



Specific and sensitive ELISA for measurement of IgE-binding variations of milk allergen β -lactoglobulin in processed foods

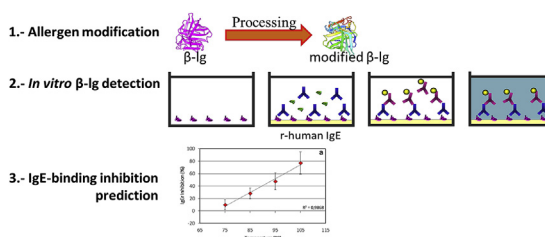
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HIGHLIGHTS

- A standardisable method for measurement of β -lg immunoreactivity changes has been developed.
- LOD for β -lg detection has been established in 114 ng mL^{-1} ($0.114 \mu\text{g mL}^{-1}$).
- β -lg concentration range to evidence changes in immunoreactivity is established between $0.078 \mu\text{g mL}^{-1}$ and $10 \mu\text{g mL}^{-1}$.
- Changes in immunoreactivity of treated samples agree with those obtained with milk allergic patients.
- The method could be also applied for β -lg detection.

GRAPHICAL ABSTRACT



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ABSTRACT

Immunochemical detection of food allergens is usually based on the use of polyclonal or monoclonal immunoglobulins G (IgG) antibodies. However, due to differences in epitopes recognition between IgG and IgE, an epitope modification during food processing can potentially alter allergenicity and detection in such different way. For that reason, the use of traditional immunological methods to anticipate or study the protein allergenicity is not recommended.

The objective of this work was to develop a standardized competitive ELISA, based on recombinant IgE antibodies to β -lactoglobulin (β -lg), to reveal and to measure changes in β -lg immunoreactivity provoked by technological processes in foods containing this milk protein.

As a result, a sensitive immunochemical method (Limit of Detection, $\text{LOD} < 0.2 \mu\text{g mL}^{-1}$) has been developed for β -lg recognition. Also, this technique has been confirmed to detect modifications in β -lg-IgE binding after food processing (thermal treatment) in a similar way to those results obtained with allergic patients in previous works. Consequently, data obtained with this assay could be used as a preliminary test to predict *in vitro* β -lg allergenic behavior in allergenicity studies in processed foods.

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1. Introduction

Cow's milk is one of the most common trigger foods causing food allergy in the first years of life. It affects around 2.5% of young children with severe consequences for the quality of life of both patient and family [1]. Symptoms of cow's milk allergy (CMA) range

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from mild to anaphylactic reactions. The development of these symptoms depends on immune mechanisms, being the one associated with Immunoglobulin E (IgE), also called Type I Anaphylactic Sensitivity the most common [2,3].

CMA can be caused by several allergenic proteins including caseins, β -lactoglobulin (β -lg) and α -lactalbumin (α -la) [4]. Among them, the β -lg is the main whey protein in the milk of many mammals and it is considered to be the major cow's milk whey allergen (Bos d 5); that is, the protein most frequently and intensively recognized by human IgE [5,6].

Up to date, the only effective treatment of any food allergy is the strict avoidance of the allergen-containing food. However, total avoidance for CMA patients is sometimes difficult. Due to the low cost of milk whey proteins, together with their worthy technological properties (gelation, emulsifying and binding properties), they are widespread used as ingredients in the food industry [7–9].

Prevention of allergies has become a clinical public health concern, and many attempts have been made for reducing allergenicity of proteins. One of the strategies for reducing allergenicity has been focused on protein modification. In IgE-mediated allergies antibodies recognize specific regions (epitopes) in the allergen molecule [10]. As food treatments have been showed that can modify the structure of allergens, a reduction in their allergenic potential could be easily addressed [11,12]. However, no standardized methods are available to predict allergen modifications due to food processing and there is still a need for improving of knowledge about influence of processing in the structure of food allergens [13]. In fact, it has been published that a deep research to develop solid and standardized methods to allow researchers meet how food processing impact in food allergenicity is still needed [14].

Traditionally, the methods for immunoreactivity prediction are based on the use of polyclonal IgG antibodies obtained from immunized animals, human IgE serum pools isolated from allergic patients or, in some cases, by monoclonal antibodies produced by hybridoma technology. Various immunological detection methods based mainly on polyclonal antibodies have been published during recent years [15,16]. Polyclonal antibodies from immunized rabbits have been used in sandwich-type ELISA for the sensitive detection of β -lg from cow's milk and food products [17,18]. However, the polyclonal and monoclonal antibodies usually used, recognize the immunogenic epitopes of the protein, those recognized by IgG. These are not necessarily the same as the allergenic epitopes recognized by the IgE antibodies that trigger the allergenic response.

