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# Detection of alkaline phosphatase by competitive indirect ELISA using immunoglobulin in yolk (IgY) specific against bovine milk alkaline phosphatase

Chao-Cheng Chen [a], Yu-Chang Tai [b], Szu-Chuan Shen [c], Yann-Ying Tu [a], Ming-Chang Wu [d], Hung-Min Chang [b,*]

[a] Department of Food Health, Chia-Nan University of Pharmacy and Science, Tainan 717, Taiwan
[b] Graduate Institute of Food Science and Technology, National Taiwan University, 59 Lane 144, PO Box 23-14, Taipei 106-17, Taiwan
[c] Department of Nutrition, I-Shou University, Kaohsiung County 840, Taiwan
[d] Department of Food Science, National Pingtung University of Science and Technology, Pingtung 91207, Taiwan

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**Abstract**

Immunoglobulin in yolk (IgY) (with a titer of $1.3 \times 10^6$) specific against bovine milk (BM) alkaline phosphatase (ALP) was obtained by intramuscularly immunizing hens on the thigh and was used as the primary antibody to conduct competitive indirect enzyme-linked immunosorbent assay (CI-ELISA) to determine BMALP in ALPs from BM and *Escherichia coli* sources. A relationship between the ELISA value and the BMALP level (0.01–10 μg/mL) in whole milk ($R^2 = 0.9019$) or in skimmed milk ($R^2 = 0.9402$) was observed. The maximal inhibition (%) of BMALP on the microtiter plate by free BMALP at 10 μg/mL whole milk (3.89 mU/μg BMALP) was about 50%, while no inhibition (%) of BMALP by free *E. coli* ALP at concentrations between 0.01 to 10 μg/mL (60 mU/μg *E. coli* ALP) was determined. At BMALP levels higher than 0.1 μg/mL, CI-ELISA was proved to be effective in differentiating between BMALP and *E. coli* ALP and quantifying BMALP in whole milk or skimmed milk in the presence of *E. coli* ALP with an activity of 0.6 U/mL. Higher inhibition (about 70%) of BMALP on the microtiter plate by free BMALP in diluted ($10^1$–$10^4$ fold) milk samples was observed. The optimal conditions for CI-ELISA in determining BMALP (0.1–10 μg/mL) from ALPs in milk samples were using $10^3$-fold diluted crude IgY specific against BMALP as primary antibody and $10^3$-fold diluted goat anti-chicken IgG–ALP conjugate as the secondary antibody.


*Keywords:* Alkaline phosphatase; Immunoglobulin in yolk; Competitive ELISA; *Escherichia coli*; Bovine milk

## 1. Introduction

Alkaline phosphatase (ALP) (E.C. 3.1.3.1.) is widely distributed in organisms, and the molecular weight of ALP monomers from microorganisms ranges between 47 (*Escherichia coli*) and 110 kDa (yeast) (Inouye & Reckwith, 1997). The main microbial source of ALP in milk is *E. coli*. This bacteria produces ALP in preiplasmic spaces to free phosphate ions from organic compounds for the production of adenosine tri-phosphates in an effort to maintain the bacterial bioactivity (Inouye & Reckwith, 1997). Native ALP in bovine milk is a glycoprotein containing sialic acid with a molecular weight of 187 kDa and an optimal pH value of 9.65. Heat

*Abbreviations:* ALP, alkaline phosphatase; IgY, immunoglobulin in yolk; CI-ELISA, competitive indirect ELISA; BM, bovine milk.

* Corresponding author. Tel.: +886 2 2363 0231x2776/21622; fax: +886 2 2362 0849.
*E-mail address:* changhm@ntu.edu.tw (Hung-MinChang).

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treatment either at 62.8°C for 30 min or at 71–75°C for 15–30 s may inactivate this enzyme and simultaneously sterilize nonsporeforming pathogenic microorganisms. Therefore, ALP activity has been conventionally an index of adequate pasteurization, and the detection of ALP activity of thermally treated liquid milk products has become a common procedure for milk quality control (International Dairy Federation, 1991; Murthy, Kleyn, Richardson, & Rocco, 1992, ch 14).

However, standard procedures such as colorimetric, fluorometric and chemiluminescent methods for ALP activity in milk are nonspecific and are unable to differentiate between bovine milk (BM) ALP and microbial ALP (Painter & Bradley, 1997). Since thermal stability of microbial ALP is higher than that of BMALP and detected ALP activity in pasteurized milk products may be due to the combined results of inadequate thermal treatment, renaturation of BMALP and/or presence of microbial ALP. The repasteurization is recommended as a check by the American Public Health Association but it is not practical since a notable reduction in milk flavor and severe browning reaction are observed. Therefore, an advanced methodology to distinguish ALP sources in milk products is urgently needed.

An immunological assay specific to BMALP may provide a better resolution. Recently, Vega-Warner, Wang, Smith, and Ustunol (1999) developed a competitive indirect enzyme-linked immunosorbent assay (CI-ELISA) to differentiate between BMALP and other proteins in milk or denatured BMALP, using antibody in rabbit serum specific against BMALP. However, no further reports on the development of an ELISA to quantify BMALP and differentiate between BMALP and microbial ALP in milk systems have been found.

