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Rapid determination of bovine lactoferrin in dairy products by an automated quantitative agglutination assay based on latex beads coated with F(ab′)$_2$ fragments

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Abstract

This study evaluated an automated immunoassay for bovine lactoferrin (LF) in dairy products based on latex beads coated with F(ab′)$_2$ fragments. Methods: F(ab′)$_2$ fragments were obtained by pepsin digestion of rabbit anti-bovine LF (IgG fraction) and polystyrene latex beads were coated with the F(ab′)$_2$ fragments. We used the beads to develop a rapid and homogeneous light scatter immunoassay employing an autoanalyzer (the Automated Latex assay). The Automated Latex assay was easy to perform and could rapidly determine bovine lactoferrin in lactoferrin-supplemented products. It was sensitive enough for testing products and showed good precision.

Introduction

Lactoferrin (LF) is an 80 kDa glycoprotein with the capacity to bind iron. Various biological functions of LF have been reported such as antimicrobial activity and immunomodulatory effects (Tomita *et al.* 2002). Recent studies have demonstrated that oral administration of LF has a host-protective effect in various animals and humans (Teraguchi *et al.* 1994, Sekine *et al.* 1997, Tanaka *et al.* 1999, Yamauchi *et al.* 2000). Bovine LF has been is isolated and purified on a large scale from cheese whey or skim milk. Purified LF has been used as a food supplement since it was first formulated into infant formula in 1986.

A single radial immunodiffusion assay and the Rocket assay were initially used to measure lactoferrin in LF-supplemented products (Laurell 1966). An enzyme-linked immunosorbent assay (ELISA), which is commercially available as a kit, is also used today. However, these methods are time-consuming and technically difficult. A simple, rapid, highly sensitive, precise, and accurate immunoassay for bovine lactoferrin is needed for the routine quality assurance testing of LF-supplemented products to prove compliance with specifications.

Latex particles have been extensively used in the development of immunoassays based on antigen-anibody reactions. Latex agglutination is usually quantified by a change of turbidity and this method is suitable for automation (Ortega-Vinuesa 1996). Latex agglutination assays in which antibody-coated latex particles undergo agglutination in the presence of antigen have been developed, which overcome most of the problems mentioned above, and are rapid, simple, and convenient to use. Such methods are currently used in routine laboratory tests for the determination of various human serum proteins. An immunoassay for bovine LF using latex beads coated with rabbit IgG and employing the slide-reverse passive agglutination method has been reported (Yamamoto *et al.* 1992).

The process is rapid, but this is a qualitative assay, rather than a quantitative method. A microparticle-enhanced nephelometric immunoassay for LF in human milk was also reported (Cuilliere *et al.* 1997). This immunoassay is based on nephelometric quantitation of the inhibition of microparticle-antigen agglutinayion and the detection limit is relatively high (approximately 0.2 μg/ml). Therefore, it is not considered suitable for the routine immunoassay of bovine LF in LF-supplemented products. We developed a more sensitive quantitative reverse passive agglutination assay using latex beads coated with $F(ab')_2$ fragments of rabbit IgG for LF-supplemented products and applied it to an automated multi-purpose analyzer.

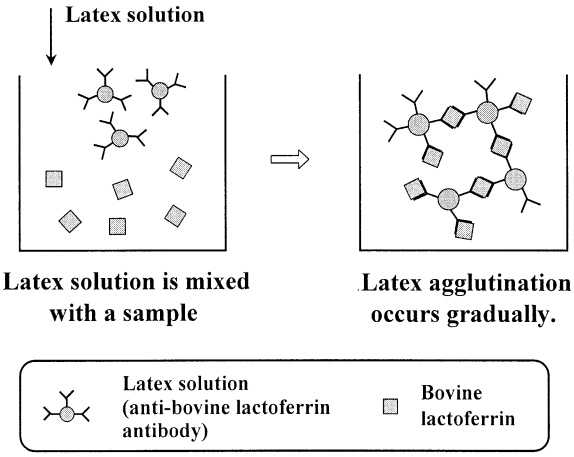

Fig. 1. Principle of the latex agglutination assay. The change in turbidity of the reaction mixture is monitored for 2 min. The increase in turbidity per minute is proportional to the concentration of bovine LF in the sample.

Materials and methods

Principle of the latex agglutination assay

The principle of the latex agglutination assay is outlined in Figure 1. Latex beads coated with rabbit anti-bovine LF antibody are mixed with a test sample and agglutination occurs gradually.

The change in the turbidity of the reaction mixture is monitored for 2 min and the increase in turbidity per minute is proportional to the concentration of bovine LF in the sample.

Immunoassays for bovine LF

Automated Latex assay

Polyclonal rabbit IgG was purified from rabbit anti-bovine LF antiserum by ammonium sulfate fractionation, followed by anion exchange chromatography. $F(ab')_2$ fragments were obtained by pepsin digestion of the polyclonal IgG and were purified by affinity chromatography. Polystyrene latex beads were coated with the $F(ab')_2$ fragments and unoccupied binding sites on the beads were filled with BSA.

The automatedimmunoassay was carried out using an automated multi-purpose analyzer (TMS-1024; Tokyo Boeki Medical Systems, Tokyo, Japan). After samples and reagents were placed in trays, each was automatically pipetted into the reaction cells, and the changes in the absorbance of the cells were monitored. Then the concentration of LF in each sample was calculated.

