SHORT COMMUNICATIONS

Effect of pH on antigen-binding activity of IgG from bovine colostrum upon heating

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Oral administration of specific antibodies from cows' milk has been found to be effective on humans suffering from intestinal infections caused by *Escherichia coli* (Mietens *et al*. 1979), *Shigella flexneri* (Tacket *et al*. 1992) and rotavirus (Mitra *et al*. 1995). Furthermore, antibodies obtained from sera of a variety of immunized mammals are valuable tools for immunochemical analysis employed for diagnostic purposes. Thus, the potential use of antibodies as a supplement for special food products or as a reagent for analytical techniques has encouraged the study of their physico-chemical and molecular properties.

Bovine colostrum, milk and whey are considered important sources for isolating immunoglobulin to prepare hyper-immune products (Larson, 1992). For practical usage of immunoglobulins as supplements to foods, their stability under different food processing and storage conditions should be determined. Several reports have been published on the resistance of bovine immunoglobulins to homogenization, heat and ultrasonic treatments (Fukumoto *et al*. 1994; Li-Chan *et al*. 1995; Domínguez *et al*. 1997; Mainer *et al*. 1997). Furthermore, various studies have been also performed to determine the effect of different conditions such as pH and medium composition, or the effect of protective agents like sugars or glycerol on the stability of immunoglobulins (Otani *et al*. 1988; Calmettes *et al*. 1991; Chen & Chang, 1998). Heat treatments are extensively used to preserve foods and the knowledge of the effects on the components is essential. Immunoglobulins are one of the most heat-stable whey proteins, probably due to the high content of intramolecular disulphide bridges (Lindström *et al*. 1994). Thermal resistance depends on the immunoglobulin class, showing higher stability in decreasing order for IgG, IgA and IgM (Mainer *et al*. 1997).

Immunochemical methods commonly used to study the effect of heat treatment on immunoglobulins are based on the reaction between immunoglobulins and the

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antibodies against them (Li-Chan *et al.* 1995; Mainer *et al.* 1997; Chen & Chang, 1998). These methods estimate the degree of denaturation of the whole immunoglobulin molecule by measuring the loss of immunoreactivity during heating. Other immunological methods have been designed to determine the effect of heat treatment on the antigen-binding region. These methods are based on the loss of the ability of immunoglobulins to bind specific antigens (Domínguez *et al.* 1997; Mainer *et al.* 1999). Using both approaches, *D* values obtained for denaturation of IgG have shown considerable differences indicating that susceptibility of immunoglobulins to heating is different when considering the whole molecule or the antigen-binding region.

Bovine IgG present in milk have been found to resist treatments of low temperature-long time (LTLT) and high temperature-short time (HTST) pasteurization (Li-Chan *et al.* 1995; Domínguez *et al.* 1997; Mainer *et al.* 1997). However, complete loss of IgG activity takes place after high thermal treatments as in canned evaporated milk and sterilized milk (Kummer *et al.* 1992; Li-Chan *et al.* 1995). The pH and medium composition also markedly influences the stability of immunoglobulins. Thus, IgG incubated at 37 °C were found to be stable under neutral pH for several hours, but its reactivity declined gradually with decreasing or increasing pH values especially for pH below 4 or above 10 (Otani *et al.* 1991; Shimizu *et al.* 1993; Chen & Chang, 1998). When immunoglobulins are heat treated in colostrum, they are more stable than in whey or PBS (Li-Chan *et al.* 1995; Chen & Chang, 1998).

Although a wealth of information about the effect of heat treatment or pH on the stability of IgG has been accumulated, the effect of the two factors combined remains unclear. The aim of this study was to determine the effect of pH on the antigen-binding activity of IgG upon heating. Kinetic parameters were also calculated for the denaturation process which enabled prediction of behaviour in the range of temperatures used in common pasteurization treatments and to select the best treatment in order to preserve biological function.

