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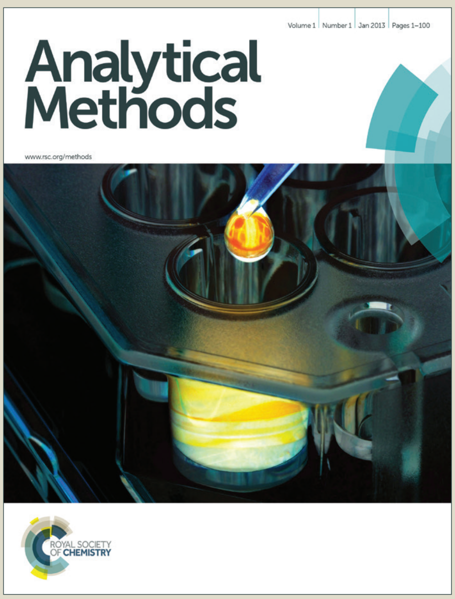

Sandwich immunoassay for lactoferrin detection in milk powder

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Abstract

Lactoferrin (LF) content in infant milk powder has been critically regulated by many governments and there is a need for convenient and reliable assays. With hybridoma techniques, fourteen monoclonal antibodies (mAbs) against LF were prepared. Two antibodies (mAb2 and mAb3), recognizing spatially distant epitopes of LF, were selected to establish a sandwich enzyme-linked immunosorbent assay (ELISA). Solution of mAb3 (1 μg/mL) was coated micro-titer plates for LF capture while mAb2 labeled with horseradish peroxidase (2.2 μg/mL) was used as detection antibody. Under optimized conditions, the proposed sandwich ELISA was evaluated linearly responding to LF standards in a range of 5-600 ng/mL and the limit of detection was defined as 3.23 ng/mL. Lactoferrin samples were able to be determined after simple dilution, and recovery in fortified milk powder averaged between 98% and 109%. The developed assay showed both high specificity (no obvious cross-reactivity with related proteins) and reproducibility (coefficient of variation ranged from 4.5% to 7.1%), indicating the utility of this sandwich ELISA in LF monitoring.

Keywords: Lactoferrin, sandwich ELISA, monoclonal antibody, milk products

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Introduction

Lactoferrin (LF) is present in high concentrations in milk and various exocrine secretions such as tears, saliva and urine[1]. LF has been shown to have antiparasitic, antifungal, and antibacterial activities[1]. Oral administration of LF is believed to be good for both infants and adults, and the observed host-protective effects have stimulated its worldwide commercial production[1]. However, the stability of LF in manufacturing decides the effectiveness of its supplements. Although LF was isolated and studied for several decades, its function hasn't totally elucidated. Chinese government has set a reasonable tolerance level of 1 g/kg for LF in infant formulas.

Thus, reliable assays for LF analysis in milk products are required. High performance liquid chromatography (HPLC) is a favorable choice for qualitative or quantitative analysis of LF. Many chromatographic columns with various solid phases have been reported for LF analysis1. However, the milk matrix is so complex and interference peaks are often observed even after numerous purification steps. Antibodies specific to LF are ideal in complex food analysis matrices. Recently, immuno-chromatography as a cleanup step was used before HPLC analysis[2,3], but the whole chromatography detection cycle is still time-consuming. Enzyme-linked immunosorbent assays (ELISAs) are sensitive, selective and convenient with a high throughput. Therefore, ELISA is very suitable for LF routine monitoring in milk products.

Early application of ELISA for LF detection was observed in 1985 [4] whereby tissue cytosol and plasma were analyzed. Various matrices such as goat milk, bovine milk and human milk have been screened for LF content using commercial ELISA kits5-7. In fact, high abundance proteins in dairy products affect seriously the accuracy of ELISA. Thus, the highly selective and sensitive antibody is the key in reliable ELISA development for LF detection.

In the present study, monoclonal antibodies (mAbs) identifying the different epitopes of LF were selected using hybridoma techniques. The antibodies were found with constant specificity and affinity. A highly sensitive and specific sandwich ELISA based on paired mAbs was developed, which was then successfully applied for LF analysis in milk powder.

