Polyclonal-Antibody-Based ELISA To Detect Milk Alkaline Phosphatase

A. Virginia Vega-Warner, Hasand Gandhi, Denise M. Smith, and Zeynep Ustunol*

Michigan State University, Department of Food Science and Human Nutrition, East Lansing, Michigan 48824

Polyclonal antibodies (PAb) prepared against bovine milk alkaline phosphatase (ALP) were used to develop a competitive indirect (CI) ELISA. Anti-ALP PAb were specific for milk ALP and did not react with ALP from *E. coli* or bovine and calf intestinal mucosa. Anti-ALP PAb were 20% cross-reactive with bovine placenta ALP. The anti-ALP antibodies also did not recognize bovine serum albumin, acid glycoprotein, ovalbumin, ferritin, and casein, although some cross-reactivity was observed with whey protein isolate. Anti-ALP PAbs reacted with deglycosylated native ALP, but did not recognize ALP denatured at 100 °C in 2% SDS or deglycosylated denatured ALP. When buffered solutions of milk ALP were heated at 70 °C, ALP activity decreased at a faster rate than ALP content determined by CI-ELISA. The ELISA differentiated between native and heat denatured ALP. Further studies are warranted to determine if an ELISA can be used to verify pasteurization of fluid milk.

Keywords: *Alkaline phosphatase; milk; antibodies; ELISA*

INTRODUCTION

Pasteurization is the most common and reliable means of thermal processing to ensure the safety of the milk supply. Pasteurization eliminates pathogenic organisms and improves the keeping quality of milk by eliminating gram negative psychrotrophic organisms (Knight and Fryer, 1989). Minimum requirements for adequate pasteurization of milk are heating at 62.8 °C for 30 min for a low-temperature long-time or 71.7 °C for 15 s for a high-temperature short time process (Pasteurized Milk Ordinance, 1993). Alkaline phosphatase (ALP) (EC 3.1.3.1; phosphatase), an enzyme native to milk, is inactivated by pasteurization. ALP activity in milk has been measured since 1935 to ensure the adequacy of the pasteurization process (Kay and Graham, 1935, Murthy et al., 1992). If ALP activity in heat-treated milk is below legal limits, it is assumed to have been properly pasteurized. In addition, the ALP activity assay has been used to detect recontamination of pasteurized milk with raw milk (International Dairy Federation, 1991).

The currently used colorimetric, fluorometric, and chemiluminescent ALP activity assays, unfortunately, are unable to differentiate between ALP from milk or microbial sources. A high ALP activity may indicate inadequate pasteurization or recontamination with raw milk after pasteurization or the presence of microbial contamination (Kay and Graham, 1935; Kleyn and Lin, 1968; Rocco, 1990). Generally, microbial ALP exhibits higher thermal stability than milk ALP. Typically, a milk sample that has tested positive is retested for ALP activity after laboratory pasteurization. Since microbial ALP exhibits higher heat stability, any remaining activity in the milk sample is then attributed to microbial sources (Murthy et al., 1992).

* Corresponding author (phone (517) 355-0285; fax (517) 353-1676; e-mail ustunol@msu.edu).

Knight and Fryer (1989) reported ALP activity in pasteurized milk stored for 4 days at 4 °C. This activity was due to heat resistant ALP produced by a pasteurization sensitive psychrotrophic organism. When the psychrotrophic counts in the milk prior to pasteurization reached more than 1.2×10^7 CFU/mL there was sufficient pasteurization resistant microbial ALP to result in ALP activity in the samples after pasteurization, thus, a failed pasteurization test. They concluded that even if milk is adequately pasteurized and properly stored, a failed pasteurization test may result due to production of sufficient quantities of microbial ALP rather than failure in the pasteurization process. Given the possibility of microbial contamination or contamination with raw milk after pasteurization and the use of microorganisms to produce fermented dairy products, there is a need to produce a rapid assay that specifically recognizes milk ALP.