Materials and methods

Production of anti-peroxidase colostrum

Five pregnant Holstein cows were immunized with 12 mg of horseradish peroxidase (Sigma, Poole, BH12 4QH, UK) dissolved in 3 ml of phosphate-buffered saline (PBS; 10 mM-sodium phosphate, 136·7 mM-NaCl, 2·68 mM-KCl buffer, pH 7·4) as described by Domínguez *et al.* (1997). The immunogen was injected subcutaneously into different areas of the back 30 and 15 days before calving. Colostrum samples were collected during two days after parturition, pooled and stored at −20 °C until used. The presence of anti-peroxidase antibodies was determined by immunodotting (Domínguez *et al.* 1997).

Preparation of colostral immunoglobulins

The anti-peroxidase colostrum was skimmed by centrifugation and caseins precipitated by addition of chymosin (E.C. 34.23.4). Whey was obtained by centrifugation at 2000 ***g*** for 20 min. at 4 °C. Then, a volume of 500 ml of whey was mixed with $(NH_4)_2SO_4$ to obtain a final saturation of 400 mg/ml and incubated for 30 min. at 4 °C. The mixture was centrifuged at 10000 ***g*** for 20 min. at 4 °C and the

pellet was dissolved and dialysed against PBS. The fraction obtained was analysed by SDS-PAGE finding that 80% of the proteins were of IgG class. Aliquots of the immunoglobulin solution were brought to pH 4·5, 5·5 and 6·5 by adding 100 mM-citric acid buffer, pH 4·1, 100 mM-citric acid buffer, pH 5·1 and 100 mM-potassium phosphate buffer, pH 6·1, respectively. The final concentration of anti-peroxidase immunoglobulin solutions at different pHs was adjusted to 0·8 mg/ml.

SDS-polyacrylamide gel electrophoresis (*SDS-PAGE*)

Electrophoresis was performed on 4 to 15% acrylamide gradient gels using a Phastsystem (Pharmacia, Uppsala, 75184, Sweden). Samples were boiled for 5 min in 10 mM-Tris-HCl buffer, pH 8·0 containing 1 mM EDTA and 25 mg SDS/ml. A molecular weight marker was included in all gels. The gels were stained with Coomassie Blue and the optical density of the electrophoresis bands determined by laser densitometry.

Heat treatment of anti-peroxidase IgG

A volume of 25 µl of anti-peroxidase IgG solutions at different pHs was introduced into glass capillary tubes (1·5 mm outer diameter, 1·1 mm inner diameter) that were sealed with a micro flame. Samples were heated at 70, 72 and 77 °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 times of heating and, immediately cooled by immersion in an ice-water bath.

Enzyme-linked immunossorbent assay (*ELISA*)

A direct competitive assay was performed according to Domínguez *et al.* (1997). Previously, anti-peroxidase antibodies for coating plates were isolated from colostrum by immunoadsorption using peroxidase immobilised in Sepharose 4B (Fuchs & Sela, 1986). Anti-peroxidase antibodies obtained were about 95% IgG as determined by SDS-PAGE. Plates were coated with 300 µl of pure anti-peroxidase IgG (1 µg/ml) dissolved in 50 mM-sodium carbonate buffer, pH 9·6, by incubation at 4 °C overnight. After washing five times with PBS containing 0·5 g Tween 20/l (PBS-Tween), a volume of 100 µl of pure anti-peroxidase IgG (0·1 to 10 µg/ml in PBS-Tween) or anti-peroxidase IgG solutions heated at different intervals (diluted 1/50 to 1/2500 in PBS-Tween) plus an equal volume of peroxidase solution (5 ng/ml) were added to wells and incubated for 2 h at 37 °C. Finally, after washing, peroxidase was developed with a substrate based on tetramethylbenzidine and the optical density determined at 450 nm using an ELISA plate reader (Labsystem Multiskan, Helsinki, 00881, Finland). A calibration curve was made by plotting absorbance versus the logarithm of the concentration of pure anti-peroxidase IgG, being linear within the range 0·1 to 10 µg/ml. Values of anti-peroxidase IgG concentration in the heated samples were calculated by interpolation of the absorbance values obtained for heat treated samples in the calibration plot. All samples were assayed (with three dilutions per plate, each dilution by triplicate) in at least two independent experiments.