Egg yolk is a potential source of immunological food supplement because of its high immunoglobulin content (approximate 15 mg IgY/mL) (Chang, Lu, Chen, & Tu, 2000), its ease of collection without sacrificing animals, and its relative abundance. The fortification of immunity by passive immunization of immunoglobulin preparations containing antibodies specific against pathogenic antigens has been reported. By immunizing hens, IgY specific against *Streptococcus mutans* (Chang, Ou-Yang, Chen, & Chen, 1999; Otake et al., 1991), rotavirus (Ebina et al., 1990), *E. coli* (Shimizu, Fitzsimmons, & Nakai, 1988) and urease of *Helicobactor pylori* (Chang, Lee, Chen, & Tu, 2002) were collected. In addition, the use of IgY for immunoaffinity chromatographies has been developed for the isolation and purification of bioactive components such as lactoferrin from bovine milk (Li-Chan et al., 1998; Tu, Chen, Chang 2001; Tu, Chen, Chang, Chang 2002), lysozyme from hen's egg white (Chen, Tu, & Chang, 2002) and γ-globulin from swine serum (Tu, 2000). Cross-reaction of antibodies from avian sources against the antigen from a mammalian source with the corresponding antigen from another mammalian animal is usually low (Kuby, 1994). Therefore, in the present study, uses this advantage to differentiate between BMALP and microbial ALP, hens instead of rabbits were immunized with BMALP to collect crude IgY, and the immunological assay for BMALP was established. In addition, the effect of dilution of milk samples on the inhibition (%) of BMALP on the microtiter plate by free BMALP or *E. coli* ALP was also investigated.

## 2. Materials and methods

### 2.1. Purification of bovine milk ALP

Bovine milk ALP was purified for standard sample according to the method described by Vega-Warner et al. (1999). Briefly, raw whole milk from the Taiwan University dairy herd was centrifuged to remove the cream and was then mixed with buffer solution containing 0.1% TritonX-100 and 25% (v/v) *n*-butanol to obtain the water-soluble portion. Subsequently, casein was removed from the water-soluble portion by adjusting the pH to about five, followed by 25% (v/v) ethanol treatment to collect the crude ALP, which was purified by Macro-prep high Q anion-exchange chromatography (Pharmacia, Uppsala, Sweden) and by Sephacryl S-200 gel (Pharmacia) permeation chromatography to obtain an enzyme product with a final yield of 28% and a purification fold (ratio of the specific activity of final ALP to that of the starting material) of 97. Purified BMALP with a specific activity of 3.89 U/mg protein, as determined by an ALP enzyme assay Kit (No. 104-LL, Sigma, St. Louis, MO, USA), was stored at −20°C in 50% glycerol for the intramuscular immunization of hens and used as standard sample added to the whole milk and skimmed milk for the following ELISA tests.

### 2.2. ALP activity assay

Enzyme activity assay was performed following the enzyme assay Kit manual (No. 104-LL, Sigma). Substrate solution (4 mg *p*-nitrophenyl phosphate/mL) was mixed homogeneously with equal volume of alkaline buffer solution (1.5 M 2-amino-2 -methyl-1-propanol, pH 10.3) at 37 °C in a water bath for 1 min. Then, 0.1 mL ALP sample was added to 1 mL of the mixture, and reacted at 37°C for 15 min, at which point the enzyme–substrate reaction was stopped by the addition of 10 mL 0.05 N NaOH. The absorbance at 405 nm was monitored with a spectrophotometer (Helios α, Spectronic Unican Co., London, Great Britain) and the enzyme activity in sample solutions was computed by means of a calibration curve (0–10 μg BMALP/mL) (3.89 U/mg protein) ($R^2 = 0.9175$). The specific activity

of *E. coli* ALP from Sigma was assayed to be 60 U/mg protein. Three samples each were tested in duplicate.

### 2.3. Preparation of IgY specific against bovine milk ALP

Three milliliters of Freund's complete adjuvant was homogenized in a syringe with 1 mL of purified bovine milk ALP in PBS (600 μg protein/mL of sterilized 0.02 M phosphate buffer saline, pH 7.0) to prepare antigens for the intramuscular injection into both legs of five hens (*im*) aged 6 months. After initial immunization, the immunization was repeated once a week for 4 weeks with antigens prepared with Freund's incomplete adjuvant in a similar manner as above (Buchta, 1991; Tsai and Cousin, 1990; Tu et al. 2001). Two-fold diluted yolk with distilled water from eggs collected at the sixth week after the initial immunization was mixed well with two volumes of 0.225% (w/v) pectin (Sigma) with a degree of esterification of 68% to prepare the crude IgY with an immunoactivity recovery of 73%, as determined by single radial immunodiffusion method (Chang et al., 2000).

