

Microwave irradiation under different pH conditions induced a decrease in β -lactoglobulin antigenicity

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Abstract Whey proteins adjusted at pH values 2, 4.6, 9 and at the natural milk pH (pH 6.8) were subjected to microwave irradiation at 300 W for 20 min or 700 W for 10 min. The protein composition of treated and native whey proteins were evaluated by Lowry's method and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The ability of treated whey to bind IgG polyclonal antibody was determined by an enzyme-linked immunosorbent assay (ELISA) using sera obtained from rabbit immunized to β -Lactoglobulin (β -Lg). Significantly higher losses in soluble protein concentrations were observed for microwave irradiated whey proteins at pH value 4.6 (35.6% at 300 W and 44.33% at 700 W) ($P < 0.0001$) compared with those irradiated at the natural milk pH (26% at 300 or 700 W). The electrophoretic patterns of these proteins revealed a considerable decrease in intensity of the band corresponding to α -lactalbumin but only a slight modification was observed for the electrophoretic profiles of β -lactoglobulin. The data obtained with a rabbit anti- β -lactoglobulin immunoglobulin indicated a low antigenic response for microwave-irradiated whey proteins at the natural milk pH (up to 29.32% as well as 300–700 W) ($P < 0.001$). The lowest antigenicity was observed for samples adjusted to pH 4.6 followed by microwave irradiation at 300 or 700 W (46.99% at 300 W and 41.16% at 700 W) ($P < 0.0001$).

Keywords Cow's milk · β -Lactoglobulin · Antigenicity · pH · Microwave irradiation · Rabbit IgG

Introduction

Several processing technologies have been designed to modify the allergenic properties of β -Lactoglobulin (β -Lg) [1]; powerful inducer of allergy in early infancy [2, 3]. However, this protein exhibits a significant stability. The relative resistance of β -Lg is highly related to its rigid spatial conformation which is stabilized by various hydrogen and disulfide bonds [4].

The effects of technological processes, especially those involving microwaves on the structural properties of whey proteins have been widely studied [5–9]. Microwave heating is different from that of the conventional heating. It is accomplished both by the absorption of the microwave energy by rotation of the dipolar water molecules and by the translation of the ionic components of food. Microwaves are shown to affect the kinetics of conformational changes of whey proteins and to accelerate their denaturation, these effects are non-thermal [5, 6, 9]. Changes in the native structure of proteins could have a profound influence on their sensitizing properties. Using rabbit polyclonal antibodies, it was found that microwave-irradiated whey proteins (β -Lg and α -La) lost a part of their IgG-binding ability, but their antigenic activities were not entirely abolished [10]. Indeed, microwave irradiation on its own is very unlikely to be sufficient to substantially reduce or entirely eliminate the antigenicity of β -Lg and should, therefore, be combined with other processes, such as pH.

It is well established that the conformation of whey proteins especially β -Lg is highly influenced by variations of pH [11–13]. Between pH 2 and 8, β -Lg exhibits a number of pH-induced structural transitions as well as changes in the association state and stability [14]. Therefore, we hypothesize that the combination of microwave treatment and pH may enhance the reduction of antigenicity. This

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study is conducted to evaluate the antigenicity of cow's milk β -Lg subjected to microwave irradiation under basic and acidic pH conditions.

Materials and methods

β -Lactoglobulin, bovine serum albumin, α -lactalbumin, acrylamide, bis-acrylamide, glycerol, glycine, TEMED (N,N,N',N'-tetramethylethylenediamine), β -mercaptoethanol, methanol, complete and incomplete Freund's adjuvant, goat anti-rabbit IgG peroxidase conjugate, OPD (diamine-orthophenylene), Tween 20, monobasic potassium phosphate and hydrogen peroxide were purchased from Sigma (France). Coomassie Blue R250, bromophenol blue, Tris, SDS (sodium dodecyl sulfate), acetic acid, ammonium persulfate, sodium citrate, di-sodium hydrogenophosphate, casein, sulfuric acid (H_2SO_4), copper sulfate, tartrate of potassium and sodium, sodium carbonate and sodium bicarbonate were obtained from Merck (France). Sodium chloride, Folin's reagent and potassium chloride were purchased from Prolabo (France). All materials and instruments of SDS-PAGE electrophoresis were purchased from Bio-Rad. Materials and instruments from Labsystem and microtiter plate (Maxisorp; Nunc, Roskilde, Denmark) were used for ELISA.

