Effect of Heat Treatment on the Antigen-Binding Activity of Anti-Peroxidase Immunoglobulins in Bovine Colostrum

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ABSTRACT

After intramammary immunization with horseradish peroxidase, bovine colostrum containing anti-peroxidase immunoglobulins (Ig) was obtained. Thermoresistance of the antigen-binding region of these Ig was studied using a direct competitive ELISA. This technique is based on the competition between the anti-peroxidase IgG coated to the plate and the anti-peroxidase IgG contained in the colostrum to bind peroxidase, the antigen, and the enzyme responsible for development of the color of the assay. Thus, the degree of denaturation of the antigen-binding region in the IgG molecule can be determined because this region is directly involved in the assay. The kinetic and thermodynamic parameters for heat-induced denaturation of IgG in colostrum were determined over a temperature range of 69 to 81°C. The denaturation of IgG was best described assuming an apparent reaction order of 1.5. D values, the time required to reduce the antigen-binding activity of IgG by 90%, were 8504, 1387, 285, and 152 s at 69, 72, 77, and 81°C, respectively. Similarly, Z value, the degrees necessary to reduce the D value in one logarithmic cycle, was estimated to be 6.6°C, and the activation energy value was 386.83 kJ/mol. These results should be taken into account in the design of heat treatments of milk in order to preserve the biological function of Ig.

(**Key words**: bovine colostrum, immunoglobulins, heat denaturation, antigen-binding activity)

Abbreviation key: **D value** = the time required to reduce the antigen-binding activity of IgG by 90%, **Z value** = the degrees necessary to reduce the D value in one logarithmic cycle.

INTRODUCTION

Intestinal infections from rotavirus, enteropathogenic and enterotoxigenic *Escherichia coli, Shigella* spp., and *Salmonella* spp. are a predominant cause of illness in infants, often producing high morbidity and mortality of infants in developing countries (3, 5, 19, 24). Human colostrum and milk contain several biologically active molecules, such as lysozyme, lactoferrin, and mainly Ig, which impart a protective effect to the neonate during the first weeks of life when the immunological system is still immature. However, breast feeding is not always possible, and, thus, it has been suggested (19, 25) that infant formulas be fortified with bovine Ig.

The efficacy of orally administered antibodies has been documented in numerous studies involving experimental models as well as clinical trials. The oral administration of Ig from colostrum and milk of immunized cows provides effective protection against enterotoxigenic *E. coli* (18, 24) and *Cryptosporidium* spp. infections (20) of human infants and adults. Moreover, Mitra et al. (19) demonstrated that the ingestion of Ig having neutralizing activity against several serotypes of rotavirus prevents the occurrence of diarrhea and reduces the duration of agent excretion in infants. Therefore, bovine Ig in colostrum and milk have the potential to be utilized as immunological supplements to infant formula and other hyperimmune foods (14).

To preserve the immunological function of Ig, the effects of processing and storage conditions on stability must be known. A substantial proportion of antibodies remains intact in pasteurized milk, powdered milk, and whey (12). However, most antibodies are denatured after severe thermal treatment, as occurs during the manufacture of canned evaporated milk and UHT-sterilized milk (9, 12). These findings indicate that the quantity of Ig in dairy products is dependent on thermal treatment.

The Ig stability to heat treatment has been studied using different experimental techniques, such as chromatographic methods (10, 16, 21), differential scanning calorimetry (13), and immunochemical methods (7, 11, 17). These techniques allowed calculation of the kinetic and thermodynamic parameters for thermal denaturation of these proteins.

The immunochemical methods that are usually employed to study the effect of heat treatment on milk Ig have been based on the reaction between the Ig in

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milk and the antibodies against them (7, 11; G. Mainer, L. Sánchez, J. M. Ena, and M. Calvo, 1997, unpublished data). These methods have determined structural changes caused by heating that occurred in different regions of the Ig molecule where the antibody-binding sites are located. Thus, the degree of denaturation of the whole Ig molecule can be estimated by measuring the loss of immunoreactivity during heating.

Also, other immunological methods have been designed to estimate the antigen-binding activity of specific Ig against bacterial lipopolysaccharides in milks after various heat treatments (11, 15). In those methods, lipopolysaccharides that had been coated to a solid support were incubated with heated milk containing specific Ig and finally with labeled antibodies against Ig. Although denaturation of the antigen-binding region of Ig is a determinant for the antigen-antibody reaction, those methods are indirect because they require incubation with a second antibody, and, thus, denaturation of the whole molecule must be considered also.