Calculation of kinetic and thermodynamic parameters

D value, the time required to reduce the antigen-binding activity of IgG by 90%, was determined for each temperature and pH, as the reciprocal of the slope corresponding to the lines obtained from semi-logarithmic plots of IgG concentration

as a function of time. z value, the deg C necessary to reduce the D value by one logarithmic cycle, was calculated from the slope of the line obtained plotting the logarithm of D values v the corresponding temperatures.

The order of reaction and the rate constants for the denaturation of anti-peroxidase IgG were calculated as previously described by Mainer *et al.* (1997). From the rate constants, the apparent activation energy was estimated, this value being used for the calculation of different thermodynamic parameters, such as change in enthalpy, entropy and free energy of activation (Mainer *et al.* 1997).

RESULTS

The effect of heat treatment on the antigen-binding activity of IgG at different pH was determined using a direct competitive ELISA. Preliminary experiments were performed to determine an appropriate range of temperatures and pH. IgG solutions at pH 4·0 heated at 72 °C completely lost the antigen-binding activity after 5 s of treatment. This time was too short to take samples for a kinetic study. Therefore, a pH range between 4·5 and 6·5 and a temperature range between 70 and 77 °C were chosen.

In order to determine if a low pH may affect the antigen-binding activity of IgG during refrigeration, IgG solutions at pH 4·5 were incubated at 4 °C for 24 h. No changes in the activity of IgG were observed. Shimizu *et al.* (1993) found no loss of activity for pig, goat and cows' IgG incubated at 37 °C and pH values above 4.

The antigen-binding activity of IgG decreased as heating time increased as shown in Fig. 1*a* for a heating experiment at 72 °C and pH 6·5. As can be seen, there were two time ranges in which the rate of reactivity loss was markedly different, being higher at the beginning of the heat treatment. To determine if aggregates are formed during the heat treatment of IgG, samples treated at different temperatures and heating times were taken and analysed by SDS-PAGE. In the case of 72 °C and pH 6·5 results showed the presence of IgG aggregates, which did not enter the gel, in the samples treated over 240 s, which corresponds with the break in the curve, but not before. This behaviour was also observed at all temperatures and pH studied. In this work, we chose to focus on the initial rates and therefore, thermodynamic and kinetic parameters at each temperature and pH studied were calculated considering only data obtained at heating times before the break of the curves.

Semi-logarithmic plots of the concentration of IgG with antigen-binding activity as a function of heating time at 70, 72 and 77 °C and at pH 4·5, 5·5 and 6·5 are shown in Fig. 1*b*, *c* and *d* respectively. The graphs show the results of one independent heating experiment, while mean values of at least two heating experiments were used to calculate all thermodynamic parameters. D values for IgG calculated at each heating temperature and pH, and z values calculated at each pH are shown in Table 1. D values decreased with increasing temperature and decreasing pH, being particularly low at pH 4·5.

The concentration of active anti-peroxidase IgG at each heating time was subjected to reaction kinetic analysis. An order of reaction $n = 1{\cdot}5$ was assumed as we found high coefficients of correlation (ranging from 0·89 to 0·95.) and ordinate intercepts close to 1 (ranging from 1·03 to 1·28) when representing values obtained for the denaturation process following this order.