New Zealand female rabbits were purchased from Centre d'Élevage du Petit Bétail de Sidi Bel Abbès. The rabbits were maintained and fed in light-cycled animal rooms.

Sample preparation

Fresh cow's milk was obtained from a local dairy farm. Whey was prepared by the precipitation of caseinates at pH 4.6 with 0.1 N HCl of skimmed milk. Caseins were removed by centrifugation at 3,500 rpm for 15 min. Supernatant (whey), re-adjusted to the natural milk pH (pH 6.8) with 0.1 N NaOH, was dialyzed overnight against running water in order to remove the lactose and mineral salts.

Adjustment of pH and microwave irradiation

Dialyzed whey (pH 6.8) was split into four batches. The first three batches were adjusted to pH values 2, 4.6 and 9 by the slow addition of either 0.1 N HCl or 0.1 N NaOH to well still-stirred solutions. The fourth batch was taken at the natural milk pH (pH 6.8). Whey samples were allowed to equilibrate for approximately 3 h at ambient temperature. Samples of whey at each pH were then exposed to microwave irradiation for 20 min at the power of 300 W and 10 min at 700 W using 2,450 Mhz Whirlpool oven and final temperatures reached were 95 and 102 °C, respectively. Following the irradiation, samples were cooled immedi-

ately in ice then freeze-dried. Non-treated whey samples (pH 6.8) served as controls.

Protein solubility

After pH adjustment and microwave irradiation, the samples were centrifuged at 10,000g for 10 min. The proteins content of the supernatants was determined using Lowry's method measuring absorbance at 750 nm [15]. Bovine serum albumin was used as a standard.

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

SDS-PAGE of whey proteins samples was performed under the denaturing conditions described by Laemmli [16]. Proteins were separated in an acrylamide gel (12.5%) and stained with Coomassie Blue R250.

Production of polyclonal antibodies (IgG anti β -Lg)

Polyclonal antibodies were obtained through subcutaneous immunization of six New Zealand female rabbits applying the immunization protocol according to Walker et al. [17]. In this procedure, 1 ml of blood from non-injected rabbits was collected by the ear-bleeding method for the serum blank. Rabbits were immunized by injecting on their shaved back 2 mg of β -Lg dissolved in 0.5 ml of physiologic serum and emulsified with 0.5 ml of Freund's complete adjuvant. The booster immunization with 1 mg of β -Lg in an incomplete Freund's adjuvant was given on days 7, 20 and 30. After 1 week, blood was collected by heart puncture and each antiserum was separated by centrifugation at 3,000 rpm for 10 min at 4 °C. All sera were kept frozen at -20 °C.

ELISA

A multi-well microtiter plate was coated with 100 μ l of treated whey (10 μ g/ml) and incubated overnight at 4 °C. The plates were then washed with phosphate buffered saline containing 0.05% Tween 20, pH 7.4. Residual-free binding sites were blocked with 200 μ l per well of 3% BSA PBS for 1 h at 37 °C. After washing, 100 μ l of serially diluted sera ($1/10^2$ – $1/10^7$) was added in duplicate to each well and incubated at 37 °C for 2 h. The plates were again washed and 100 μ l of goat anti-rabbit IgG peroxidase conjugate (1:3,000) was added to the wells and the plates were left for 1 h 30 min at 37 °C. After further washing, peroxidase activity was assayed by the addition of 50 μ l H_2O_2 (30%, 0.25 ml/l) associated with diamine-orthophenylene (0.5 mg/ml) in 0.05 M citrate buffer, pH 5.1. The stained wells were kept in the dark at room temperature for 30 min. The reaction was stopped with 6 N H_2SO_4 (50 μ l per well)

after which absorbance was measured at 492 nm with an automated ELISA reader (ELx 800). Positive titers were given as the last dilution of optical density, which was above the background.

