Journal of Food and Drug Analysis

Volume 14 | Issue 4 Article 3

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Journal of Food and Drug Analysis, Vol. 14, No. 4, 2006, Pages 379-384 藥物食品分析 第十四卷 第四期

Affinity Measurement of Lactoferrin (LF)-Anti-LF Immunoglobulin in Yolk (IgY) Complexes by Competitive Indirect Enzyme-Linked Immunosorbent Assay (CI-ELISA)

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(Received: September 6, 2005; Accepted: June 30, 2006)

ABSTRACT

Competitive indirect enzyme-linked immunosorbent assay (CI-ELISA) was employed to perform the affinity measurement of dissociation constant (Kd) and affinity constants (Ka) for bovine milk lactoferrin (LF) and IgY (immunoglobulin in yolk) specific against LF using antisera from rabbit and hen as references. In liquid phase equilibrium measurements by CI-ELISA, the elevation in antigen level in antigen-antibody mixtures decreased the ELISA values, suggesting the increased competition of free LF in solution with that coated on plate for LF-specific IgY purified by immunoaffinity chromatography (purified IgY). From the Klotz plots of the binding of LF to purified IgY, Kd and Ka were determined to be about 2.6×10^{-8} M and 0.5×10^{8} M^{-1}, respectively. The Kd values of 1500-fold diluted crude IgY, diluted sera from hen and rabbit were determined to be very close to that of purified IgY, revealing that CI-ELISA under liquid phase equilibrium by CI-ELISA was appropriate for the affinity measurement of IgY samples.

Key words: dissociation constant, affinity constant, indirect competitive ELISA, immunoglobulin in yolk, lactoferrin

INTRODUCTION

Lactoferrin (LF) is a single-polypeptide glycoprotein of about 80 KDa, resembling to transferrin in structure and displaying a high affinity to ferric ions. It is abundant in bovine milk and shows remarkable antimicrobial effect against gram-negative bacteria and viruses[(1)] by depriving iron from environment[(2-4)]. Ferric ions binding sites in N- and C-terminals of LF are in close vicinity and composed of three anionic ligands, aspartate and two tyrosines, and one neutral histidine[(5)].

LF content in bovine colostrums (about 1 g/L) is determined to be four to five times higher than that in normal milk (200 mg/L)[(6)]. Recent reports indicated that the antibacterial activity of LF was enhanced when in combination with immunoglobulin G (IgG), immunoglobulin A (IgA) or lysozyme[(7-9)]. Therefore, formula fortification infant milk with LF is suggested due to the antimicrobial activity as well as the enhancement of iron bioavailability to intestinal cells[(10)]. Recent report on LF indicates its anti-inflammatory effect as a result of the binding competition on CD14 (surface designation 14) of macrophages or/and monocytes with LPS (lipopolysaccharide)[(11)]. In addition, *in vitro*, LF markedly inhibits the release of cytokines, such as IL (interleukine)-1, IL-6 or TNF (tumor necrosis factor)-α, as a result of the stimulation of monocytes and lymphocytes by LPS[(11)]. Shinoda *et al.*[(12)] also have stated that LF from either bovine milk or breast milk presented potent stimulation on the release of IL-8 from human polymorphonuclear leukocytes. Therefore, preparation LF from bovine milk for food and pharmaceutical uses has become a subject of intensive researches[(13-15)].

Monoclonal antibody immunoaffinity chromatography has been proved satisfactory for the isolation of corresponding antigen such as LF, a minor component in milk[(16)]. However, some major drawbacks such as difficulty, lengthiness, and costliness in preparing monoclonal antibody specific against certain antigen[(16)] limit the use of those antibodies in food industries. IgY, a polyclonal antibody from hen yolk, shows some major advantages over IgG such as a high content of immunoglobulin in egg yolk[(17)], the large amounts of chicken eggs available, and the ease of IgY collection without eventual sacrifice of animals, which have made IgY a potential source for use in immunochemical assays as well as for therapeutic applications[(18-21)]. However, to use IgY for the further, some basic properties of IgY specific against certain antigen need to be explored urgently.

Some parameters such as binding capacity (Qm) and dissociation constant (Kd) of immunoaffinity gels for the corresponding antigen or antibody have been described

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previously. By using Sepharose® gel immobilized with LF, IgY and rabbit serum IgG specific against LF were isolated with a satisfactory purification efficiency of about 2400. The Qm and Kd of LF bound (LF-) Sepharose® 4B immunoaffinity gel to LF-specific IgY was 0.81 mg/mL wet gel (or 1.620 mg/mg LF) and 6.4×10^{-6} M, as determined by Langmuir-type adsorption isotherms[22]. Similar results were obtained when lysozyme (LS)-specific IgY was isolated by LS bound Sepharose immunochromatography[23].