Values of activation energy decreased markedly with pH (Table 1). Thermo-

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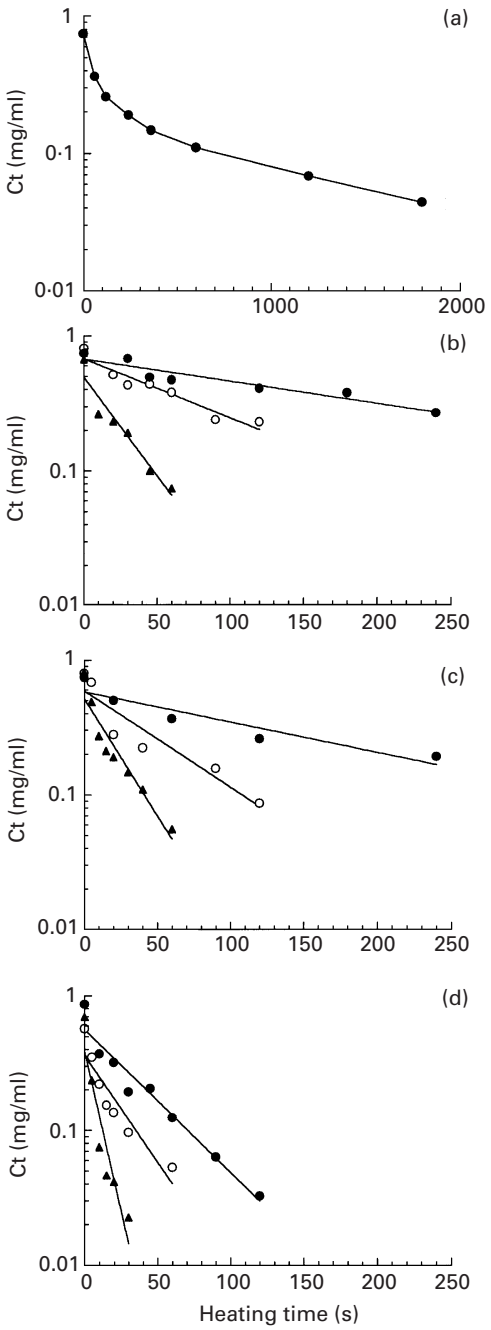

Fig. 1. Effect of heat treatment at 72 °C (*a*, *c*), 70 °C (*b*) and 77 °C (*d*) on denaturation of antigen-binding region of bovine IgG. Ct is the concentration of anti-peroxidase IgG determined by ELISA at each heating time. pH 6·5 (●), 5·5 (○) and 4·5 (▲).

dynamic parameters, calculated from the activation energy are shown in Table 2. For a given pH, values of the change in enthalpy of activation and the entropy of activation were similar for all temperatures studied. However, those values were lower as pH decreased at any treatment temperature.

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Table 1. D, *z and* E_a *values for thermal denaturation of the antigen-binding region of bovine IgG at different temperatures and pH*

(Values are means of at least two determinations)

	D, s				
pH	70 °C	72 °C	77 °C	z, deg C	E_a, KJ/mol
6·5	423±9	248±9	90±1	10·6	315·78
5·5	216±9	163±5	54±6	11·3	230·35
4·5	65±1	43±2	19±3	13·2	204·67

D = time required to reduce the antigen-binding activity of IgG by 90 %; z = deg C necessary to reduce the *D* value by one logarithmic cycle; E_a = energy of activation.

Table 2. *Results of the application of the kinetic parameters to the denaturation of the antigen-binding region of bovine IgG at different temperatures and pH for n = 1·5*

Temperature	pH	$k \times 10^{-3}$, s^{-1}	$\Delta H^{\#}$, KJ/mol	$\Delta S^{\#}$, KJ/mol	$\Delta G^{\#}$, KJ/mol
	6·5	3·17	312·70	0·627	97·64
70 °C	5·5	7·96	227·49	0·385	95·43
	4·5	40·92	201·59	0·323	90·80
	6·5	6·64	312·91	0·627	96·95
72 °C	5·5	14·76	227·48	0·385	94·65
	4·5	59·38	201·57	0·323	90·13
	6·5	29·81	312·87	0·627	93·42
77 °C	5·5	41·56	227·44	0·385	92·69
	4·5	169·95	201·53	0·323	88·48

k = rate constant; $\Delta H^{\#}$ = change in enthalpy of activation; $\Delta S^{\#}$ = change in entropy of activation; $\Delta G^{\#}$ = change in free energy of activation.

DISCUSSION

Protein aggregates were found in solutions of bovine immunoglobulins heated at temperatures above 60 °C (Lindström *et al.* 1994) and 65 °C (Shimazaki, 1990) for times over 20 min. In the present study, prolonged heating (greater than 240 s at pH 6·5) also caused protein aggregation. *D* and z values were obtained from the initial linear portion of the time-dependent denaturation curve.

The increased susceptiblity of IgG to heat denaturation at acid pH, specially at pH values close to 4·5 may be due to the conformational change produced as a result of variation in the charge on the protein surface (Shimizu *et al.* 1993; Lindström *et al.* 1994), which finally results in the denaturation of IgG (Calmettes *et al.* 1991; Chen & Chang, 1998).