Statistical analysis

Experiments were repeated six times and the results were expressed as mean $\pm$ standard error (SE). Statistical data analysis was performed using Student's *t* test with $P < 0.05$ as the minimal level of significance.

Results and discussion

Solubility of treated whey proteins

Figure 1a shows the effect of pH on the soluble whey protein concentrations before irradiation (non-irradiated samples). At acid and alkaline pH, the proteins content of

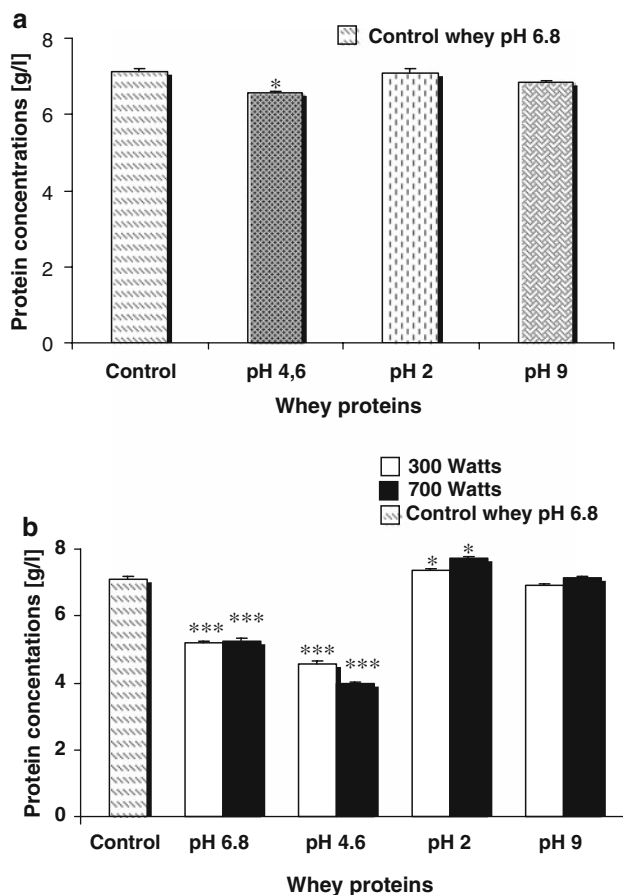


Fig. 1 Protein concentrations of whey proteins at different pH conditions before (a) and after microwave irradiation (b). Values are given as mean $\pm$ SE (standard error) from six independent measurements (* $P < 0.01$, ** $P < 0.001$ and *** $P < 0.0001$)

supernatants before microwave treatment were not significantly different from the control whey (at neutral pH). However, at pH 4.6 a slight, but significant decreased in soluble protein concentration was seen for 7.47% ($P < 0.01$).

As shown in Fig. 1b, microwave irradiation applied at 300 and 700 W of whey proteins at the natural milk pH induced a significant decrease ($P < 0.0001$) in the soluble protein content for 26.79 and 26.06%, respectively. At this pH, the secondary and tertiary structures of heated proteins are unfolded and the hydrophobic groups interact and reduce water binding [18]. The hydrophobic interactions lead to aggregation, followed by coagulation and precipitation [19]. Soluble protein concentration was also dramatically reduced after microwave irradiation of whey proteins at pH value 4.6 ($P < 0.0001$) for 35.6% at 300 W and for 44.33% at 700 W. This decrease in protein solubility at pH values close to the isoelectric point of β -Lg (pH 5.2; [20]) can be attributed to a reduction in the electrostatic repulsion between the protein molecules [21, 22] and agrees with the fact that the aggregation of heated whey proteins is pH-dependent [23]. Whey proteins were more soluble when the microwave irradiation was conducted at pH 2 ($P < 0.01$) confirming the high solubility of proteins at low pH (acidic) [24]. Similar results were observed with whey proteins heated conventionally suggesting that β -Lg dimer, the most abundant protein present in whey, dissociated to monomers below pH 3.5 [25].