The immunochromatographic method can be employed to measure directly the interaction of the antibody with the corresponding immobilized antigen (or vice-versa). It does not allow the measurement of the true equilibrium Kd since the antigen and antibody are in separate phases, and moreover, the antibody (or antigen) may denature as a result of binding to the gel matrices. To avoid this, Friguet *et al*.[24] have developed a measurement for the true affinity constants of antigen-monoclonal antibody equilibria in solution by competitive indirect enzyme-linked immunosorbent assay (CI-ELISA). They found that the obtained Ka values are very close to those obtained by conventional affinity measurements by immunoprecipitation and fluorescence transfer. In addition, Loomans *et al*.[25] have indicated that a liquid-phase equilibrium method of CI-ELISA is effective in evaluating the binding capacity and affinity of synthetic peptides of the β-chain of human chorionic gonadotropin for the respective monoclonal antibody.

The affinity measurement of antigen-IgY complexes in liquid phase equilibrium has not been reported, yet. CI-ELISA was performed to measure Ka (affinity constant) and Kd of LF-IgY specific against LF complex in order to evaluate the further use of IgY in immunochemical assays. First, sera from hens and rabbits immunized with LF were obtained and IgY specific against LF was isolated by LF-immunoaffinity chromatography from yolk of collected eggs. Then, a serially diluted antibody samples were individually incubated with free LF to reach equilibrium prior to CI-ELISA. Finally, Ka (M) and Kd (M^{-1}) of each antibody sample for LF were determined and discussed.

MATERIALS AND METHODS

I. *Preparation of Antiserum and IgY Specific against LF*

Hen antiserum, rabbit antiserum, and IgY raised against LF were prepared by following the method described by Tu *et al*.(22). In brief, 1 mL of bovine LF (L 4765, Sigma, St. Louis, MO) (500 μg protein/mL of sterilized 0.02 M phosphate buffer saline, pH 7) was emulsified with 3 mL of Freund's complete adjuvant for the initial immunization, while those for the second and further immunizations were prepared in the same way except the use of Freund's incomplete adjuvant[26-27]. Subsequently, five hens (160-day old) were intramuscularly injected on four spots of each leg (0.5 mL antigen emulsion/leg), and boasted every other week for six weeks. Hen serum from blood and yolks from eggs, collected over a 4- and 16-week experimental period after the initial immunization, were kept frozen (-20°C) prior to the determination of changes in binding activities of IgY in yolks or hen sera to LF by the ELISA as described below.

Each of the two New Zealand rabbit (weighted 1.8 kg each) was subcutaneously injected on the backs with the same antigens used for hens during the immunization period. Before each immunization, blood (2-3 mL) were drawn from the vein of each rabbit over a 6- and 11-week period after the initial immunization and was left at room temperature for 30 min to clot. Then, the serum was collected by centrifugation (2000 ×g, 4°C, 20 min), heated for 30 min at 56°C for the inactivation of complements and stored at -20°C until use. Rabbit serum was also collected for the determination of changes in binding activities of IgG to LF by the ELISA described as followed.

II. *Purification of IgY by LF-immunoaffinity Chromatography*

First, IgY in yolk was partially purified with pectin with approximate 70% recovery according to the method previously reported by Chang *et al*.[17]. Then, 5 mL of crude IgY (9.0 mg/mL) was applied to a LF-Sepharose® 4B immunoaffinity chromatography column, prepared by the method described by Tu *et al*.[22], in order to purify LF-specific IgY. Concentration and desalting of the pooled antibody fractions were simultaneously carried out by centrifugation (5000 ×g, 4°C, 30 min) using Millipore ultra-free membrane, followed by lyophilization. The purified IgY specified against LF (purified IgY) was stored at -20°C for the later use.

III. *Determination of Antibody*

Indirect ELISA was performed to determine the changes in binding activity of IgG from hen serum, rabbit serum as well as of IgY in yolk to LF during the immunization period[22,27-28]. A hundred microlitters of 10×8^6 diluted serum or yolk was added to the wells coated with LF (5 μg/each well) and then reacted with 2000-fold diluted peroxidase-conjugated goat anti-rabbit IgG (Sigma) or rabbit anti-chicken IgG (Sigma). Absorbance of sample was at least three times higher than that of the control (serum or IgY of eggs from animals before initial immunization). The value of the maximal dilution fold of serum or yolk sample was used as antibody titer[27]. The titers of hen serum and IgY of eggs collected over a 4- and 16-weeks period was determined to be 16.8×10^7 and 9.4×10^7, respectively, while that of rabbit serum collected over a 6- and 11-week period was 16.8×10^7[22]. The antisera and IgY were used in the liquid phase equilibrium affinity measurement described below.