### 2.4. Development of competitive indirect (CI) ELISA for milk ALP

CI-ELISA (Vega-Warner et al., 1999) was performed to determine the inhibition (%) of free BMALP and *E. coli* ALP in whole milk (30.03 mg protein/mL) and skimmed milk (30.61 mg prtein/mL) on BMALP coated on a microtiter plate. Both milk samples used were the commercial products from a local supermarket, containing no ALP activity as determined by the enzyme assay Kit (No. 104-LL, Sigma).

BMALP solution (50 μg/mL 0.1 M carbonate buffer, pH 9.6) at 100 μL/well was used to coat a microtiter plate, which was incubated at 4 ± 1°C overnight. After washing it four times with PBS (0.15 M phosphate buffer saline, pH 7.0)/0.05% Tween-20 (PBS–Tween), each well was supplemented with 100 μL of 0.5% gelatin–PBS and kept for incubation at 37 ± 1°C for 30 min. Wells were washed four times with 200 μL PBS–Tween and then supplemented with 50 μL of BMALP and/or *E. coli* ALP (0.01–10 μg/mL) in sample solutions (PBS, distilled water-diluted or undiluted commercial skim milk or whole milk) as competitive antigens. Subsequently, to each well was added 50 μL of $10^3$ fold diluted crude IgY (specific against BMALP) with PBS, followed by the incubation at 37 ± 1°C for 90 min. Wells were washed six times with PBS–Tween. Then to each well was added 100 μL of $10^3$-fold diluted ALP-conjugated goat anti-chicken IgG (KPL Co.) (0.5 μg/mL) with PBS. Incubation occurred at 37 ± 1°C for 90 min. Each well was again washed with PBS–Tween for six times; then to each well was added 100 μL of freshly prepared substrate [5-bromo-4-chloro-3-indolyl phosphate (BCIP)] solution (1 mg BCIP/mL) (KPL Co.), which was the mixture of BluePhos Microwell Phosphatase solution A and B (1/1, v/v). The plates were allowed to complete the reaction for 30 min at 37 ± 1°C before the addition of 100 μL of 2.5% 2Na–EDTA to stop the reaction. The color developed was read at 650 nm with an ELISA reader (Emax, Molecular Devices Co., San Francisco, CA, USA).

Inhibition of BMALP on ELISA plate by free ALPs in sample solutions was determined according to Vega-Warner et al. (1999) by the following equation: Inhibition $(\%) = (A_o - A_c)/A_o \times 100\%$ Where, $A_o = A_{650\ nm}$ of sample without ALP; $Ac = A_{650\ nm}$ of sample with 0.01–10 μg ALP/mL.

Cross-reactivity was defined as the concentration of ALP required for 50% inhibition divided by the concentration of competitive antigen required for 50% inhibition, multiplied by 100 (Deshpande, 1996).

Antibody titer (Buchta, 1991; Tsai & Cousin, 1990) was performed to determine the changes in binding activity of IgY to BMALP during the immunization period of hens, except for the addition of the competitive antigens to the BMALP coated wells. Absorbance of sample was at least three times higher than that of the control. Yolks obtained from eggs laid by hens before the initial immunization were used as controls. Three samples were tested in duplicate.

### 2.5. Protein assay

Protein content was determined according to the method described by Bradford (1976). The microtiter plate protocol of Bio-Rad protein assay was conducted using chicken serum IgG (Sigma) as the standard solution (50–500 μg/mL) for the standard curve (24) ($R^2 = 0.98$). Three samples each were tested in duplicate.

## 3. Results and discussion

### 3.1. Productivity of IgY specific against BMALP

Hens were immunized intramuscularly on their both thighs with antigen containing BMALP with a specific activity of 3.89 U/mg protein. The level of antibody in serum against this antigen began to rise after the initial immunization, reaching a peak at approximately the 7th week with an ELISA value as high as 1.383 and 1.078 (Absorbance at 650 nm) for $10^5$-fold diluted serum and yolk, respectively. The titer of the corresponding serum and yolk samples was determined to be about $1.3 \times 10^8$ and $2.1 \times 10^7$ (data not shown), respectively. It is significant that the hen serum titer was higher and the yolk titer was almost equal to that ($1.2 \times 10^7$) of serum of rabbit immunized with purified BMALP with a specific activity as high as 37 U/mg protein, as indicated by

Vega-Warner, Gandhi, Smith, and Ustunol (2000). The immune response induced by antigen from alien species is usually stronger than that induced by antigen from kindred species (Kuby, 1994), suggesting that the use of hens is suitable in preparing antibodies specific against BMALP.

The increasing trend of antibody level in yolk was similar to that reported by Chang et al. (2002) using urease from *Helicobacter pylori* as antigen and by Tu, Chen, and Chang (2001). IgY is converted from hen serum IgG with minor modification, which displays a titer lower than that of serum IgG against specific antigen (Otani, Matsumoto, Saeki, & Hosono, 1991; Shimizu et al., 1988; Warr, Magor, & Higgins, 1995). Yolks of eggs collected at the 6th week after initial immunization with a titer of about $1.3 \times 10^6$ were used to prepare the crude IgY specific against BMALP for the following BMALP detection in milk samples with different dilution folds.