An immunological assay specific for bovine milk ALP may provide a method to differentiate milk from microbial ALP. We previously reported on the successful purification and production of polyclonal antibodies (PAb) against purified ALP (Vega-Warner *et al*., 1999). As shown by western blot analysis, the PAbs were cross-reactive with bovine milk and placenta ALP but did not cross-react with ALP from calf or bovine intestinal mucosa or *Escherichia coli*, or with other milk proteins. The overall objective of this study was to develop a PAb based enzyme-linked immunosorbent assay (ELISA) to detect milk ALP, determine the specificity of the PAbs used in the ELISA toward ALP from various sources, milk proteins and glycoproteins, by assessing their competitiveness, and begin evaluating the suitability of the ELISA to quantify native and heat denatured ALP by comparing the ability of the ELISA and an enzyme assay to detect changes in ALP upon heating. We hope that this work will lead to the development of an ELISA

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to quantify ALP in milk as an index of adequate pasteurization.

MATERIALS AND METHODS

Materials. Polyethylene sorbitan monolaurate (Tween 20), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS), 3,3′,5,5′-tetramethylbenzidine (TMB), alkaline phosphatase (ALP) enzyme assay kit no. 104-LL, bovine serum albumin, acid glycoprotein, casein, ovalbumin, and ALP from bovine milk, bovine placenta, bovine intestinal mucosa, calf intestinal mucosa, and *E. coli* were purchased from Sigma Chemical Co. (St. Louis, MO). K-blue was from Neogen Corp. (East Lansing, MI). Ferritin was obtained from Boehringer Mannheim (Indianapolis, IN), and BIPRO LE whey protein isolate (>95% protein) was from Davisco Foods International Inc. (Le Sueur, MN). Polystyrene microtiter ELISA plates (Immunolon-2 Removawells) were purchased from Dynatech Laboratories (Alexandria, VA), the goat anti-rabbit immunoglobulin G (IgG) peroxidase was from Organon Teknika Corp. (West Chester, PA), and the deglycosylation kit was from Bio-Rad Laboratories (Hercules, CA). All other chemicals were of reagent grade or better.

Development of Competitive Indirect (CI) ELISA for Milk ALP. Antibodies were prepared against purified ALP in New Zealand white female rabbits as reported previously and stored at −80 °C until used (Vega-Warner et al, 1999). Microtiter wells were coated with 100 μL of 0.5 μg of ALP/mL in 0.1 M carbonate buffer, pH 9.6, and held overnight at 4 °C. The plate was washed 4 times with 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2 (PBS), containing 0.05% (v/v) Tween 20 (PBS-Tween). Three hundred microliters of 0.5% (w/v) ovalbumin in PBS (OV−PBS) was added to each well and the plate was incubated for 30 min at 37 °C to block nonspecific binding. The plate was washed 4 times with PBS-Tween, and 50 μL of competitive antigen and 50 μL of PAb (diluted 1/2000 in OV−PBS) were added to each well before incubation for 1 h at 37 °C. After washing 4 times with PBS-Tween, 100 μL of goat anti-rabbit IgG peroxidase (diluted 1:500 in OV−PBS) was added to each well. After incubation for 30 min at 37 °C, the plate was washed 8 times with PBS-Tween. Peroxidase activity was determined at 405 nm with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Wang et al., 1992) and at 650 nm with 3,3′,5,5′-tetramethylbenzidine (TMB, Harlow and Lane, 1988) or K-Blue substrate using a Vmax Kinetic Microplate Reader (model 1234, Molecular Devices Corp., Menlo Park, CA). A standard curve using ALP in OV−PBS was prepared in each plate. The minimum level of detection was defined as 2 times the standard deviation of the mean response of the zero standard (Deshpande, 1996).

