both steps was < 15 min. The two steps were then repeated with the same sensors in a cyclic manner, which showed an identical pattern of sensor responses (refer to the inset for six repetitive responses). The same experimental procedure was applied to analyze the sample not containing CBP (Control). The sensor under this condition did not respond to the presence of calcium and displayed only a background level. Thus, the CBP-antibody complex was clearly formed only in the presence of calcium, discriminating the dose response from that obtained in the absence of calcium. This result indicated that calcium induced CBP binding to the immobilized antibody in the association step, which was caused by a probable conformation change of the protein molecule as described previously.[29]

Dose response of the sensor. The complex formation of CBP with the antibody was used to generate different signals from the sensor according to the concentration change of calcium in the sample. Standard samples containing different concentrations of calcium (50–5,000 μM) were prepared by diluting the stock in casein medium and adding them to microwells. The antibody-coated sensors in triplicate were then used to sequentially measure the standard samples for 15 min after adding a defined amount of CBP (Figure 4, step 3). After each measurement, the sensors were regenerated by transfer into calcium-deficient medium to maintain a constant quantity of CBP (Step 4). The same sensors were then reused to repetitively monitor the analyte by moving them back and forth between each CBP-containing standard sample and regeneration medium. The sensor consequently produced signals in a stepwise manner directly proportional to the analyte dose (Analyte). As a control, the same setting was used except for the absence of CBP and, in this case, the signal remained at background level (Control). The distinct sensors were able to be reused to reproducibly measure different samples, suggesting that the immunosensor could be used in a semi-continuous mode for automated sample analysis.

To draw a dose-response curve, the intensity produced on each sensor at the end of a time interval was determined as the signal value and the mean of triplicate measurements was then

plotted against the calcium concentration on a semi-log chart. The detection limit was calculated as the analyte dose (0.091 mM; 3.6 μg/mL) corresponding to the signal obtained by multiplying the standard deviation of the blank by three.[30] The sigmoidal pattern was linearized by log-logit transformation (Figure 4, inset),[31] revealing a dynamic range of 0.1–5 mM (4–200 μg/mL) for calcium in the sample. As the casein medium tended to aggregate at 10 mM calcium, the upper limit of detection was restricted to 5 mM. A high correlation (R^2 > 0.98) was observed between the measured and estimated values in the standard curve.

Real Sample Testing in a Semi-continuous Manner

The analytical performance was sufficient for detecting analyte present in various foods. The concentrations of calcium contained in foods are approximately 117 mg/100 g milk, 5.4–33.1 mg/g cheese,[32] < 10–250 mg/100 g vegetables,[33] and 2.5–7.0 mg/100 g fruits.[34] The samples are diluted to bring the concentrations within the dynamic range of the sensor prior to measurements, which could also alleviate potential side effects caused by the sample matrix. For example, we selected milk for real sample testing using the novel immunosensing technique. Although milk contains a number of components, the matrix effects could simply be reduced by five-fold diluting the milk with a buffer medium without further pre-processing steps.[35]

Testing with milk. We chose three milk products (manufactured by Maeil [54.9 mM Ca], Seoul [24.9 mM Ca], and Namyang [24.9 mM Ca] in Korea) to prepare real samples, which were used to determine calcium concentrations by employing the label-free immunosensor and to compare the sensor performance with that of the reference system based on the $KMnO_4$ titration method.[27] The reference method has been conventionally adopted for calcium analysis in the food processing industry. The milk samples were formulated by serially diluting each product in a range of 1/25 to 1/200 with casein medium and then sequentially measured by reusing the same label-free sensors in triplicate after adding CBP as described. Identical samples were analyzed using the $KMnO_4$ titration method at the same time according to a procedure reported previously.[27] The two measurements for each sample were plotted on each axis of a graph, which revealed high correlations (R^2 > 0.99) between the two analytical system performances for all milk samples (Figure 5). High variations were shown (CV = 11.6%, maximum) for milk samples diluted at a low ratio (e.g., 1/25), i.e., at

high calcium concentration range in the plot, although those for the other samples were acceptable (CV = 1.53–3.54%). These results suggest that the novel immuno-sensing approach can be applied to the food analysis for calcium with minimal sample handling such as dilution.