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IV. *Affinity Measurement*

CI-ELISA was performed to determine the affinity of antibody solution (purified IgY, crude IgY, hen serum or rabbit serum) for LF[24-25]. Briefly, wells of ELISA plate were coated overnight with 100 μL of 10 nM LF. At the same time, LF at various levels (0.078-1280 nM) was first incubated in 2 mM EDTA/0.1 M potassium phosphate buffer (pH 7.8) with certain levels (0.6, 6.0, 15.0 or 30 nM) of purified LF-specific IgY overnight at ambient temperature to reach equilibrium. Then, 100 μL of this equilibrium mixture was transferred to the LF-coated wells. After 1 hr of incubation at 20°C, wells were washed with PBS supplemented with 0.05% Tween-20 and the bound IgY was detected by adding 100 μL of 2000-fold diluted peroxidase conjugated goat anti-rabbit IgG or rabbit anti-chicken IgG (10 μg conjugate/mL) (Sigma) (10 μg conjugate/mL) in 0.5% gelatin-PBS. After incubation at 37°C for another 90 min, wells were then washed with PBS-Tween followed by the addition of 100 μL of freshly prepared substrate solution (40 μg phenylene diamine/mL 0.01% H_2O_2/citrate buffer, pH 4.6). After incubation at ambient temperature for 30 min, 2 N H_2SO_4 solution was applied to each well to stop the enzyme reaction. The developed color was quantified at 490 nm by using an ELISA reader (Emax; Molecular Device, San Francisco, CA). Absorbance of sample was at least three times higher than that of the control (serum or IgY of eggs from animals before initial immunization). Each samples was tested in duplicate.

The affinity of hen and rabbit sera for LF was determined in the same way except the dilution fold was 6000, 15000 or 30000 for antisera and 1500 or 3000 for crude IgY. Absorbance of sample was at least three times higher than that of control. Each sample was tested in duplicate. Sera from rabbits and hens, and yolks and crude IgY solution obtained from eggs laid by hens before the initial immunization were used as controls.

Dissociation constant (Kd, M) and association constant (Ka, M^{-1}) of antigen-antibody complexes was determined according to the equation[24] below:

$$A_o / A_o - A = 1 + \mathrm{Kd} / a_o \quad (1)$$

where A_o and A, absorbance of total antibody (IgY or IgG in serum) incubated in the absence and presence of a given concentration of antigen, respectively; a_o, total concentration of antigen in antigen-antibody mixture; Ka, the inverse value of Kd

V. *Determination of Protein*

Protein content was determined according to the method described by Bradford[29]. Microtiter plate protocol of Bio-Rad protein assay was conducted using chicken serum IgG (Sigma) as the standard solution (5-500 μg/mL) for the standard curve ($r^2 = 0.9875$)[30]. Molar concentration of IgY (MW 220 KDa) and LF (MW 77 KDa) was used in the present study. Each sample was tested in duplicate.

RESULTS AND DISCUSSION

I. *Liquid Phase Equilibrium Affinity Measurement*

Purified IgY at various levels was incubated with free LF at various concentrations overnight to reach equilibrium. To each well previously coated with LF was added the equilibrium mixture, and the bound IgY was determined by a CI-ELISA. As shown in Figure 1, despite the level of IgY used in the IgY-LF mixture, ELISA value (absorbance at 490 nm) decreased with the increasing level of LF in mixture, indicating an increased competition of free LF in solution with that coated on plate for anti-LF IgY.