Antibody Specificity. The CI-ELISA was used to determine the anti-ALP PAb specificity to ALP (0.01−10 μg of protein/mL) from bovine milk, bovine placenta, bovine intestinal mucosa, calf intestinal mucosa, and *E. coli*. Bovine serum albumin, acid glycoprotein, casein, ovalbumin, ferritin, and whey protein isolate in concentrations ranging from 0 to 5 μg/mL were also used as the competitive antigen to test anti-ALP PAb specificity. Suitable dilutions of each protein listed were made in OV−PBS and 50 μL was added as the competitive antigen in the CI-ELISA.

The CI-ELISA developed was also tested for its ability to recognize and differentiate between native and heat denatured ALP. In this experiment, solutions (1 mL) of ALP (0.01−1000 μg/mL) in 0.1 M carbonate buffer, pH 9.6, were divided into two parts. One part (0.5 mL) was used as native ALP. The other part (0.5 mL) was denatured by heating at 72 °C for 2 min in a water bath, followed immediately by cooling in an ice bath.

For all of the above experiments, percent inhibition was calculated as

$$\% \text{ inhibition} = [(A_0 - A_c)/A_0] \times 100$$

Where, A_0 = absorbance of sample without any ALP; A_c = absorbance of sample with known concentration of ALP.

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).

Enzymatic Deglycosylation of ALP. Native and denatured ALP was enzymatically deglycosylated by using a deglycosylation kit (Bio-Rad). ALP was denatured in 2% SDS and 1 M β-mercaptoethanol by heating at 100 °C for 5 min. For deglycosylation, 12 μL of native or denatured ALP solution was mixed with 4 μL of the reaction buffer (250 mM sodium phosphate, pH 6.0) and incubated with 2 μL of NaNase II (α II-3,6 *N*-acetylneuraminidase) and 2 μL of O-glycosidase DS (*endo*-α-*N*-acetylgalactosaminidase) at 37 °C for 1 h. After incubation, 10 μL of deionized water and 10 μL of pH adjustment buffer (0.5 M sodium phosphate dibasic) were added to the reaction mixture followed by addition of 2 μL of PNGase F (peptide-*N*-[*N*-acetyl-β-glucose aminyl]asparagine amidase). The solution was incubated at 37 °C for 24 h. The CI-ELISA was used to evaluate the specificity of anti-ALP PAb with native and denatured deglycosylated milk ALP.

Effect of Heating on Quantification of ALP by CI-ELISA. Solutions (1 mL) of bovine milk ALP (10 μg/mL) in 50 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS), pH 7.0, in 10 × 75 mm glass tubes, sealed with Teflon tape, were heated to 70 °C for 2 h in a Polystat circulating water bath (model 1286-52, Cole-Parmer Instrument Co., Chicago, IL) connected to a temperature programmer (model 1268-62, Cole-Parmer). A resistance temperature detector probe (RTD, 1.6 mm diameter, ±0.5 °C accuracy) connected to a Solomat MPM 200 Modumeter (Solomat Partners LP, Glenbroon Industrial Park, Stanford, CT) was inserted in the center of a glass tube containing the ALP solution. Tubes were removed at specific time intervals and immediately cooled in an ice−water bath. Cooled ALP solutions were centrifuged at 5000*g* for 10 min at 4 °C. Supernatants were analyzed for ALP concentration by CI-ELISA and enzyme activity.

Determination of Protein Concentration and Enzyme Activity. A micro Bio-Rad protein assay at 280 nm with bovine serum albumin as the standard was used to determine protein concentration. An enzyme kit (no. 104-LL, Sigma) was used to determine ALP activity (U/L). Briefly, the buffered reaction mixture (pH 10.3) was incubated at 37 °C for 1 min and release of *p*-nitrophenol was determined at 405 nm by using an extinction coefficient of 18.5 μmol/L cm (Bergmeyer, 1991). One unit of activity was defined as the amount required to liberate 1 μmol of *p*-nitrophenol/min.