We selected casein as the inert protein in the medium for calcium analysis, which offered the most reproducible results, particularly in real sample testing with a typical calcium resource, milk.[36] As casein is the major milk protein consisting of heterogeneous molecular sizes in range of 1K to 10K Da, it could be the most effective to protect antigen-antibody binding from a non-specific interaction.[37] The protein-containing medium further provides a matrix similar to that of milk and, therefore, may be appropriate for analyzing food components without profoundly altering the aqueous environment. However, casein is negatively charged (pI = pH 4.6)[38] and can aggregate in the presence of a high content of cationic components via charge interaction, which could eventually cause aggregation and further precipitation.[39] Casein may form protein micelles during the assay in certain types of buffers such as phosphate.[40] For this reason, Tris buffer was used to formulate the assay medium throughout this study.

Analytical selectivity to calcium. Because milk contains many different ionic components that may cross-react with CBP, the label-free immunosensor was tested for selectivity to measure calcium contained in milk. We initially selected eight different ions in addition to calcium in the order of their amounts present in raw cow milk[25] and tested them by using microwell-based immunoassay with CBP immobilized on the solid surfaces. The analytical protocol was the same as that used for the antibody screening except for the inclusion of each test ion in the media for reaction and washing. As the initial testing for phosphorus, magnesium, potassium, and aluminum (mM range) showed no cross-reaction with CBP (Supplementary Figure 1) as reported previously,[41] the other four multivalent cations (zinc, iron, manganese, and copper) were further examined with the immunosensor (Figure 6). We prepared test samples for each ion to contain a 10 times higher dose than that present in raw milk (0.03–3 μM [25]). Triplicate immunosensing for calcium (1 mM) was first carried out to produce a reference signal in the presence of CBP (Analyte, step 3) and, after 15 min, the sensors were regenerated in the calcium-depleted medium (step 4). The same sensors were repetitively reemployed to test the four ions in sequential manner according to the identical

protocol; however, not indicating any significant signals. The same testing procedure was repeated except for the absence of CBP in sample, which consistently revealed only background levels (Control).

The real sample testing results demonstrated that this immunosensing approach can be used for measuring free ionized calcium in dairy products, such as milk, containing other multivalent cations (e.g., zinc, iron, manganese, and copper) in traceable amounts (a few μM or lower[25]). Because the listed cations showed cross-reactivity with CBP in the mM range or higher,[25] such calcium quantification for other foods (e.g., vegetables or fruits) might interfere depending on the quantitative constitution of the reactive ions. Calcium binding to CBP, serving a role of stabilizing the protein, induces ligand-specific conformational changes in protein.[42,43] Many of the binding proteins employ the EF-hand calcium binding motif, of which binding parameters are controlled, in part, by the structure of its calcium binding loop, termed the EF-loop.[44] The structural basis provides selectivity regarding cationic charge as well as size.[45] Nevertheless, the four cations showing reactivity with CBP hold oxidation states of +2 or +3, and an atomic mass ranging from about 55 to 65, which are very close to the properties of calcium (+2 and 40 for the charge and mass, respectively). Ionic molecules with a similar charge and size to those of calcium may be discriminated for the binding only by lowering their relative concentrations as displayed by the preferential calcium binding to CBP in nature.[46]

Conclusions

The molecular interaction between tiny molecules, free ionized calcium, and CBP was semi-continuously traced on the Octet mass-sensitive sensor associated with a monoclonal antibody, specific to the conformation of CBP-calcium binding complex, and immobilized on the sensor surfaces. As the antibody served as capture binder sensitive to CBP complex formation with calcium, the immunosensor produced an amplified signal from the label-free sensor in response to the analyte concentration change in the sample. Such performance would be difficult to achieve for molecular substances of this size without employing a specific antibody. However, CBP also reacts with other multivalent cations such as zinc, iron, manganese, and copper, having properties comparable to calcium regarding charge and size.

Although those substances may potentially interfere with the accurate measurement of the target analyte, such effect was not shown in real sample testing with dairy products due to their presence in traceable amounts. Our novel approach of label-free biosensing for calcium suggests that food components can be analyzed with minimal handling, and should eventually lead to further advances in automated analysis. This sensor could also be applied to clinical diagnosis to measure the analyte present in a range of 1.21 ± 0.04 mM in blood.[47] To this end, we are investigating a needle-type immunosensor, retaining a constant CBP within the sensor compartment enclosed by a semi-permeable membrane, for continuous monitoring.