Materials and Methods

Chemicals and instruments

Bovine LF, horseradish peroxidase (HRP), bovine serum albumin (BSA), casein, α-lactalbumin, β-lactoglobulin and Freund's adjuvant were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Aladdin Chemical Co., Ltd (China) and all cell fusion reagents were purchased from Sunshine Biotechnology Co., Ltd. (Nanjing, China). All solutions were prepared with ultrapure water obtained from a Milli-Q Ultrapure System and optical density (O.D.) was detected using an MK3 microplate reader (Thermo Labsystems; Chicago, IL, USA).

Antibody production and HRP-labeled antibody preparation

Female Balb/C mice (8 weeks old) were immunized with emulsified LF (100 µL per mouse). Mice were immunized at three-week intervals with net immunization dose of 100 µg for first injection and 50 µg for the following booster injections. After four injections, the mouse with the highest anti-sera titer was selected for subsequent cell fusion. Positive hybridomas secreting LF-specific antibodies were selected and sub-cloned using limited dilution measure[8]. All chosen cell lines were expanded and injected into mice primed with Freund's adjuvant at a dose of 2×10^{8} cells/mouse. After two weeks, ascite fluid was collected from mice and purified using a caprylic acid-ammonium sulfate method[9]. The purified antibody solution from a different cell line was numbered and divided into smaller aliquots. All antibodies were labeled with HRP using a previously reported procedure[10]. The antibody and HRP conjugates were fully dialyzed against 0.01 M phosphate-buffered saline (PBS, pH 7.4) and examined via direct ELISA.

Paired antibody selection

In order to develop a sensitive sandwich immunoassay, the selection of matching detection and capture antibodies is extremely important. All antibody combinations were tested for the differences in epitop recognizing. HRP-labeled antibodies were used as the detection antibody and the unlabeled antibodies were used as the coating antibody. Conjugation of HRP and antibody was assessed by color development.

Capture and detection antibodies were diluted to the appropriate concentrations (OD reading of 1.8-2.2.) with buffer (PBS containing 0.1% BSA and 0.05% Tween 20). 100 µL of capture antibody was added to all wells of microtiter plates, which were then stored overnight at 4°C. After removal of the solution in the wells, plates were washed three times using washing buffer (PBS containing 0.05% Tween 20; PBST). Potential free binding sites of the wells were blocked with gelatin buffer (0.2% gelatin in 0.01 M sodium carbonate; 200 µL/well). After incubation at 37°C for 2 h, plates were again washed three times. Positive control containing 200 ng/mL LF in PBS was added to assay wells whilst PBS buffer was added into control wells as a negative control (50 µL/well). Next, capture antibody solution was added to all wells (50 µL/well) and plates were then incubated at 37°C for 30 min. After a further washing cycle, 100 µL substrate solution (0.01% TMB in 0.1 M citrate phosphate buffer containing 0.1% hydrogen peroxide, pH 5.0) was added to each well and plates were then incubated at room temperature in the absence of light for 15 min to allow color development. Stop solution (2 M sulfuric acid) was then added into wells (50 µL/well) before determination.

OD value ratios between positive and negative controls were calculated as P/N[10]. The optimum combination would be two antibodies that bind different antigenic determinants of LF with the highest P/N value. The selected antibody pair was used for development of a sandwich immunoassay.

Evaluation of sandwich ELISA for LF

Using selected capture and detection antibodies, a sandwich ELISA was developed to detect LF. A calibration curve was established using LF standard solution of 5-600 ng/mL against OD values. Other common milk proteins, including BSA, casein, α-lactalbumin and β-lactoglobulin were tested for cross-reactivity at high concentrations (15, 30, 60 and 120 μg/mL; diluted in PBS). All OD values were compared against negative control to get P/N values.