### 3.2. Determination of the optimal condition for CI-ELISA

Experimental conditions for competitive ELISA were determined by varying the concentrations of primary antibody (IgY against BMALP) and enzyme-conjugated secondary antibody to detect the inhibition (%) of BMALP on ELISA plate by free BMALP or *E. coli* ALP (0.01–10 μg/mL) in PBS. It was observed that the inhibition of BMALP increased with the increasing level of BMALP in PBS (Fig. 1). It reveals that higher level of free homologous antigen exhibits stronger competition with antigen on ELISA plate for the same specific antibody, resulting in the lower absorbance at 650 nm of sample solution and higher inhibition (%) (Vega-Warner et al., 1999).

The inhibition of BMALP was as high as about 75% in the presence of 10 μg BMALP/mL PBS, regardless of the dilutions of crude IgY ($1–2 \times 10^3$ folds) and conjugate ($0.5–1 \times 10^3$ folds) used (Fig. 1). Vega-Warner et al. (1999) pointed out that the inhibition of BMALP was about 80% at a BMALP level of about 7 μg/mL PBS, using rabbit serum IgG as antibody source. This suggests a relatively higher specificity between immunoglobulin (IgY) from avian source and BMALP than that between rabbit serum IgG and BMALP. Changes in the trend of inhibition (%) of BMALP by various levels of free BMALP and *E. coli. ALP* in Fig. 1(a) ($1 \times 10^3$-fold diluted crude IgY, $0.5 \times 10^3$-fold diluted conjugate) and Fig. 1(b) ($1 \times 10^3$-fold diluted crude IgY, $1 \times 10^3$-fold diluted conjugate) were similar. The inhibition of BMALP

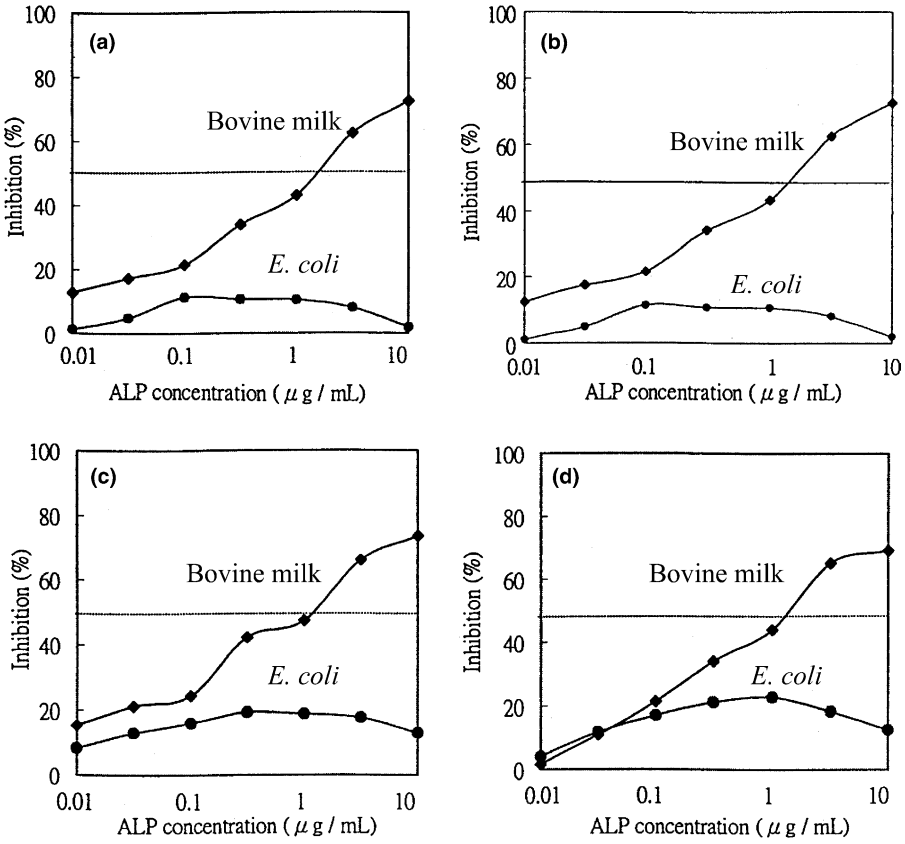


Fig. 1. Effect of concentrations of IgY specific against bovine milk ALP and conjugate on the inhibition (%) of bovine milk ALP by various levels of bovine milk ALP or *E. coli* ALP in PBS (0.15 M phosphate buffer, pH 9.5). Reference line is used to indicate 50% inhibition: (a) $1 \times 10^3$ fold diluted crude IgY, $0.5 \times 10^3$ fold diluted conjugate; (b) $1 \times 10^3$ fold diluted crude IgY, $1 \times 10^3$ fold diluted conjugate; (c) $2 \times 10^3$ fold diluted crude IgY, $0.5 \times 10^3$ fold diluted conjugate; (d) $2 \times 10^3$ fold diluted crude IgY, $1 \times 10^3$ fold diluted conjugate.