Statistical Analysis. All experiments were replicated 3 times. Standard error of the mean was determined for each analyte in the CI-ELISA by using one way analysis of variance (JMP software, version 3, SAS Institute, Inc., Cary, NC). Within-run ($n = 32$) and between-run ($n = 8$) precision of the CI-ELISA was assessed by repeated analysis at three ALP concentrations (2, 5, and 8 μg/mL) to cover the useful linear range of the assay (Deshpande, 1996). Tukey's test (SigmaStat software, version 2.0, Jandel Corp., San Rafael, CA) with the mean square error term at 5% significance level was used to analyze results from the heating experiment. ALP concentration and activity at each time interval were compared with the corresponding values at zero time, to find the minimum holding time to get a significant decrease in ALP activity.

RESULTS AND DISCUSSION

Development of CI-ELISA for Milk ALP. A CI-ELISA to quantify milk ALP was developed. The three substrates were compared during the initial stages of ELISA development, to find the substrate providing maximum sensitivity. The minimum detection limits, defined as 2 times the standard deviation of the mean response of the zero standard (Deshpande, 1996), for milk ALP using ABTS, K−Blue, and TMB as substrate in the CI-ELISA were 1, 0.5, and 1 μg/mL, respectively, indicating that K-Blue gave an assay with the lowest detection limit (Figure 1). Thus, K-Blue was used as the

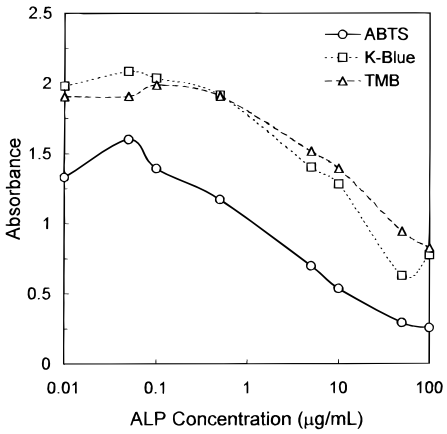

Figure 1. Detection of milk alkaline phosphatase (ALP) by competitive-indirect ELISA using ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), K-Blue, and TMB (tetramethylbenzidine) substrates. Absorbance was read at 405 nm for ABTS and 650 nm for K-Blue and TMB. Standard errors of means ($n = 4$) were 0.07, ABTS; 0.07, K-Blue; 0.05, TMB.

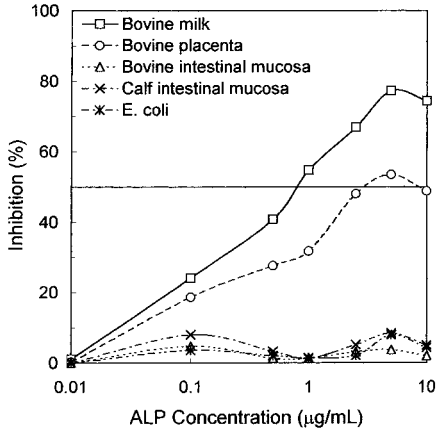

Figure 2. Specificities of milk alkaline phosphatase polyclonal antibodies to alkaline phosphatase (ALP) from various sources as determined by competitive-indirect ELISA. Reference line is used to indicate 50% inhibition. Cross-reactivity is defined as the concentration of ALP required for 50% inhibition divided by the concentration of the competitive antigen required for 50% inhibition multiplied by 100. Standard errors of means ($n = 4$) were 3.75%, bovine milk; 3.86%, bovine placenta; 3.42%, bovine intestinal mucosa; 3.04%, calf intestinal mucosa; 4.07%, *E. coli*.

substrate in the CI-ELISA for the rest of this work. The intraplate or between run ($n = 8$) coefficient of variation values were 2.4%, 3.3%, and 2.9% for ALP concentration of 2, 5, and 8 μg/mL. The interplate or within-run ($n = 32$) coefficient of variation values among plates were 7.5%, 9.2%, and 6.4% for the three ALP solutions.