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Figure legends

Figure 1. Analytical concept for calcium using a CBP conformation-specific antibody. The immunosensor was constructed by immobilizing the antibody on the surfaces of label-free, mass-sensitive sensor and placing CBP in the sample medium. In the presence of calcium in the sample (A), the calcium binding to CBP (Reaction 1, complex 1) causes a conformational change in the protein, which then induces complex 1 binding to the antibody (Reaction 2, complex 2). Upon its absence in the sample (B), the analyte is dissociated from CBP, which is sequentially uncoupled from the antibody (right). Therefore, the sensor can produce a signal directly proportional to the calcium concentration.

Figure 2. Production of genetically modified GGBP as a CBP and antibodies specific to a particular CBP conformation. The CBP produced from *E. coli* was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (A), indicating that the genetic recombinant was produced mostly in the culture supernatant, and the protein was recovered in a purified form (Eluted) via chromatography on a Ni-NTP column. To achieve the concept proposed in Figure 1, monoclonal antibodies against the CBP as an immunogen were raised and tested under differential binding conditions with or without calcium in the medium (B). The 3-6F antibody clone showing specific binding to the CBP-calcium complex was eventually screened.

Figure 3. Construction of label-free immunosensor sensitive to calcium binding protein (CBP) binding to calcium. The 3-6F antibody was immobilized on the sensor tip (Step 1) and the residual surfaces were then blocked (Step 2). Upon adding CBP to the positive sample, calcium-induced CBP binding to the antibody was monitored by the sensor (Analyte, Step 3), but, in the absence of calcium, the binding complex appeared to dissociate in the next step (Step 4). Steps 3 and 4 were repeated many times using the same sensors, which showed cyclic responses (see inset). No response was obtained without CBP (Control).

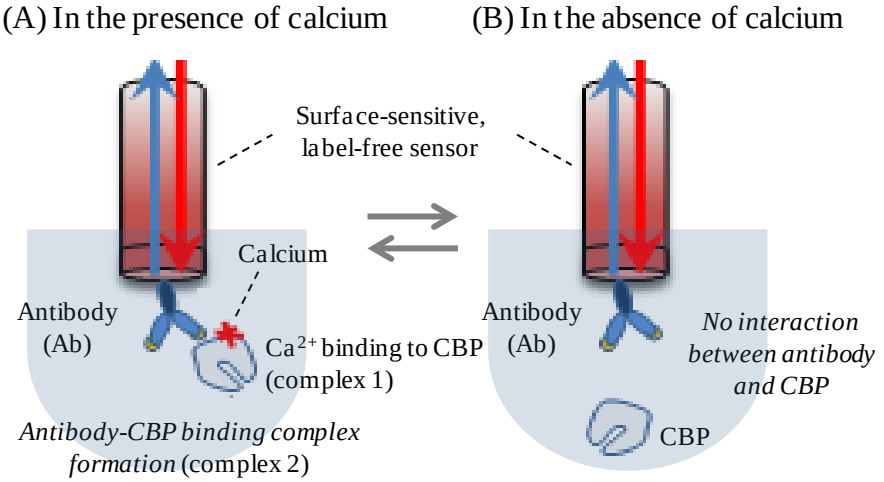
Figure 4. Dose responses of the immunosensor to calcium. After antibody immobilization, triplicate sensors were simultaneously used to measure standard samples containing different calcium doses in a sequential manner. In the presence of CBP in sample (Step 3), each sensor consistently produced a signal proportional to the analyte concentration. As they were able to

be regenerated upon depletion of calcium (Step 4), the same sensors were reused to analyze the next samples by repeating steps 3 and 4. The sensor signals determined at the respective endpoints were plotted against the calcium concentration and then transformed to a linearized standard curve via log-logit transformation (inset). The data showed linearity with a correlation coefficient > 0.98.

Figure 5. Real sample testing with the label-free immunosensor and comparison of the performance with that of the reference $KMnO_4$ titration method. Three milk products from different sources were purchased and respectively serially diluted in Tris-Casein-TW to prepare real samples. They were analyzed in triplicate using the immunosensing technique and the reference method at the same time. The two values for the same sample were plotted on each axis, revealing high correlations ($R^2 > 0.99$) for the three different products.

Figure 6. Testing for interference by various multivalent cations cross-reacting with CBP other than calcium. Because cations, such as zinc, iron, manganese, and copper, are also present in milk, their contributions to signal generation were determined at each concentration 10-fold higher than that in milk (indicated in the figure). Triplicate immunosensors were used to measure the analyte signal at 1 mM calcium (Analyte, step 3) and then regenerated in the absence of the analyte (step 4). They were then reused to sequentially test the samples containing the respective cations in the same manner, not indicating any significant signal production.