Thus, due to the probable dissimilarities between IgG and IgE epitopes recognition, it would be useful to count on a method based on IgE antibodies for allergenic properties identification, in order to relate protein detection with real potential allergenicity of β -lg-containing foodstuff. However, the high variability between IgE from sera collected from sensitized persons makes the standardization of the detection methods very difficult for commercial purposes.

Nevertheless, today it is possible to produce human recombinant antibodies [19] that would ensure the specific detection of the harmful food content for a sensitized person [20]. Previous works dealt with the development of a recombinant human IgE, which could be used for standardized detection of β -lg, thanks to its homogeneity and specificity [21].

However, beyond recombinant-IgE development, the standardization and validation of a consistent method for protein modification estimation must ponder many other parameters, such as food matrix composition and the intensity of food processing since they can have a great impact on the elicitation of allergic reactions and protein modification [2,22]. The alteration in protein structure by food processing can lead to epitope destruction,

modification, masking or unmasking [23–25], and due to the mentioned differences between IgG and IgE epitopes, allergenicity and detection can be altered in a different way. This fact strengthens the value of a common and standardized method to easy and fast estimation of changes in IgE bonding to β -lg.

Thus, in this work, our objective was to implement an analytical technique which allows to evidence changes in the immunoreactivity of the β -lg protein due to different processing conditions: the detection of the protein in its native and thermally-treated forms has been studied to check the tangible potential of this method to be used for detecting potential modifications of β -lg recognition by IgEs and consequently, to be used as a preliminary *in vitro* prediction of β -lg allergenic behavior in processed foods.

2. Materials and methods

2.1. Materials

β -lactoglobulin (β -lg) lyophilized powder (L0130 $\geq$ 90% purity) from bovine milk was purchased from Sigma-Aldrich chemicals (St. Louis, MO). Bovine serum albumin (BSA) fraction V was obtained from Fisher Scientific (Leics, UK). Goat anti-human Kappa-HRP IgG (2060–05) was collected from SouthernBiotech (Birmingham, Alabama, USA) and Ultra TMB-ELISA substrate (tetramethylbenzidine) was acquired from Thermo Scientific (Rockford, USA). Raw milk was ordered to a local supplier (Vascolac, Zamudio, Spain). Microtiter polystyrene MaxiSorp 96 U-well plates were obtained from NUNC (Roskilde, Denmark).

2.2. Methods

2.2.1. Optimization of direct ELISA for isolated β -lactoglobulin

Anti β -lactoglobulin (β -lg) recombinant Immunoglobulin E (IgE) was obtained as previously described [21] and was kindly donated by VTT Technical Research Centre of Finland (Espoo, Finland). In order to test the optimal assay conditions, IgE/ β -lg proportions and reagent concentrations for subsequent competitive method development, a preliminary direct ELISA was performed. For that, microtiter plates were coated with 100 μ L per well of a 5 μ g mL⁻¹ or 10 μ g mL⁻¹ β -lg solution in Na₂CO₃/NaHCO₃ 0.05 M (carbonate buffer, pH 9.6). After incubation overnight at 4 °C, the wells were washed three times with PBST (14 mM NaCl, 2 mM KCl, 1 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.4 -phosphate buffered saline –PBS–, with 0.05% [v/v] Tween 20). Then, wells were blocked with 300 μ L of 1% (w/v) BSA in PBS and incubated for 1 h at room temperature (RT). After washing three times with PBST, wells coated with the two different concentrations of β -lg, were incubated with 100 μ L of serial dilutions of IgE (Fab) ranging from 1/3000 to 1/24000 in PBS for 1 h. Plates were then washed three times with PBST and incubated with 100 μ L of different dilutions of the goat anti-human Kappa-HRP, from 1/2000 to 1/16000, in PBS for 1 h at RT. Finally, plates were washed three times with PBST and TMB substrate was added in a volume of 100 μ L per well and incubated for 15 min at RT. The enzyme reaction was stopped by adding 50 μ L of 2 M H₂SO₄ per well, and the absorbance was determined at 450 nm using a microplate reader Thermo Scientific™ Varioskan Flash (Waltham, MA, USA). Each sample (each β -lg/IgE/anti-human Kappa-HRP combination) was tested in triplicate in two independent experiments.

2.2.2. Optimization of the competitive indirect ELISA method

Competitive indirect ELISA (ciELISA) procedure was adapted then, using the best conditions obtained in the previous step. Microtiter plates were coated with 100 μ L per well of a 5 μ g mL⁻¹ β -lg solution and blocked as previously described. After washing three

times with PBST, plates were incubated with 100 μL of a mixture (1:1) of serial double dilutions of samples with a known concentration of $\beta\text{-Ig}$, ranging from $10\text{ }\mu\text{g mL}^{-1}$ to $0.039\text{ }\mu\text{g mL}^{-1}$, and IgE (Fab) at 1/4000 (final concentrations), all diluted in PBS. As negative controls, 100 μL of a mixture (1:1) of PBS (no $\beta\text{-Ig}$) and IgE (Fab) at 1/4000 (final concentration) were also assayed. The competitive reaction took place in the wells for 2 h at RT. Plates were then washed three times with PBST and incubated with 100 μL of goat anti-human Kappa-HRP 1/8000 in PBS for 1 h at RT. Finally, plates were washed three times with PBST and 100 μL per well of TMB substrate were added and incubated for 15 min at RT. After color development, the reaction was stopped by adding 50 μL of 2 M H_2SO_4 per well, and the absorbance determined at 450 nm using a microplate reader Thermo Scientific™ Varioskan Flash. Each sample was incubated by duplicate in at least three independent experiments.