Specificity of Antibodies to ALP from Other Sources. The specificity of the anti-ALP PAb in the CI-ELISA was examined by evaluating competitiveness with ALP from different sources. 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). Anti-ALP PAb did not cross-react with ALP from bovine intestinal mucosa, calf intestinal mucosa, or *E. coli*. However, the PAb showed 20% cross-reactivity with bovine placenta ALP (Figure 2).

These results were consistent with our earlier work (Vega-Warner et al., 1999) where some cross-reactivity was observed between bovine milk anti-ALP PAb and bovine placenta ALP on western blots prepared from native and SDS PAGE gels. Western blot analysis did not show any cross-reactivity between bovine milk anti-ALP PAb and ALP from bovine intestinal mucosa, calf intestinal mucosa, or *E. coli*. McComb et al. (1979) reported that the amino acid composition of the ALP depends on its source. *E. coli* ALP possesses a large number of alanine residues and very few aromatic amino acid groups compared to ALP from other sources. Alkaline phosphatase from mammalian sources usually contains carbohydrate, whereas *E. coli* ALP does not. These results suggest that CI-ELISA may differentiate between microbial and bovine milk sources of ALP. ALP activity methods currently used to ascertain phosphatase activity in milk are unable to differentiate between ALP from milk, microbial, or other sources. If a pasteurized milk sample shows high values for ALP activity by the currently used enzymatic methods, it is recommended that the sample be repasteurized and tested again for ALP activity. If ALP activity is detected again, then the ALP activity is reported to be due to heat resistant microbial ALP (Murthy et al., 1992). However, this approach is not always successful. Murthy and Kaylor (1990) reported that it is not always possible to differentiate between bovine milk and microbial ALP by repasteurization, because both heat-labile and heat-stable ALP can be produced by microorganisms. Although, further studies with other microbial sources of ALP are necessary, our results suggest that the CI-ELISA may eliminate the false positive results which occur with current methods (Kay and Graham, 1935; Kleyn and Lin, 1968; Rocco, 1990). For example, if a pasteurized milk sample that tests positive for ALP by the enzyme activity assays is assayed by the CI-ELISA, a positive result for ALP will confirm the presence of bovine milk ALP. A negative result, however, will confirm the absence of milk ALP and suggests the presence of microbial ALP.

Specificity of Antibodies to Selected Milk Proteins and Glycoproteins. The specificity of anti-ALP PAb was also evaluated with selected milk proteins that may interfere in the CI-ELISA. Anti-ALP PAb was not reactive with bovine serum albumin or casein. Whey protein isolate was the only effective competitor among the proteins tested. A concentration of 4.9 μg/mL was required for 50% antibody binding inhibition (Figure 3), indicating 21.2% cross-reactivity (as defined previously) with the anti-ALP PAb. In our earlier work using Western blot analysis, whey protein isolate showed some cross-reactivity with the bovine milk ALP anti-ALP PAb as observed in this study (Vega-Warner et al, 1999). We believe that this cross-reactivity may be due to ALP or other glycoproteins present in the whey protein isolate. Milk contains a variety of glycoproteins which include α_1-acid glycoprotein, M_1-glycoproteins, and M_2-glycoproteins (Whitney, 1988). Milk ALP is also a glycoprotein (Sharma and Ganguli, 1969; Peereboom, 1968). To test the competitiveness of glycoproteins in the CI-ELISA, α_1-acid glycoprotein and ferritin (two milk glycoprotein) and ovalbumin (another common glycoproteins) were tested for reactivity with anti-ALP PAb. Ferritin, ovalbumin, and α_1-acid glycoprotein showed no reactivity with anti-ALP PAb (Figure 3), suggesting that glycoproteins did not interfere with the CI-ELISA, again suggesting that our anti-ALP PAb were specific for milk ALP.