Obviously, the concentration of purified IgY (0.6 nM) was too low to be appropriate for the present study. ELISA value was minimized to be about 0.05-0.2 in the presence of high level (1280 nM) of free LF in mixture, revealing that the binding of IgY to LF coated on plate was almost completely inhibited (Figure 1). Friguet *et al.*[24] have indicated that when antibody at a constant concentration was incubated with antigen at various quantities in solution for a long period of time (overnight), they should reach equilibrium. Accordingly, a classical indirect ELISA allowed the determination of Kd of the antigen-antibody equilibrium in solution by estimating the amount of free antibody remaining in solution, provided that antigen was previously coated in wells. In addition, the concentration of required antibody in mixture should be as small as possible[24], lower or close to the value of Kd,

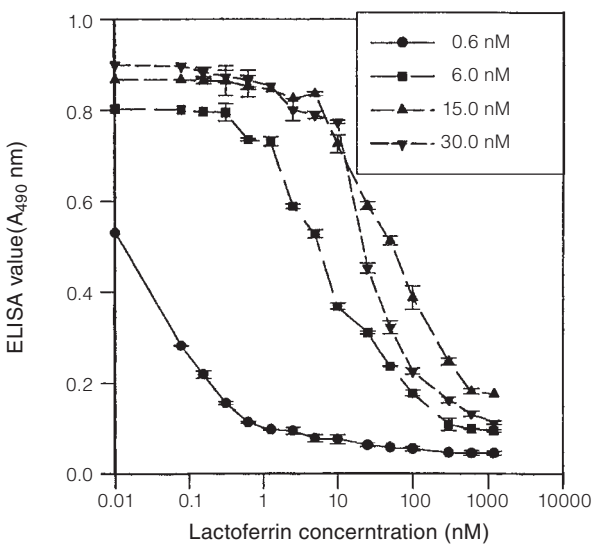

Figure 1. Liquid phase equilibrium affinity measurements between lactoferrin (LF) and IgY specific against LF in solution by the competitive indirect ELISA method. IgY specific against LF was purified by a LF bound Sepharose® immunoaffinity chromatography.

to determine binding equilibrium. Therefore, we minimized the total antibody concentration to about 6×10^{-9} M (6 nM) in the present study. In addition, the antigen level ($0.078\text{-}1280 \times 10^{-9}$ M) in the present study was in large excess over the total antibody level in the antigens-antibodies mixture, which was deduced from a preliminary ELISA calibration (Figure 1). Friguet *et al.*[24] have conducted the affinity measurement using indirect ELISA at the antigen concentration of $0.4\text{-}200 \times 10^{-9}$ M and antibody concentration of 0.3×10^{-9} M. It was suggested that the total antigen concentration should be excessively higher than the antibody concentration.

II. *Klotz Plot of the Binding of LF to Anti-LF IgY and the Determination of Kd and Ka*

According to equation (1) the Kd and Ka values can be deduced from the slope, by plotting the inverse value of the fraction of bound antibody ($A_o / A_o - A$) against the inverse value of total antigen ($1/a_o$) (Figure 2). It was found that purified IgY (except 0.6 nM) with a concentration of 6.0, 15.0, and 30.0 nM showed Kd values between 1.3×10^{-8} M and 4.4×10^{-8} M with an average value of about 2.6×10^{-8} M (Table 1). Ka, the inverse value of Kd, was 0.77, 0.23, and 0.44×10^{8} M^{-1} when the concentration of purified IgY was 6.0, 15.0, and 30.0 nM, respectively (Table 1). Frigu *et al.*[24] have pointed out that Kd value deduced from the slopes calculated by linear regressions are 3.8×10^{-9} M for 46-9 IgG2b monoclonal antibody/reduced holo-β_2 antigen equilibrium and 1.29×10^{-8} M for D_4-B_6 monoclonal antibody/F2-AEDANS [N-acetyl-N'-(5-sulfo-1-naphtyl) ethylene diamine] antigen equilibrium (the monoclonal antibodies directed against β_2 protein from IgG fraction by 46-9 and D_4-B_6 clones, respectively). IgY, a polyclonal antibody, purified by LF-immunoaffinity chromatography thus showed similar affinity to the corresponding antigen as that of the above two types of monoclonal antibodies to the corresponding antigens. However, LF-purified IgY complex showed much higher Ka values than the complexes of monoclonal antibodies and their respective antigens (epitope-peptides or N-terminally extended synthetic peptides) (10^2-10^4 M^{-1})[25]. The Kd value of LF-Sepharose® 4B immunoaffinity gel to LF-specific IgY was 6.4×10^{-6} M, as determined by Langmuir-type adsorption isotherms[22], higher than that of LF-IgY in Table 1, suggesting the superiority of affinity measurement in liquid phase equilibrium. Different values of Kd in Table 1 derived from the various levels (6-30 nM) of IgY specific against LF in IgY-LF mixtures could be due to the variation in experimental conductions and antibody concentrations.