Figure 1

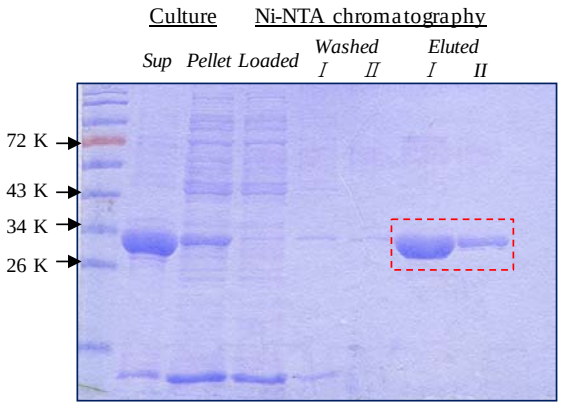


Reaction 1: $CBP + Ca^{2+} \rightleftharpoons$ complex 1

Reaction 2: Complex 1 + Ab $\rightleftharpoons$ complex 2
(Produces signal on the sensor)

Figure 2

(A) Production of GGBP as a CBP by genetic recombination



(B) Screening of monoclonal antibodies specific to the CBP conformation

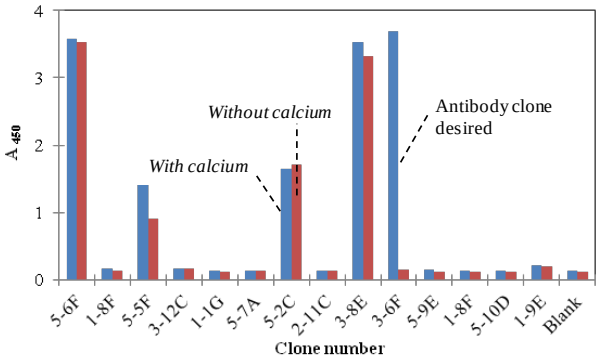