In the present study, a direct competitive ELISA, using peroxidase as antigen, has been designed to estimate the effect of heat treatment on the antigen-binding region. This technique presents the additional advantage over methods that had been mentioned earlier as being more direct and accurate to estimate denaturation of that region because only the part of the molecule that interacts with the antigen is involved in the assay. This work could facilitate the design of heat treatments that preserve the structural integrity and biological activity of Ig.

MATERIALS AND METHODS

Immunization

Horseradish peroxidase (Sigma Chemical Company, Poole, United Kingdom) was used as the antigen to immunize pregnant Holstein cows. About 8 mg of peroxidase were dissolved in 2.5 ml of 0.15 *M* NaCl and 0.1 *M* potassium phosphate buffer, pH 7.4, then mixed with 2.5 ml of complete Freund's adjuvant, and injected subcutaneously into the mammary gland 20 d before parturition. Ten days later, cows were injected again with the same amount of peroxidase that had been homogenized with incomplete Freund's adjuvant. Colostrum samples were collected during the 2 d after calving, and the presence of anti-peroxidase antibodies was determined by immunoblot (2).

Colostrum Samples

Colostrum samples from second milking of the 2nd d and containing anti-peroxidase antibodies were skimmed by centrifugation at 2000 × *g* for 15 min at 4°C and stored at –20°C until used.

Heat Treatment

Skimmed colostrum (25 μl) was introduced into glass capillary tubes (1.5 mm o.d., 1.1 mm i.d.) that were sealed with a microflame and checked by immersion in warm water. Colostrum was treated at four different temperatures (69, 72, 77, and 81°C) by immersion of capillaries in a temperature-controlled water bath (± 0.1°C). Heated samples, in duplicate, were removed from the bath at different intervals and immediately cooled by immersion in an ice water bath.

Isolation of Anti-Peroxidase Antibodies

Horseradish peroxidase was insolubilized in Sepharose 4B (Pharmacia, Uppsala, Sweden) that had been previously activated with BrCN (6). Then, 50 ml of colostrum from immunized cows were applied to the immunosorbent, and the column was washed with 0.5 *M* NaCl and 0.05 *M* potassium phosphate buffer, pH 7.4. Retained antibodies were eluted with 0.5 *M* NaCl and 0.1 *M* glycine buffer, pH 2.8, and immediately neutralized with 0.1 *M* Tris·HCl buffer, pH 8.5 (6). The antibodies obtained were about 95% IgG as determined by SDS-PAGE using 4 to 15% acrylamide gradient gels (Pharmacia Biotech, Uppsala, Sweden). Specificity of isolated antibodies was determined by immunodot analyses (2).

ELISA

A direct competitive assay was performed in 96-well polystyrene microtiter ELISA plates (Greiner Labortechnik, Frickenhausen, Germany) that were coated with 300 μl per well of anti-peroxidase IgG (1 μg/ml) in 0.05 *M* sodium carbonate buffer, pH 9.6. After incubation at 4°C overnight, the plates were washed five times with 0.15 *M* NaCl and 0.1 *M* potassium phosphate buffer, pH 7.4, containing 0.05% (wt/vol) Tween 20 (PBS-Tween). Next, 100 μl of unheated colostrum (diluted 1:100 to 1:5000 in PBS-Tween) or colostrum heated at different intervals (diluted 1:100 to 1:2500 in PBS-Tween) plus 100 μl of peroxidase solution (2.5 ng/ml in PBS-Tween) were added to the wells and incubated for 2 h at 37°C. Finally, after samples were washed five times with PBS-Tween, plates were incubated with 200 μl per well of substrate [compound of 0.1% (vol/vol) of 3-3′, 5-5′ tetramethylbenzidine (Sigma Chemical Company) and 0.1% (wt/vol) dimethylsulfoxide dissolved in 0.1 *M* citrate buffer, pH 6, containing 0.05%

(vol/vol) H_2O_2. The reaction was stopped by the addition of 50 μl per well of 2 *M* H_2SO_4, and the optical density of each well was determined at 450 nm using an ELISA plate reader (Labsystem Multiskan, Helsinki, Finland). All samples were assayed (with three dilutions per plate, each dilution by triplicate) in at least two independent experiments.