by 0.01–10 μg *E. coli* ALP/mL PBS was all between 0% and 10%. On the other hand, comparing to those (0–10%) in Fig. 1(a) and (b), the inhibition (10–20%) of BMALP by the same level of *E. coli* ALP in Fig. 1(c) ($2 \times 10^3$-fold diluted crude IgY, $0.5 \times 10^3$-fold diluted conjugate) and Fig. 1(d) (2 $10^3$-fold diluted crude IgY, $1 \times 10^3$-fold diluted conjugate) appeared to be higher. This reveals that $2 \times 10^3$-fold diluted primary antibody was unfavorable for the detection of BMALP in ALPs from bovine milk and *E. coli* sources. Vega-Warner et al. (1999) also indicated that the bigger difference between the inhibitions of BMALP by free BMALP and by free *E. coli* ALP in the sample solution was more beneficial for differentiating BMALP from different sources. Therefore, based on the above findings and in the view of cost, 1 $10^3$-fold diluted crude IgY and $1 \times 10^3$-fold diluted enzyme-conjugated secondary antibody (Fig. 1(b)) appears to be favorable for the following CI-ELISA to determine the inhibition of BMALP by ALPs in a milk system.

### 3.3. Determination of BMALP in milk systems by CI-ELISA

In an attempt to determine the sources and the level of the residual ALP activity in commercial liquid milk products such as whole milk (30.03 mg protein/mL) and skimmed milk (30.61 mg protein/mL), CI-ELISA was applied to test the inhibition of BMALP on ELISA plate by free ALPs. As can be seen in Fig. 2, the ELISA value decreased ($R^2 = 0.9402$) with the increasing level (0.01–10 μg/mL) of BMALP in the pasteurized commercial whole milk. Accordingly, BMALP level in fresh raw whole milk from Taiwan University dairy herd was determined to be about 26.3 mU/mL (6.75 μg/mL) with the CI-ELISA developed in the present study (data not shown). Therefore, ALP content ranged between 0.01 and 10 μg/mL for determining the inhibition of BMALP by various levels of free BMALP or *E. coli* ALP in the following experiments. In detection of the inhibition of BMALP in the presence of various levels (0.01–10 μg/mL) of BMALP (0.04–38.9 mU/mL) in whole milk, the inhibition (%) was intensified as the concentration of BMALP increased, reaching about 48% at 10 μg BMALP/mL (Fig. 2).

It is noteworthy that *E. coli* ALP, in the presence or absence of BMALP, at concentrations between 0.01 and 10 μg/mL (about 0.6–600 mU/mL) did not display BMALP inhibition (%) (no cross-reactivity) under the present CI-ELISA conditions using crude IgY specific against BMALP as the primary antibody. Accordingly, any detection of ALP activity in whole milk could be completely due to the presence of BMALP (Fig. 1) at levels between 0.01 and 10 μg/mL (0.0389–38.9 mU/mL). Though less sensitive than the Fluorophos method (<0.01 mU/mL) (Painter & Bradley, 1997), the CI-ELISA developed in this study is able to differentiate between BMALP and ALPs from *E. coli* sources when BMALP in milk was higher than 0.1 μg/mL (0.389 mU/mL) (Fig. 2). As a result, microbial ALP in milk products was quantifiable when Scharer method or Fluorophos method (Painter & Bradley, 1997) was combined to determine the total ALP activity in milk products.

For the detection of BMALP in skimmed milk system, various levels of BMALP in pasteurized skimmed milk were prepared and the relationship between the ELISA value and BMALP level was determined. It was found that the ELISA value decreased with an increasing level (0.01–10 μg/mL) of BMALP in skimmed milk with a correlation coefficient ($R^2$) of 0.9019 (Fig. 3). Similar to the results in Fig. 2, the inhibition of BMALP on ELISA plate also increased with increasing levels of free BMALP in skimmed milk, up to about 50% at 10 μg BMALP/mL. Apparently, the compositional difference between these two milk products influenced insignificantly the CI-ELISA results when used as back-

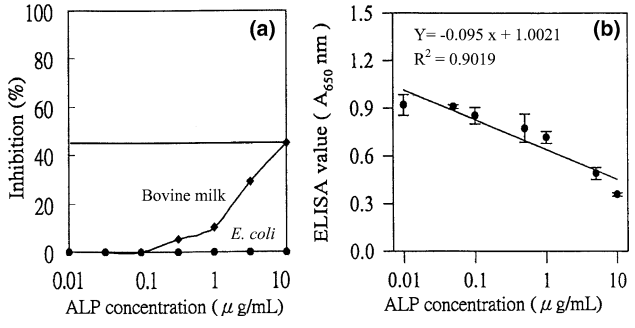


Fig. 2. The inhibition (%): (a) of bovine milk ALP by various levels of bovine milk ALP or *E. coli* ALP in whole milk (30.03 mg protein/mL) and the linear relationship; (b) between ELISA value and the level of bovine milk ALP in whole milk. Reference line (A) is used to indicate 50% inhibition. Commercial pasteurized whole milk was used as the ALP-free milk. Inhibition (%) = $(A_o - A_c)/A_o \times 100\%$; $A_o$, $A_{650\ nm}$ of sample without bovine milk ALP; $A_c$, $A_{650\ nm}$ of sample with certain level of ALP.