Figure 3

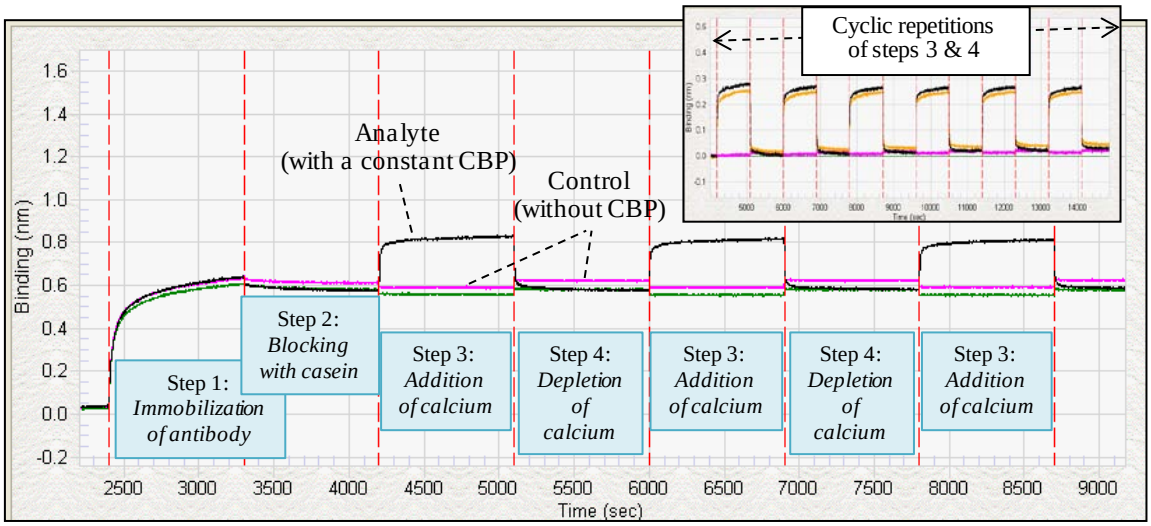


Figure 4

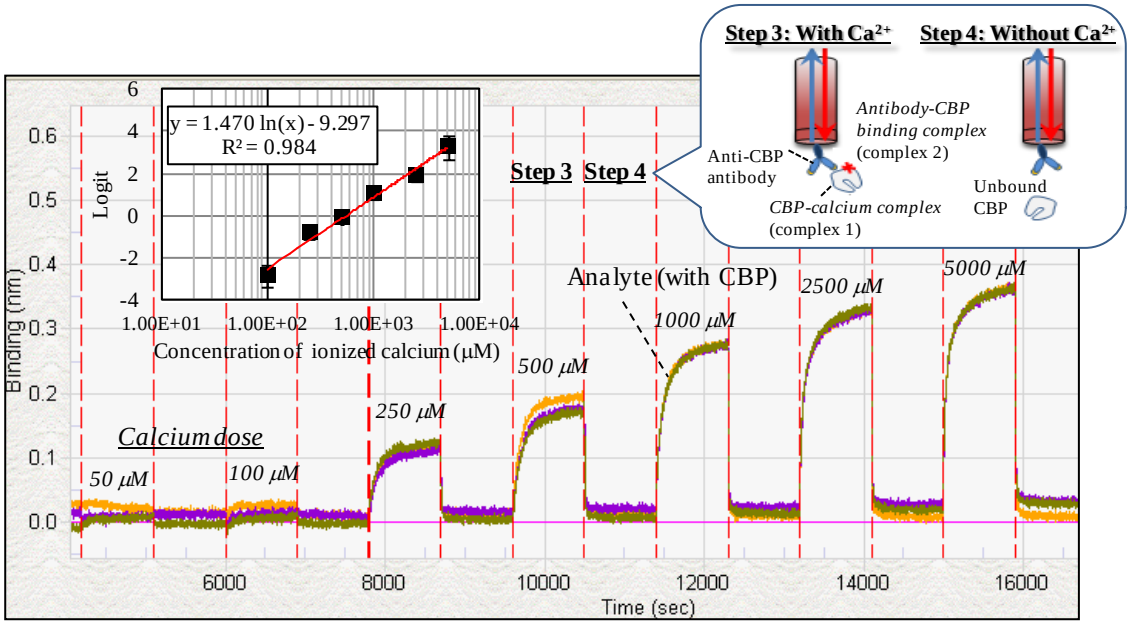


Figure 5

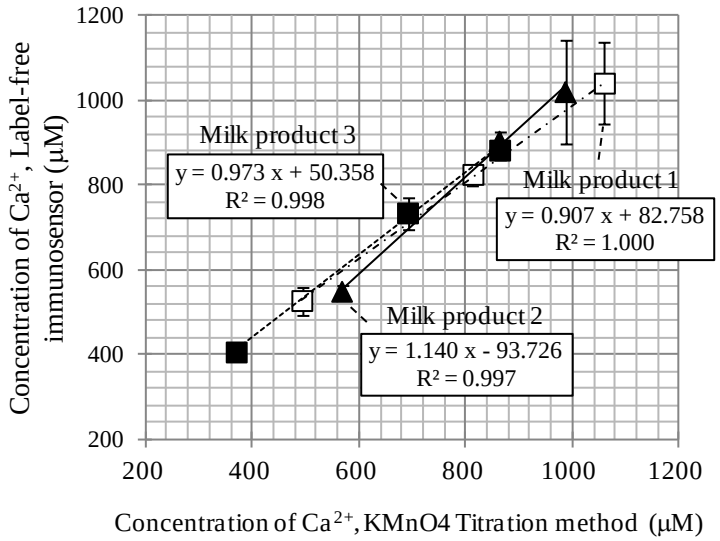
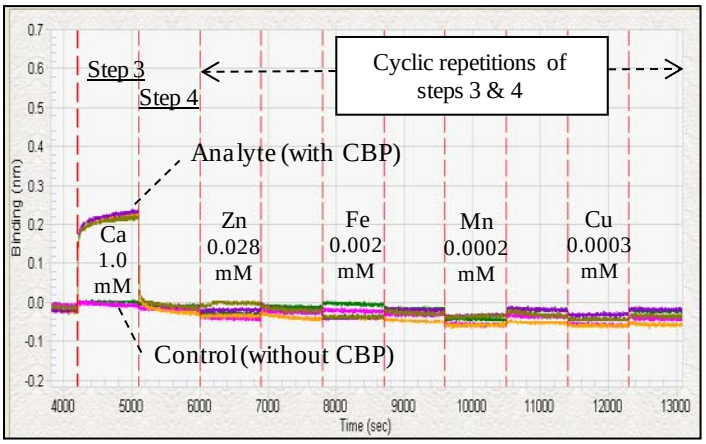


Figure 6



Graphic Abstract

Highlights:

Label-free immunosensing based on the conformational change of CBP depending on analyte concentration was explored for semi-continuous analysis of Ca^{2+}.