SDS-PAGE analysis of whey proteins samples

Figure 2a shows SDS electrophoresis of whey proteins (pH 6.8) after microwave irradiation at 300 and 700 W. No difference was observed between these profiles and those obtained with untreated whey. The same results were obtained in case of samples irradiated either at pH 2 (Fig. 3a) or at pH 9 (Fig. 3b). In contrast, for whey adjusted at pH 4.6, the intensity of the band corresponding to α -La decreased considerably after microwave irradiation at 300 and 700 W (Fig. 2b). This can be explained by the appearance of a molten globule structure of this protein at acid pH, which influences its stability [23].

Antigenicity of treated whey proteins against IgG anti β -Lg

The antigenicity of treated whey proteins expressed as a percentage is illustrated in Fig. 3. Our results show that the adjustment of pH of whey had no significant effect on β -Lg antigenicity (Fig. 3a) confirming the robustness of the protein structure [26, 27]. However, a significant decrease in immunoreactivity ($P < 0.001$), almost 29.32% in comparison with untreated control sample was observed for whey proteins (pH 6.8) exposed to microwave irradiation at 300

Fig. 2 SDS PAGE of whey proteins at different pH values before and after microwave irradiation at the natural milk pH (a), at pH 4.6 (b), at pH 2 (c) and at pH 9 (d). Individual lanes show the microwave irradiation powers (W) at pH 6.8, 4.6, 2 or pH 9 received for each sample. Kit: Molecular weight standard: bovine serum albumin (BSA, 68,000 Da), casein (Cas 24,000 Da), β -Lactoglobulin (β -Lg, 18,000 Da), and α -Lactalbumin (α -La, 14,000 Da)

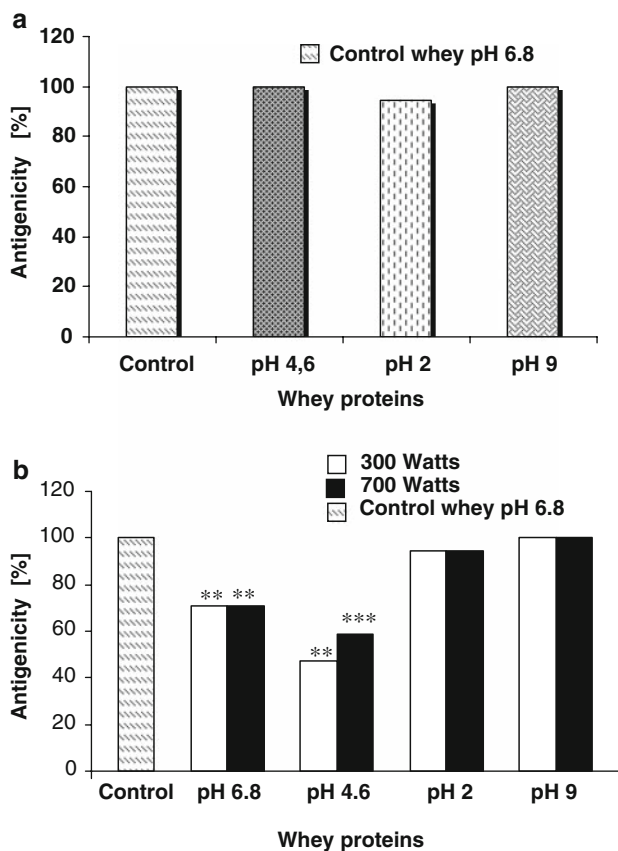
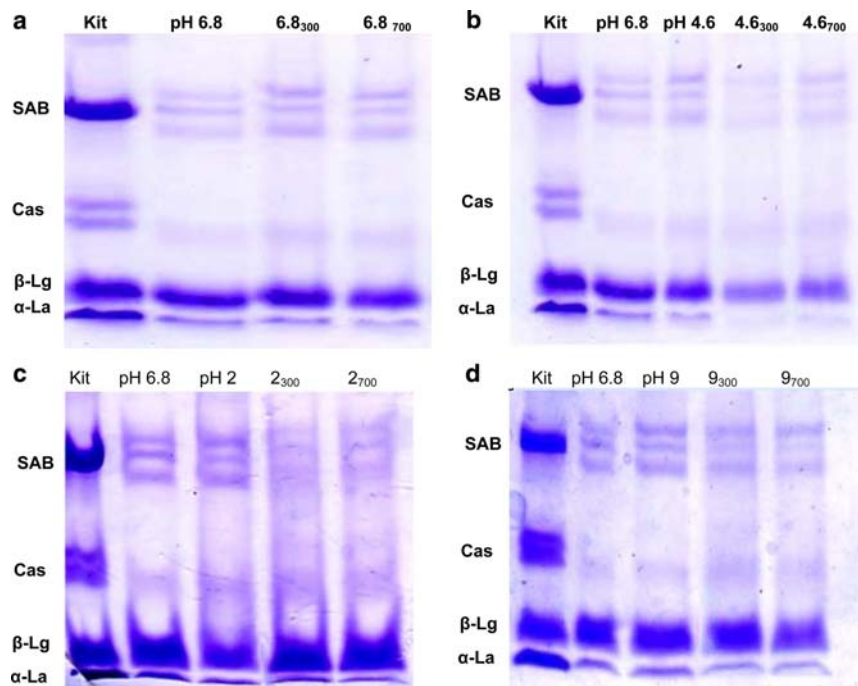