A single brand of bovine milk powder was purchased from a local market, which claimed an LF level of 0.4 g/kg. Samples of milk powder (0.5 g per aliquot) were fully dissolved in 10 mL PBS. Aliquot (1 mL) was then added to 99 mL assay buffer (PBS + 0.2% Tween) and the resultant solutions were analyzed using the sandwich ELISA. Milk powder samples were fortified using LF standards at levels of 0.5, 1 and 2 g/kg. LF content was detected using calibration curve and recovery rates were calculated by deduction measure.

Ethical issue statement of animal testing

All experiments were performed under the guidance of animal welfare committee of Jiangnan University. The care of laboratory animal and the animal experimental operation conformed to Wuxi Administration Rule of Laboratory Animal. The housing facility and environment are in compliance with Chinese standard GB 14925(Laboratory Animal-Requirements of Environment and Housing Facilities).

Results and discussion

Antibody characterization and matched pair screening

A total of 14 cell lines were sub-cloned from cell fusion cycle and mAbs numbered 1 to 14 were harvested. All antibodies were found with high titer to LF (>1:10^6). The greater P/N values mean the more different epitopes recognized by two tested antibodies[9,10]. As shown in Table 1, the observed maximum P/N value was 28.35 using paired antibodies (mAb2 used as the detection antibody and mAb3 used as capture antibody). The paired mAbs to spatially distant epitopes allowed us to develop a sandwich-formatted ELISA for LF.

Establishment of sandwich ELISA

With checkerboard measure, we were able to determine the optimal concentration for both coating antibody mAb3 (1 μg/mL) and detection antibody mAb2-HRP (2.2 μg/mL). Using LF standard, a linear curve was plotted for OD values vs. logarithmic LF concentration. Figure 1 shows that good linearity (R^2=0.99) was observed across the 5-600 ng/mL concentration range. The limit of detection (LOD) was defined as the targeted analyte concentration when the P/N value was 2.1[1], and assay sensitivity was calculated as 3.23 ng/mL. In specificity tests, the tested proteins were all conventional milk ingredients; casein, in particular, is the major protein in milk (0.8-1.2%, w/v). As such, higher concentrations of related proteins (up to 120 μg/mL) were examined. P/N values for tested proteins across all concentrations varied from 1 to 1.3 (less than 2.1).

Assays using polyclonal antibody (pAb) were reported with similar detection range[2,5]. However, pAb recognizes several epitopes of LF whereas mAb binds to only one specific part of LF. A recent identifying assessment for mAb and pAb has been reported. It demonstrated that the diagnostic accuracy of mAb testing of objectives was superior to pAb testing[11]. The specificity tests of ELISA here further verified the highly selectivity of mAb based immunoassays.

Milk powder detection

The LF level of milk powder was detected as 0.44 ± 0.02 g/kg using the calibration curve above (Figure 1), which is very close to the claimed value of 0.4 g/kg. Milk powder additivity was tested, via simple treatment, and the data is shown in Table 2. Using a deduction measure (measurement results minus background value 0.44 g/kg), recovery rates were calculated and ranged from 98% to 109% with a coefficient of variation (CV) of 4.5-7.1%. The dilution steps should be based on the expected concentration of the analyte in order to fall within the concentration range of the standards (5-600 mg/L). The sensitivity of this sandwich immunoassay for LF is superior to a previous immunoassay (quantitative range of 12-780 mg/L)[12] ,commercial ELISA kit (quantitative range of 7.8-500 mg/L, Bethyl Laboratories,Inc.,Cat.No.E10-126)[13,14], immunosensors (0.2 mg/L)[15-17] and chromatography measure (0.02-0.4mg/mL)[18-20].The high sensitivity of this mAb-based ELISA enables us to detect LF in bovine milk powder at a high dilution factor, which greatly brings down possible matrix interferences.

Conclusion

Quantification of LF content in infant milk powder is necessary for a producer before it enters into the market. The ELISA presented here provides a sensitive and accurate method to quantify LF level in samples using simple dilutions. The sandwich-formatted ELISA was characterized high specificity and sensitivity. Application of milk powder detection was conducted indicating favorable recovery and stability.