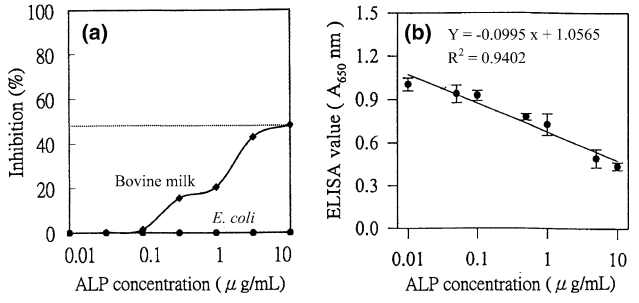


Fig. 3. The inhibition (%): (a) of bovine milk ALP by various levels of bovine milk ALP or *E. coli* ALP in skimmed milk (30.61 mg protein/mL) and the linear relationship; (b) between ELISA value and the level of bovine milk ALP in skimmed milk. Reference line (A) is used to indicate 50% inhibition. Commercial pasteurized skimmed milk without ALP activity was used.

ground solution to determine BMALP (Figs. 2 and 3). Thus BMALP content in skimmed milk ranging from 0.1–10 μg/mL was determinable using the method developed in the present study.

### 3.4. Effect of dilution of milk system on the detection of BMALP

Various levels (0.01–10 μg/mL) of BMALP or *E. coli* ALP were added to $10^1$–$10^4$-fold diluted (3 mg protein/L- 3 g protein/L) milk systems to investigate the effect of background milk concentration on the inhibition of BMALP by free ALPs in diluted milk. Similar to those in Fig. 1, increased BMALP level in diluted whole milk increased the inhibition of BMALP on ELISA plate (Fig. 4). The inhibition of BMALP was about 70% (Fig. 4(a) and (b)) in the presence of 10 μg BMALP/mL, which was higher than that (48%) in non-diluted whole milk, as shown in Fig. 2. This reveals that the reduction ($10^1$–$10^2$-fold dilution) in whole milk concentration facilitates the competitive inhibition of BMALP on ELISA plate for primary antibodies by free BMALP molecules in whole milk.

On the other hand, the inhibition (10–20%) of BMALP on ELISA plate by free *E. coli* ALP (0.01–10 μg/mL) in $10^4$-fold diluted whole milk (Fig. 4(d)) was apparently higher than that (0–10%) in $10^1$–$10^3$-fold diluted whole milk (Fig. 4(a)–(c)). This suggests that low milk concentration ($10^4$-fold dilution) of background milk sample was apparently unfavorable for the quantification of BMALP in mixtures of ALPs from different sources.

Similar results were obtained when skimmed milk with 0.01–10 μg BMALP/mL added was tested (Fig. 5). Though the inhibition (0–3%) of BMALP by *E. coli* ALP in $10^1$–$10^2$-fold diluted skimmed milk was apparently lower than that (0–10%) observed in Fig. 4(a) and (b), major trends in changing the inhibition of BMALP by free ALPs were similar (Fig. 5). Apparently, milk concentration affected the inhibition of BMALP by *E. coli* ALP, and non-diluted whole milk or skimmed milk is suggested as the most effective in determining BMALP in milk samples. ALP from E. coli source presents no inhibition on BMALP on microtiter ELISA plate (Figs. 2 and 3) and it appears to be beneficial for differentiating between BMALP and ALPs from other sources.