Fig. 3 Antigenicity of whey proteins at different pH values before (a) and after microwave irradiation (b) as determined by ELISA. Values are given as mean $\pm$ SE (standard error) from six independent measurements (** $P < 0.001$ and *** $P < 0.0001$)

and 700 W for 20 and 10 min, respectively. The lowest immunoreactivity was observed for samples adjusted to pH 4.6 followed by microwave irradiation at 300 or 700 W (46.99% at 300 W and 41.16% at 700 W) ($P < 0.0001$) (Fig. 3b). This reduction in antigenic response of β -Lg may be explained by different phenomena. There is evidence that microwave irradiation speeds up rates of folding and unfolding for β -Lg [5] and that conformational changes of this protein is highly dependent on pH [11–13]. This could lead to the loss of tertiary protein structures that play an important role in maintaining conformational epitopes [28] and, therefore, leads to a decreased antigenic potential.

Conclusions

The impact of microwave irradiation of whey proteins under different pH conditions on the β -Lg protein was examined by Lowry's method and SDS-PAGE. A significant decrease in soluble protein concentration was seen after microwave irradiation (at 300 or 700 W) of whey proteins at the natural milk pH (pH 6.8). A more pronounced decrease was found at pH value 4.6. At this pH value, the electrophoretic profiles of whey proteins revealed a considerable attenuation of the band corresponding to α -La. Changes in the immunoreactive properties of treated whey proteins were determined by ELISA with the use of anti β -Lg rabbit IgG. Subjecting whey proteins at the natural milk pH (pH 6.8) to microwave irradiation has been shown to decrease the antigenic properties of β -Lg (up to 29.32% as

well as 300–700 W). At pH value 4.6, microwave irradiation of whey proteins resulted in a severe loss of the immunoreactivity of β -Lg (up to 46.99% at 300 W and 41.16% at 700 W).

The effect of microwave irradiation under different pH conditions which might decrease antigenicity of β -Lg has been discussed in this work, but it remains unknown whether this treatment is capable of reducing the β -Lg allergenicity. Thus, more studies are needed to fully understand the effect of this treatment on the allergenic properties of β -Lg.

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