Deglycosylation Studies. Antibodies can recognize a variety of chemical structures including protein and

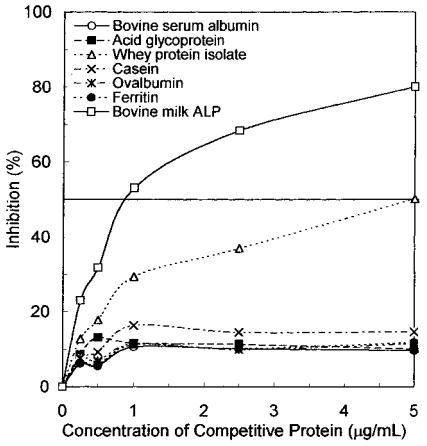

Figure 3. Specificities of milk alkaline phosphatase polyclonal antibodies to milk proteins and glycoproteins as determined by competitive-indirect ELISA. Reference line is used to indicate 50% inhibition. Cross-reactivity is defined as the concentration of alkaline phosphatase (ALP) required for 50% inhibition divided by the concentration of the competitive antigen required for 50% inhibition multiplied by 100. Standard errors of means (n = 4) were 3.38%, bovine serum albumin; 2.17%, acid glycoprotein; 3.67%, whey protein isolate; 3.48%, casein; 2.86, ovalbumin; 2.93, ferritin; 2.94%, milk ALP.

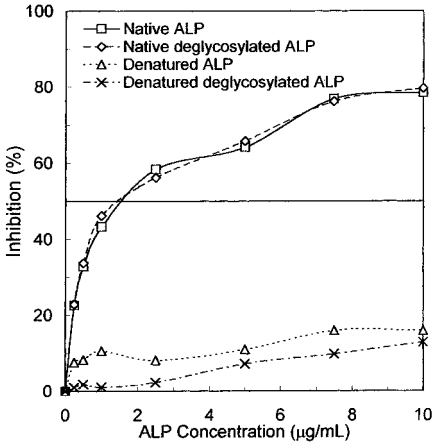

Figure 4. Specificities of milk alkaline phosphatase polyclonal antibodies to native deglycosylated, native heat denatured, and heat denatured deglycosylated alkaline phosphatase (ALP) as determined by competitive-indirect ELISA. Reference line is used to indicate 50% inhibition. Cross-reactivity is defined as the concentration of ALP required for 50% inhibition divided by the concentration of the competitive antigen required for 50% inhibition multiplied by 100. Standard errors of means (n = 4) were 2.53%, native milk ALP; 3.30%, native deglycosylated milk ALP; 1.42%, denatured milk ALP; 1.31%, denatured deglycosylated milk ALP.

sugar moieties on an antigen (Harlow and Lane, 1988). Thus, the affinity of the anti-ALP PAb to the protein or sugar molecules was evaluated by determining competitiveness of native deglycosylated, denatured deglycosylated, and denatured ALP. PAb were highly reactive with deglycosylated native ALP showing 100% cross-reactivity (Figure 4), suggesting that the apoprotein was required for antibody recognition. ALP denatured at 100 °C in 2% SDS or deglycosylated denatured ALP were not effective competitors. Exposure to SDS and high temperature led to complete denaturation and destruction of the epitopes on ALP recognized by anti-ALP PAb. The inability of the anti-ALP PAb to recognize denatured ALP suggests that the CI-ELISA may be a useful tool to differentiate between native and denatured ALP

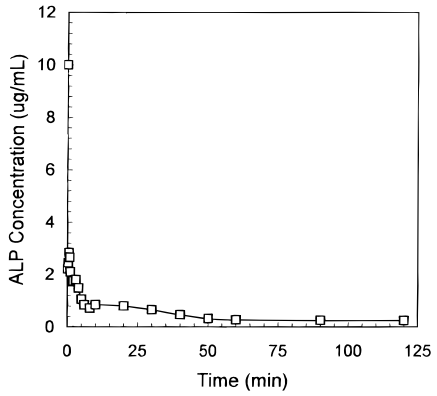

Figure 5. Milk alkaline phosphatase (ALP) quantification with competitive-indirect ELISA after heating at 70 °C for 2 h. ALP was in 50 mM 3-[*N*-morpholino]propanesulfonic acid solution, pH 7.0.

populations, i.e., as would be present in raw and pasteurized milk, respectively.