Equation (1) also allows the determination of Ka even if the specific antibody concentration is unknown[24]. Kd values of LF-crude IgY and LF-antiserum complexes were thus estimated. It was noteworthy that the Kd value of 1500-fold diluted crude IgY was 2.3×10^{-8} M (Table 2), close to that (2.6×10^{-8} M) of purified IgY shown in Table 1. The agreement of the Kd values between purified IgY and crude IgY made CI-ELISA under liquid phase equilibrium a valuable tool for affinity measurement.

Furthermore, the Kd and Ka values of LF-hen serum, LF-rabbit serum and LF-crude IgY complexes by CI-ELISA were deduced and compared. Kd value of 6,000-fold

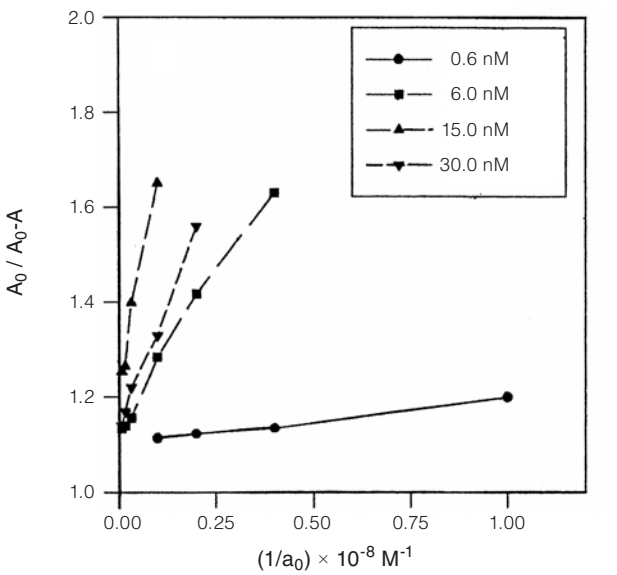

Figure 2. Klotz plots of the binding of lactoferrin (LF) to anti-LF IgY measured by the competitive indirect ELISA.

a_0 : the total antigen
A_0 : the absorbance measured for the antibody in the absence of antigen
A : the absorbance measured for bound antibody

Table 1. Dissociation constants (Kd, $\times 10^{-8}$ M) and affinity constants (Ka, $\times 10^{8}$ M^{-1}) of lactoferrin against anti-lactoferrin IgY as determined by competitive indirect ELISA.

Concentration of IgY (nM)	Kd	Ka
6.0	1.298 ± 0.066	0.770 ± 0.003
15.0	4.394 ± 0.219	0.228 ± 0.010
30.0	2.116 ± 0.106	0.437 ± 0.020
	(2.603)	(0.478)

Each value is the average ± standard deviation of three determinations
Value in the parentheses represents the averaged figure of different IgY concentrations

Table 2. Dissociation constants (Kd, $\times 10^{-8}$ M) and affinity constants (Ka, $\times 10^{8}$ M^{-1}) of lactoferrin against hen antiserum, rabbit antiserum, and crude IgY as determined by competitive indirect ELISA.

Antibody sample	Kd	K_a
Hen antiserum 6000 ×[a]	2.246 ± 0.113	0.445 ± 0.022
Rabbit antiserum 15000 ×[a]	2.514 ± 0.105	0.398 ± 0.018
Crude IgY[a] 1500 ×[a]	2.305 ± 0.107	0.434 ± 0.021

Each value is the average ± standard deviation of three determinations
[a]dilution fold
[b]IgY in yolk was partially purified with pectin

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diluted hen serum and 15,000-fold diluted rabbit serum were 2.2 and 2.5 × 10^{-8} M, respectively, in Table 2, which were close to that of purified IgY presented in Table 1. It was interesting to note that the affinity constant of LF-purified IgY complex was similar to that of LF-crude antibody sample complexes, and most importantly, they were very close to that of antigen-corresponding monoclonal antibody complexes.

CONCLUSIONS

Liquid phase equilibrium affinity measurements between LF and IgY or antiserum in solution by CI-ELISA were conducted to study the association-dissociation equilibrium. The concentration of free antibody at equilibrium gave reliable values of the real dissociation or affinity constants of the system in solution. The high sensitivity of indirect ELISA allowed the measurement of very low concentration of free antibody. Based on this fact, polyclonal antibody of IgY, with abundant sources and easy use, appears to be suitable for the immunochemical assay and immunochromatographic separation of minor and bioactive components from raw materials.

ACKNOWLEDGEMENTS

The study was supported by a grant from the National Science Council, Taiwan, ROC (NSC 89-2313-B041-010).

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