The heating conditions employed in this work includes temperatures and times used in HTST pasteurization. From the results it is possible to predict that HTST treatment at 72 °C for 15 s would denature about 5 %, 8 % and 31 % of IgG in PBS at pH 6·5, 5·5 and 4·5, respectively.

D values determined in this work are lower than those reported for IgG in PBS by Li-Chan *et al.* (1995) and Chen & Chang (1998), this may be partly due to differences in the immunological technique used. Techniques used by these authors are based on the ability of immunoglobulins to react with specific antibodies. In this work, the ELISA technique used is based on the competition for binding peroxidase,

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between the anti-peroxidase IgG coating the plate and the anti-peroxidase IgG which remain reactive in heated samples. Thus, peroxidase is simultaneously the antigen and the enzyme responsible for the development of the colour of the assay. Besides, as this study was performed using polyclonal antibodies directed against different antigenic determinants of the antigen and obtained from different animals, it could be considered that the antiserum employed contains an heterogeneous population of IgG with different antigen-binding regions. Therefore, results obtained in this work for IgG against a specific antigen could be extended to the behaviour of IgG directed against other antigens. Using this technique, we estimated the degree of denaturation of the antigen-binding region in the IgG molecule because only this region is involved in the assay.

Differences in the results obtained in this work and those reported by Li-Chan *et al.* (1995) and Chen & Chang (1998) indicate that IgG subjected to heat denaturation seem to lose the ability to bind to the antigens (biological activity), earlier than their ability to elicit response from their specific antibodies. Similar findings have been reported for the thermal treatment of IgG in colostrum or milk using these two approaches. *D* values based on the loss of the virus neutralizing activity (Mainer *et al.* 1999) or on the loss of the antigen-binding activity (Domínguez *et al.* 1997) are lower than those based on the loss of immunoreactivity with antibodies (Fukumoto *et al.* 1994; Li-Chan *et al.* 1995; Mainer *et al.* 1997). These results are supported by the studies performed by Rizzo *et al.* (1992) who found that Fab fragment, where the antigen-binding region is located, is structurally altered earlier in the IgG molecule. Likewise, Rotterman *et al.* (1994) reported that CH1 region of Fab fragment is the first to denature when IgG are subjected to heat treatment.

On the other hand, heating experiments performed with IgG in colostrum (Domínguez *et al.*, 1997) have shown that the loss of the antigen-binding activity of these proteins is linear and *D* values greater than those determined in this work for IgG in PBS at similar pH (6·5). This difference has suggested a protective effect of other milk components such as proteins and salts that cause a delay in denaturation (Linström *et al.* 1994; Li-Chan *et al.* 1995; Chen & Chang, 1998) and prevent aggregation of IgG during heat treatment (Xiong, 1992; Lindström *et al.* 1994). However, further experiments should be performed to clarify this point since few data points were taken early in the heat treatments studies of IgG in colostrum (Domínguez *et al.* 1997).

The apparent reaction order (1·5) of denaturation of anti-peroxidase IgG is similar to that reported for IgG in milk (Law,1995; Domínguez *et al.* 1997; Mainer *et al.* 1997), although an order of reaction 1 (Fukumoto *et al.* 1994; Li-Chan *et al.* 1995) and 2 (Lucisano *et al.* 1994) has been also indicated for IgG.

Changes in activation energy suggested that the antigen-binding region was less stable at acid pH where less energy was needed for denaturation. Higher denaturation rate constants indicate that acid pH favours the unfolding of that region. Furthermore, the high values of the energy of activation and the enthalpy of activation as well as the positive value of the entropy of activation suggest that a large change in protein conformation and extensive protein unfolding predominates over aggregation processes during the initial heating of IgG. Similar behaviour has been reported for other milk proteins in the range of temperatures studied in this work (Dannenberg & Kessler, 1988; Sánchez *et al.* 1992).

These results should be considered when selecting heat treatments of acidified milk or milk products in order to preserve the antigen-binding activity of IgG and thus its biological function when they are used in supplemented foods.

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