Calculation of Thermodynamic Parameters

The **D value**, the time required to reduce the antigen-binding activity of IgG by 90%, was determined for each heating temperature and calculated by regression analysis, as the reciprocal of the slope corresponding to the lines obtained from semi-logarithmic plots of IgG concentration as function of the time. The **Z value**, the degrees necessary to reduce the D value in one logarithmic cycle, was calculated by regression analysis from the negative reciprocal slope of the line obtained when the logarithm of D values versus corresponding temperatures were plotted (23).

Calculation of Kinetic Parameters

Order of reaction. The reaction of protein thermal denaturation behaves in a way analogous to a general rate reaction of order n according to the equation

$$-dc/dt = kc^n$$

where –dc/dt = the rate of protein denaturation, k = rate constant, c = protein concentration at each time, and n = order of reaction.

Considering the order of reaction $n \neq 1$, k_n is dependent on the initial protein concentration, and this equation yields

$$(C_t/C_0)^{1-n} = 1 - (n - 1)\ kC_0^{n-1}t$$
$$= 1 - (n - 1)\ k_n t$$

where $k_n = kC_0^{n-1}$, the apparent rate constant, and k = true rate constant. When the experimental points are plotted according to this equation, straight lines are obtained by linear regression from which the apparent rate constant k_n, the coefficient of correlation r, and the value of the ordinate intercept b (time t = 0) are calculated (1).

Kinetic parameters. The apparent activation energy has been calculated from the Arrhenius equation. This value allows the determination of different thermodynamic parameters, such as change in enthalpy of activation, change in entropy of activation, and change in free energy of activation as previously described (23).

RESULTS AND DISCUSSION

An ELISA method has been designed to study the effect of thermal treatment on the antigen-binding activity of IgG. This assay is based on the competition between the anti-peroxidase IgG remaining in heated colostrum samples and the pure anti-peroxidase IgG coating the plate to bind peroxidase, which is simultaneously the antigen and the enzyme responsible to develop the color of the assay. Using this technique, the effect of heat treatment on the specific activity of Ig can be determined because these proteins act as antibodies and not as antigens.

A calibration curve was obtained using the relationship between absorbance and logarithm of the concentration of anti-peroxidase IgG dissolved in PBS-Tween; the relationship was linear within the range 0.1 to 10 μg/ml. The concentration of anti-peroxidase IgG in raw colostrum was determined using this calibration curve and was found to be 0.62 mg/ml. Afterward, a calibration curve was obtained using raw colostrum containing anti-peroxidase IgG, and values of anti-peroxidase IgG remaining in heated colostrum were interpolated from this plot. No effect of the colostrum components was observed at the dilutions employed in this study.

Preliminary experiments were performed to determine an appropriate range of temperatures. No loss in antigen-binding activity of IgG occurred after 60 min of incubation at 65°C, and thus, the temperature range of 69 and 81°C was chosen. Findings were similar for denaturation of the whole molecule on human and bovine IgG after 30 min of incubation at 63°C (8, 12). The range studied in this work includes the times and temperatures used in HTST pasteurization; therefore, the data could be valid for predicting activity of IgG after HTST processing.

The degree of antigen-binding activity of IgG decreases as temperature and holding time increase (Figure 1). The graphs show results of every independent experiment, and mean values were used to calculate all thermodynamic parameters. The D values that were calculated for heating temperatures of 69, 72, 77, and 81°C and the Z value are shown in Table 1.

The D values that were obtained in this experi-

ment are slightly lower than those reported by Li-Chan et al. (11) and G. Mainer, L. Sánchez, J. M. Ena, and M. Calvo (1997, unpublished data) and much lower than those determined by Fukumoto et al. (7) using immunochemical methods based on the reaction between Ig and antibodies against them. Differences among D values are higher for the lower temperatures studied; for the higher temperatures, reported values are quite similar. The relationship between the logarithm of D values and the heating temperatures to determine Z value is shown in Figure 2. The high values reported by Fukumoto et al. (7) have been attributed to the possible inaccuracy from the limited sensitivity of the radial immunodiffusion method that was used to analyze low IgG concentrations. In contrast, the use of the ELISA method has enabled a more accurate determination of decreasing IgG concentration under increasingly severe conditions of heating temperature or time (9, 11). Some of the differences could also be attributed to the thermal treatment. When milk is heated, the heating and cooling times may vary greatly depending on the sample volume and the type of container used to heat the samples. In the present study, 25-μl aliquot portions of the milk samples were heated into glass capillary tubes, but larger volumes (from 100 μl to 2 ml) have been used in other studies (11), resulting in longer heating and cooling times to equilibrate samples. The long heating and cooling times could have led to the inactivation of IgG during the transient temperature stage of the process that could not be accounted for in the calculation of the thermodynamic parameters (1). Also, the design of the assay employed in this work is inherently different from the design of the other studies. As has been previously indicated, the method described here estimates the antigen-binding activity of IgG by measuring the decrease in the amount of IgG that are able to bind peroxidase during heat treatment. This method is possible because only the IgG region responsible for binding the antigen is involved in the assay. Thus, the lower D values in this work than in other studies suggest that the antigen-binding region may be more susceptible to heat denaturation than the whole molecule. These findings agree well with results of studies performed by Roterman et al. (22), who indicated that structural alterations in heated IgG are mainly located in Fab fragments, where the antigen-binding region is located, rather than in the Fc fragment.