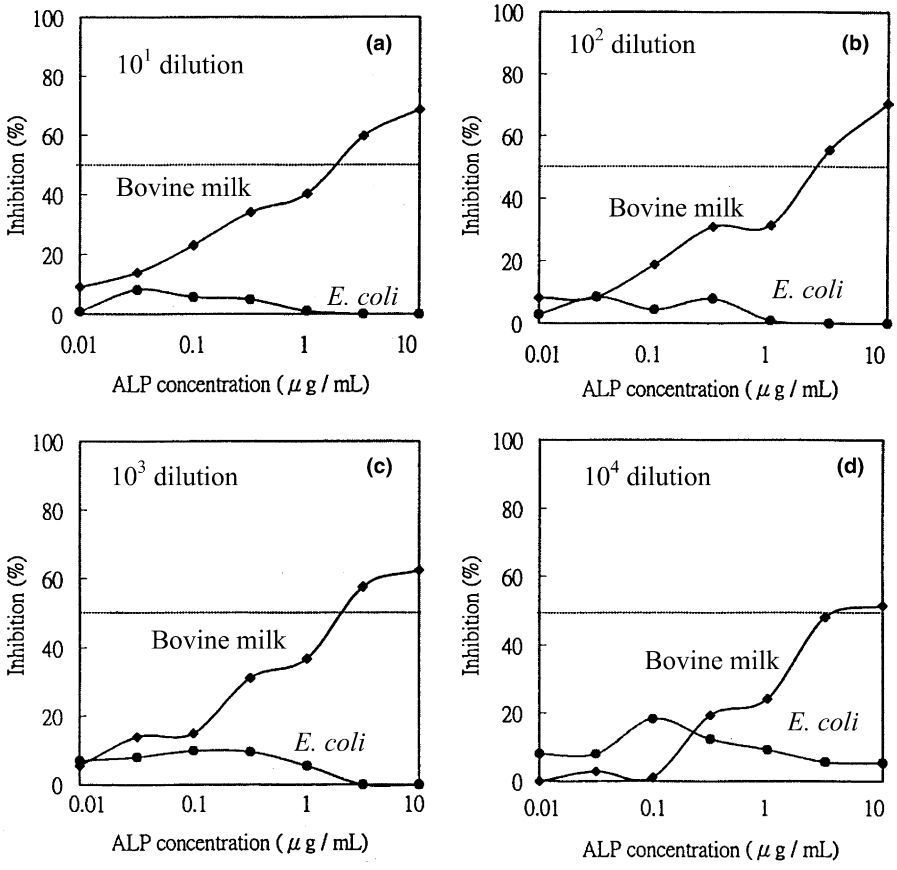


Fig. 4. Comparison of the inhibition (%) of bovine milk ALP by various levels of bovine milk ALP or *E. coli* ALP in $10^1$-fold (a), $10^2$-fold (b), $10^3$-fold (c), and $10^4$-fold (d) diluted whole milk. Reference line is used to indicate 50% inhibition. Commercial pasteurized whole milk without ALP activity was used.

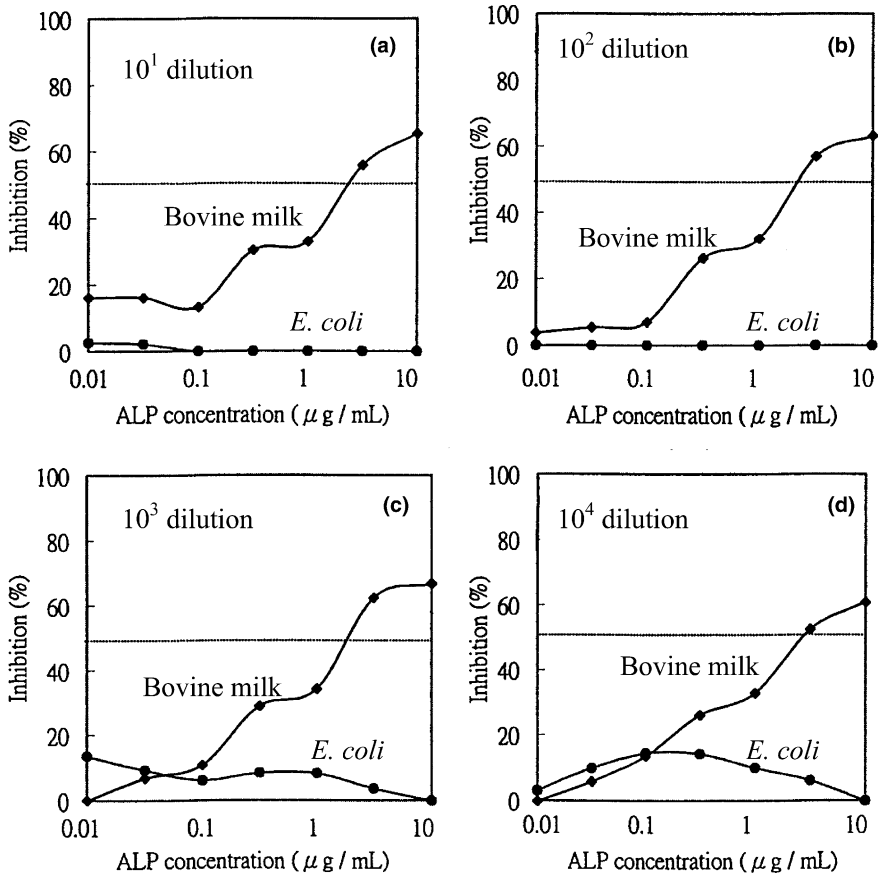


Fig. 5. Comparison of the inhibition (%) of bovine milk ALP by various levels of bovine milk ALP or *E. coli* ALP in $10^1$-fold (a), $10^2$-fold (b), $10^3$-fold (c), and $10^4$-fold (d) diluted skimmed milk. Commercial pasteurized skimmed milk without ALP activity was used.

A combination of CI-ELISA and other ALP activity assays such as the Scharer method or the Fluorophos method could be practical to determine the presence and magnitude of ALP from microbial sources. The CI-ELISA developed in the present study may be able to differentiate between BMALP and ALPs from other sources, and its application to the dairy industry is recommended.

## References


Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Buchta, R. (1991). Ovine lactoferrin: isolation from colostrum and characterization. *Journal of Dairy Research, 58*, 211–218.