Effect of Heating on ALP. The ALP concentration significantly decreased ($p < 0.05$) from 10 to 2.2 μg/mL in the time (7 s) it took to heat native ALP solution to 70 °C (Figure 5). ALP concentration decreased ($p < 0.05$) further with an increase in the holding time (Figure 5). About 2.5% of the initial ALP concentration was present after the buffered solutions were heated at 70 °C for 60 min or more. This decrease in protein content as measured by the CI-ELISA is due to the conformational change in the protein epitope due to heating and the inability of the anti-ALP PAb to recognize ALP. Painter and Bradley (1997) concluded that some ALP activity is retained after most pasteurization treatments, which is supported by our results. ALP activity, however, decreased at a faster rate than the epitope denaturation due heating. After the ALP solution reached 70 °C (7-s heating time), ALP activity decreased from 76.25 to 6.70 U/L, only 8.8% of ALP activity remaining. No ALP activity was observed in buffered solutions held at 70 °C for 30 s or more.

Denaturation Studies. To explain the difference between CI-ELISA and activity assays, we next investigated the recognition of native and heat-denatured ALP by the anti-ALP PAb. The anti-ALP PAb were somewhat reactive with heat-denatured ALP (Figure 6). A cross-reactivity of 2.7% was calculated. The heat treatment used in this experiment may not have been sufficient to completely denature ALP, as was observed earlier when ALP solution was heated to 100 °C for 5 min in the presence of 2% SDS. The denatured enzyme may have retained some epitopes, which was recognized by the anti-ALP PAb, thus resulting in this small reactivity with the anti-ALP PAb. This recognition of denatured ALP by anti-ALP PAb may explain why a small quantity of ALP was detected by CI-ELISA after heating at 70 °C for 2 h in the previous experiment (Figure 5).

In summary, the anti-ALP PAb did not react with ALP from different microbial and animal sources. Also, no reactivity of the anti-ALP PAb was observed with various milk proteins and glycoproteins, except for whey protein isolate, which may have residual amounts of ALP. The CI-ELISA developed in this study differentiated between native and heat denatured ALP. However, further studies are warranted to determine if the CI-ELISA may be used to verify proper pasteurization of fluid milk products.

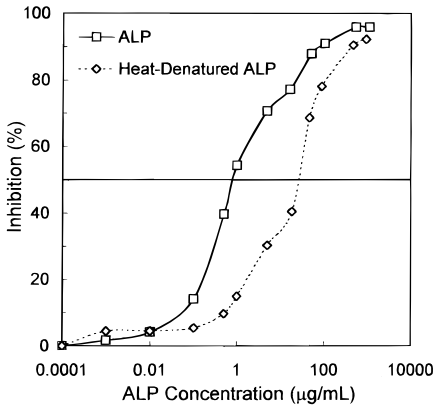

Figure 6. Specificities of bovine milk alkaline phosphatase polyclonal antibodies to native and heat denatured milk alkaline phosphatase (ALP) as determined by competitive-indirect ELISA. Milk ALP was denatured by heating at 72 °C for 2 min. Reference line is used to indicate 50% inhibition. Cross-reactivity is defined as the concentration of ALP required for 50% inhibition divided by the concentration of the competitive antigen required for 50% inhibition multiplied by 100. Standard errors of means ($n = 3$) were 2.06%, milk ALP; 1.21%, heat denatured milk ALP.

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