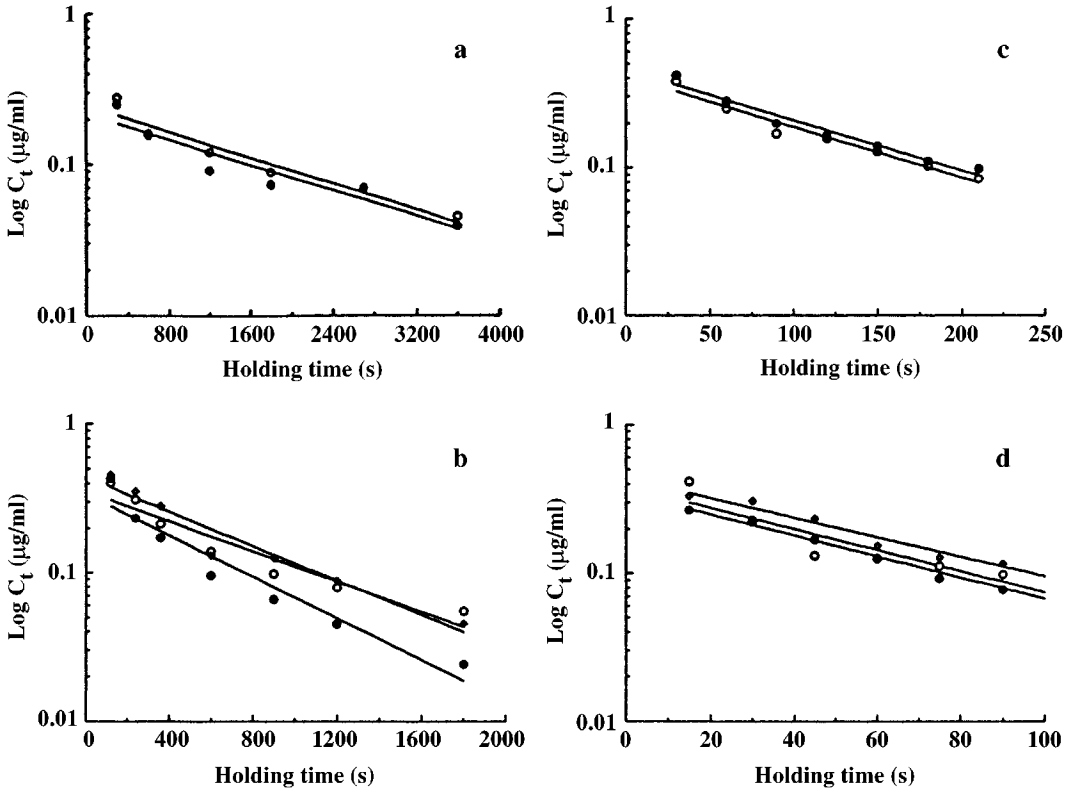

Figure 1. Effect of heat treatment on denaturation of antigen-binding region of IgG in bovine colostrum at 69°C (a), 72°C (b), 77°C (c), and 81°C (d). C_t = Protein concentration at each holding time.

TABLE 1. The D and computed Z value for thermal denaturation of the antigen-binding region of IgG in bovine colostrum at different temperatures.[1]

Temperature	D Value
	(s)
69°C	8504
72°C	1387
77°C	285
81°C	152

[1]D Value = Time required to reduce the antigen-binding activity of IgG by 90%; Z value = degrees necessary (6.6°C) to reduce the D value in one logarithmic cycle.

TABLE 2. Results of the application of the kinetic parameters to the denaturation of the antigen-binding region of IgG in bovine colostrum at different temperatures.[1]

Temperature	k	b	r
	(/s)		
69°C	2.15×10^{-4}	1.04	0.96
72°C	14.48×10^{-4}	1.19	0.96
77°C	96.11×10^{-4}	1.05	0.99
81°C	214.37×10^{-4}	1.08	0.98

[1]k = Rate constant; b = ordinate of the intercept.