The sample was diluted with 0.9% NaCl solution containing 2.0% BSA. Then an aliquot of the sample solution (15 μl) was injected into 200 μl of Tris-NaCl buffer containing the stabilizer and the activator (reagent 1), and agitation was performed. After 6 min, 30 μl of the coated latex bead solution (reagent 2) was injected. The change in the absorbance of the reaction mixture was measured from 30 to 150 sec after addition of the latex beads, and the percent increase in turbidity at 800 nm was proportional to the concentration of bovine LF in the sample.

ELISA

Affinity-purified goat anti-bovine LF (Bethyl A10-126A-2) was diluted to 0.5 μg/ml with 0.1 mol/l sodium carbonate (pH 9.6), and 50 μl aliquots were added to each well of a microplate. After incubation for 2 h at room temperature, each well was washed four times with PBS containing 0.05% Tween. Then 200 μl of PBS containing 1% gelatin was injected into each well and incubated for 1 h at room temperature. After washing, aliquots (50 μl) of sample solutions were added to the wells and incubated for 1.5 h at room temperature. After washing, HRPO-conjugated goat anti-bovine LF (Bethyl A10-126P-4) was diluted 2000-fold with PBS containing 0.05% Tween and 1.0% gelatin, and a 50 μl aliquot was added to each well. After incubation for 1 h at room temperature, each well was washed. A O-phenylenediamine (OPD) tablet (Sigma) was dissolved in distilled water, and 100 μl of the resulting solution was added to each well. After incubation for 15 min, 20 μl of 8 mol/l H_2SO_4 was added to each well. Finally, the absorbance of each well was read at a wavelength of 492 nm using a microplate reader.

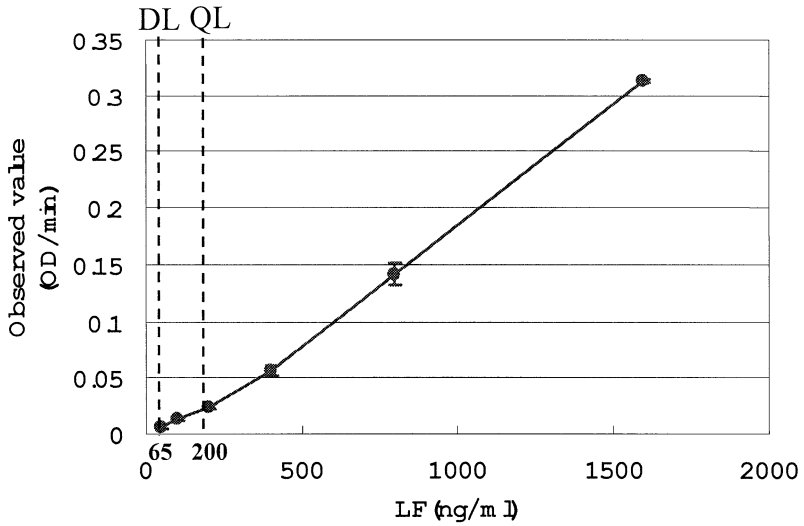

Fig. 2. Standard curve, detection limit (DL), and quantitation limit (QL) of the Automated Latex assay Values are the mean (and SD) of 18 measurements.

Results

Linearity, range, detection limit, and quantitation limit of the ELISA and Automated Latex assay

Using the Automated Latex assay, measurement of LF concentrations from 50 ng/ml to 1600 ng/ml was carried out in triplicate every day for 6 days (Figure 2). When the standard curve for the Automated Latex assay was plotted, linearity was good from 200 to 1600 ng/ml. One-way analysis of variance was carried out with the main facter being the concentration of lactoferrin (50 to 200 ng/ml), and the variance within each group (experimental error) was estimated as the SD of a blank (0 ng/ml). The detection limit was defined as being 3.3 SD above the y-intercept derived from the regression line (50 to 200 ng/ml) and the quantitation limit was defined as being 10 SD above the y-intercept. The detection limit was 65 ng/ml and the quantitation limit was 200 ng/ml.

Using the ELISA, measurement of LF at concentrations from 0.25 ng/ml to 64 ng/ml was carried out. When the standard curve was plotted, linearity was good from 1.5 to 12 ng/ml. The detection limit was 0.5 ng/ml and the quantitation limit was 1.5 ng/ml.

Immunoassay of bovine LF spiked into dairy products

Fifity milligrams of LF was spiked into 100 g of lF-free commercial infant formula. For the Automated Latex assay, a 2000-fold dilution was used as the test solution. Assay of LF was done as six replicate measurements on each of 3 days. All of the assay samples were freshly prepared on the day of measurement. The mean values were 51 to 52 mg per 100 g.

Discussion

It only took 8 min to complete the Automated Latex assay. An assay was initiated every 15 sec by the auto analyzer used in this study, so it took only 1 h to assay 200 samples. For speed and simplicity, the Automated Latex assay is also preferable to the ELISA. When the specificity of the Automated Latex assay was tested with human LF, values at 0 to 2000 ng/ml were below the cut-off level and a nonspecific reaction to human LF was not seen (data not shown). Evaluation of the effect of LF-free infant formula on the automated Latex assay showed that a nonspecific reaction to dairy products did not occur (data not shown). When the detection limit and quantitation limit of the Automated Latex assay and ELISA were compared, the ELISA was highly sensitive. Considering the range of LF levels in commercial products, however, the Automated Latex assay was sensitive enough to measure LF in LF-supplemented products. However, when the test requires greater sensitivity, the ELISA is considered to be superior.

From these results, it is considered that the Automated Latex assay is appropriate for the routine meas-

urement of bovine LF in various LF-supplemented products.

The reagents for the Automated Latex assay of bovine LF are now commercially available as a kit.

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