Chang, H. M., Lee, Y. C., Chen, C. C., & Tu, Y. Y. (2002). Microencapsulation protects immunoglobulin in yolk (IgY) specific against *Helicobactor pylorie* urease. *Journal of Food Science, 67*, 15–20.

Chang, H. M., Lu, T. C., Chen, C. C., & Tu, Y. Y. (2000). Isolation of immunoglobulin from egg yolk by anionic polysaccharides. *Journal of Agricultural and Food Chemistry, 48*, 995–999.

Chang, H. M., Ou-Yang, R. F., Chen, Y. T., & Chen, C. C. (1999). Productivity and some properties of immunoglobulin specific against *Streptococcus mutans* serotype c in chicken egg yolk (IgY). *Journal of Agricultural and Food Chemistry, 47*, 61–66.

Chen, C. C., Tu, Y. Y., & Chang, H. M. (2002). Isolation of immunoglobulin in yolk (IgY) specific against hen egg white lysozyme by immunoaffinity chromatography. *Journal of Agricultural and Food Chemistry, 50*, 5424–5428.

Deshpande, S. S. (1996). Assay development, evaluation, and validation. In *Enzyme immunoassay: from concept to product development*. New York: Chapman and Hall.

Ebina, T., Tsukada, K., Umezu, K., Nose, M., Tsuda, K., & Hatta, H. (1990). Gastroenteritis in sucking mice caused by human rotavirus can be prevented with egg yolk immunoglobulin (IgY) and treated with a protein-bound polysaccharide preparation (PSK). *Microbiology and Immunology, 34*, 617–629.

Inouye, H., & Reckwith, J. (1997). Synthesis and processing of an Escherichia coli alkaline phosphatase precursor in vitro. *Proceedings of National Academy and Science, 74*, 1440–1444.

International Dairy Federation. (1991). Alkaline phosphate test as a measure of correct pasteurization. *Bulletin of IDF, 262*, 33–35.

Kuby, J. (1994). *Immunology* (2nd ed.). New York: W.H. Freeman Co., p. 79.

Li-Chan, E., Ler, S. S., Kummer, A., & Akita, E. M. (1998). Isolation of lactoferrin by immunoaffinity chromatography using yolk antibodies. *Journal of Food Biochemistry, 22*, 179–195.

Murthy, G. K., Kleyn, D. H., Richardson, T., & Rocco, R. M. (1992). Alkaline phosphatase methods. In R. T. Marshall (Ed.), *Standard methods for examination of dairy products* (pp. 413–431). Washington, DC: American Public Health Assoc.

Otake, S., Nishihara, Y., Makimura, M., Hatta, H., Kim, M., & Yamamoto, T. (1991). Protection of rats against dental caries by

passive immunization with hen-egg-yolk antibody (IgY). *Journal of Dental Research, 70*, 162–166.

Otani, H., Matsumoto, K., Saeki, A., & Hosono, A. (1991). Comparative studies on properties of hen egg yolk IgY and rabbit serum IgG antibodies. *Lebens. mittel-Wissen. Schaft+ Technology, 24*, 152–158.

Painter, C. J., & Bradley, R. L. (1997). Residual alkaline phosphate activity in milks subjected to various time-temperature treatments. *Journal of Food Protection, 60*, 525–530.

Shimizu, M., Fitzsimmons, R. C., & Nakai, S. (1988). Anti-*E. coli* immunoglobulin Y isolated from egg yolk of immunized chickens as a potential food ingredient. *Journal of Food Science, 53*, 1360–1366.

Tsai, G. J., & Cousin, M. A. (1990). Enzyme-Linked immunosorbent assay for detection of molds in cheese and yogurt. *Journal of Dairy Science, 73*, 3366–3378.

Tu, Y. Y., (2000). Purification of lactoferrin from cow milk and γ-globulin from pig blood by yolk immunoglobulin-immunoaffinity chromatography. Ph.D dissertation, Graduate Institute of Food Science and Technology, National Taiwan University.

Tu, Y. Y., Chen, C. C., & Chang, H. M. (2001). Preparation of immunoglobulin in yolk (IgY) and rabbit serum immunoglobulin (IgG) specific against lactoferrin by immunoaffinity chromatography. *Food Research International, 34*, 783–789.

Tu, Y. Y., Chen, C. C., Chang, J. H., & Chang, H. M. (2002). Characterization of lactoferrin (LF) from colostral whey using anti-LF antibody immunoaffinity chromatography. *Journal of Food Science, 67*, 996–1001.

Vega-Warner, A. V., Wang, C. H., Smith, D. M., & Ustunol, Z. (1999). Milk alkaline phosphatase purification of polyclonal antibodies. *Journal of Food Science, 64*, 601–605.

Vega-Warner, A. V., Gandhi, H., Smith, D. M, & Ustunol, Z. (2000). Polyclonal-antibody based ELISA to detect milk alkaline phosphatase. *Journal of Agricultural and Food Chemistry, 48*, 2087–2091.

Warr, G. W., Magor, K. E., & Higgins, D. A. (1995). IgY: clues to the origins of modern antibodies. *Immunology Today, 16*, 392–398.