The concentration of denatured IgG at each measurement time was subjected to kinetic analysis. Using the general rate equation indicated in the Materials and Methods section, it is possible to reach the overall order of the reaction of the process under investigation. The plot of the concentration ratio $(C_t/C_o)^{1-n}$ versus the reciprocal of the absolute temperature yielded a straight line when n = 1.5. The apparent rate constant k_n could then be obtained from the slope. In addition, the rate constant k, the coefficient of correlation r, and the value of the ordinate intercept b (at time t = 0) were calculated (Table 2).

The high coefficient of correlation and the intercept of the ordinate ~1 indicate that the value of n = 1.5 is suitable at the temperatures studied to obtain a formal mathematical description of the reactions. The reaction order obtained for the thermal denaturation of antigen-binding site of IgG was quite similar to that reported by Law (10) and G. Mainer, L. Sánchez, J. M. Ena, and M. Calvo (1997, unpublished data) for denaturation of the whole IgG molecule. However, an order of reaction of 1 (7, 11) or 2 (16, 21) also has been reported for IgG.

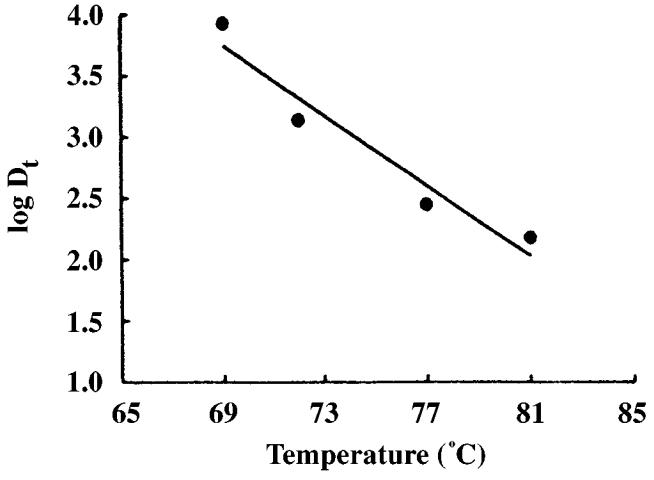

Figure 2. Semi-logarithmic plot of D values, the time required to reduce the antigen-binding activity of IgG by 90%, as a function of temperature of heating for thermal denaturation of antigen-binding region of IgG in bovine colostrum.

Nevertheless, when the natural logarithm of the rate constant was plotted versus the reciprocal of the absolute temperature, the relationship was linear over the temperature range studied, which permits the determination of value for the activation energy. Then, from the value of the activation energy, thermodynamic parameters, such as the change of enthalpy of activation, entropy of activation, and free energy of activation, were calculated. Values of the change in enthalpy of activation and entropy of activation were similar for all temperatures studied: 389.9 and 0.82 kJ/mol, respectively. However, values of the free energy of activation decreased slightly as the heating temperature increased, ranging from 104.31 kJ/mol for 69°C to 94.5 kJ/mol for 81°C. The high values for the activation energy and enthalpy of activation and the positive values of the entropy of activation indicate the existence of a marked change in the conformation and a lower state of order of the antigen-binding site of IgG. Therefore, the values are typical of a reaction mechanism in which the denaturation of the antigen-binding region is the rate-determining process that predominates over an aggregation process. Similar results have been reported (4, 23) for other milk proteins in the range of temperatures studied. In contrast, lower values for activation energy and enthalpy along with a negative entropy value have been observed for some milk proteins at higher temperatures, suggesting the predominance of an aggregation process in which intermolecular bonds are formed, and, therefore, the state of order of the system increases (4). The activation energy estimated in this work, 386.83 kJ/mol, is higher than that reported by others (11, 16) and may indicate that the antigen-binding region of IgG is more stable because a larger amount of energy is needed in order

to start its denaturation. However, the higher values of the rate constant suggest that, once the unfolding of the region has started, denaturation occurs more quickly than for the whole IgG molecule.

Therefore, the ability of IgG to bind the antigen and, thus, to maintain its immunological activity probably has been overestimated until now. In conclusion, these considerations should be taken into account in the design of heat treatment of milk in order to preserve the biological function of Ig when they are added to formula milk or other hyperimmune products.

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