Acknowledgements

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Captions

Figure 1. Standard curve for LF based on sandwich ELISA

Table 1. Pair-wise screening of 14 monoclonal antibodies

Table 2. Results of spiked tests in milk powder

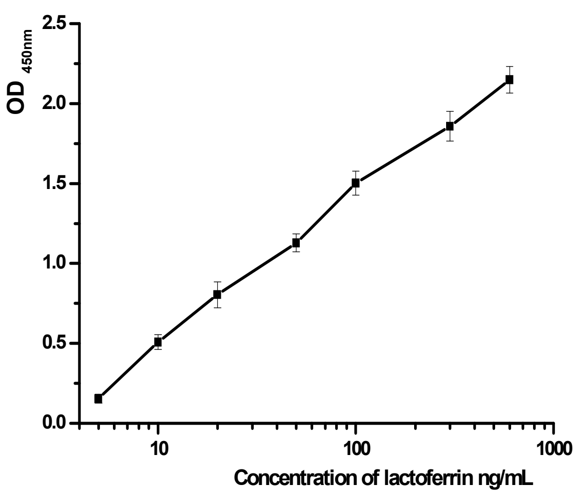

Figure 1. Standard curve for LF based on sandwich ELISA. Each point in curve represents mean of six determinations and the error bar indicates standard

deviations

Table 2. Results of spiked tests in bovine milk powder (Each fortified level was repeated eight times)

Fortified levels g/kg	Detected levels g/kg	Recovery* %	CV %
0.5	0.93±0.07	98%	7.1
1	1.51±0.09	109%	6.3
2	2.47±0.11	103%	4.5

* All recovery data were calculated by deduction of the background level of lactoferrin in milk powder (0.44 g/kg)

Table 1. Pair-wise screening of 14 monoclonal antibodies (Each data is the mean of five repeats. All data was OD value ratios between positive well and negative controls)

Coating	HRP labeled antibody													
antibody	**mAb1**	**mAb2**	**mAb3**	**mAb4**	**mAb5**	**mAb6**	**mAb7**	**mAb8**	**mAb9**	**mAb10**	**mAb11**	**mAb12**	**mAb13**	**mAb14**
mAb1		6.26	7.44	2.48	1.23	1.44	1.62	1.46	3.33	1.16	9.32	1.02	1.25	3.59
mAb2	23.95		15.82	18.76	4.31	13.47	2.58	3.90	9.78	14.22	13.90	2.75	6.53	5.98
mAb3	20.58	28.35		14.23	1.58	4.39	1.21	1.29	2.80	13.95	9.83	0.96	2.22	6.78
mAb4	1.38	5.03	1.57		0.86	0.76	1.21	1.23	1.99	0.91	3.50	0.79	0.89	2.17
mAb5	0.75	0.85	0.86	0.85		0.89	0.97	0.98	0.96	1.11	1.24	1.19	0.80	1.00
mAb6	1.37	3.85	2.16	2.15	1.63		2.00	3.59	1.07	1.09	2.31	1.15	0.99	1.41
mAb7	2.72	3.13	1.67	2.02	1.72	2.24		1.71	0.86	1.12	1.02	1.06	1.05	1.00
mAb8	3.33	3.70	1.87	2.24	1.56	3.78	1.56		1.23	1.33	1.90	1.22	1.00	0.96
mAb9	12.74	17.12	4.43	3.51	2.78	2.86	2.79	2.47		3.26	4.21	2.82	2.86	6.30
mAb10	1.67	5.61	5.70	1.41	1.17	1.11	1.23	1.13	2.81		4.55	1.13	1.48	4.15
mAb11	18.74	18.68	12.45	7.81	5.22	5.84	3.62	4.50	7.52	3.82		3.37	4.28	7.25
mAb12	4.39	2.15	2.40	1.26	1.23	2.07	1.41	1.56	1.72	1.44	2.34		1.34	2.05
mAb13	0.64	0.67	0.70	0.71	0.67	0.73	0.63	0.60	1.03	0.84	0.82	0.87		0.94
mAb14	17.54	10.51	5.90	3.13	2.28	2.65	2.14	2.16	2.46	2.30	1.97	1.81	2.30	2.74