2.2.3. Thermal treatments

In order to study the effect of the matrix conditions and thermal processing on the detection of $\beta\text{-Ig}$ by the ciELISA developed, the samples were prepared and subjected to different treatments as follows.

Protein solutions of $2000\text{ }\mu\text{g mL}^{-1}$ of $\beta\text{-Ig}$ were prepared dissolving protein powder in $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ 0.1 M (phosphate buffer, pH 6.8), and $\text{C}_2\text{H}_4\text{O}_2/\text{C}_2\text{H}_3\text{NaO}_2$ 0.1 M (acetate buffer, pH 4.6). Skimmed milk (SM) was obtained from raw whole milk by centrifugation of milk samples at 2500 rpm and 4°C for 20 min in an Ortoalresa Digitor 20R centrifuge with a rotor RT191 (Madrid, Spain). Skimmed milk was then carefully separated from solid fat remaining at the top phase. Acid whey (AW) was obtained by acidifying raw skimmed milk by adding 1 M HCl until the pH reached a value of 4.6. Precipitated caseins were separated from AW by centrifuging once at 2500 rpm at 4°C for 20 min.

For thermal treatments, 300 μL of each sample were packed in heat resistant bags and thermally sealed. Samples were treated in a Lauda Controller E300 Baths EcoLine 012 (Lauda-Königshofen, Germany) at 75, 85 and 95°C for 20 min. For treatments over 100°C (105°C for 20 min) an autoclave Suredry APR-95 (Biscay, Spain) was used. After treatments, all samples were placed in ice cold water for fast cooling and analyzed by competitive ELISA method as described below. All samples were treated by duplicate and subsequently analyzed by triplicate.

2.2.4. Validation of the method for $\beta\text{-Ig}$ -immunoreactivity changes in raw and thermally-treated samples

Considering that milk contains on average $3000\text{ }\mu\text{g mL}^{-1}$ of $\beta\text{-Ig}$ (Wal, 1998), standard curves of SM and AW were firstly studied (in serial double dilutions) together with isolated $\beta\text{-Ig}$ standard curve, in order to adjust the proper sample dilution for analysis.

Afterwards, the ciELISA method in both untreated and treated samples was carried out similarly as previously assessed. Briefly, microtiter plates were coated, washed and blocked as described above (section 2.2.2). After washing three times with PBST, plates were incubated with 100 μL of a mixture (1:1) of the corresponding diluted sample of non-treated/treated $\beta\text{-Ig}$, SM or AW and 1/4000 of IgE (Fab) (final concentration) in PBS. All samples were diluted to obtain a theoretical concentration of $10\text{ }\mu\text{g mL}^{-1}$ (according to absorbance obtained in standard curves previously described) and then serial double dilutions from $5\text{ }\mu\text{g mL}^{-1}$ to $0.313\text{ }\mu\text{g mL}^{-1}$ were made. As negative control, 100 μL of a mixture (1:1) of PBS (no $\beta\text{-Ig}$) and 1/4000 IgE (Fab) dilution was also analyzed. The competitive reaction took place in the wells for 2 h at RT. Plates were then washed three times with PBST and incubated with 100 μL of 1/8000 goat anti-human Kappa-HRP in PBS for 1 h at RT. Finally, plates were washed three times with PBST and TMB substrate was added

in a volume of 100 μL per well and incubated for 15 min at RT. The reaction was stopped by adding 50 μL of 2 M H_2SO_4 per well, and the absorbance was determined at 450 nm using a microplate reader Thermo Scientific™ Varioskan Flash.

Percentage of inhibition of IgE binding to the treated proteins with respect to untreated protein (native commercial $\beta\text{-Ig}$, untreated SM and AW, respectively) was calculated using the following equation (adapted from Ref. [25]):

$$\text{IgE binding inhibition (\%)} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{0\%}}{\text{OD}_{100\%} - \text{OD}_{0\%}} \times 100$$

Where $\text{OD}_{\text{sample}}$ is defined as the value of absorbance of the treated sample; $\text{OD}_{0\%}$ as the value of control (untreated sample); and $\text{OD}_{100\%}$ as the absorbance of the negative (no $\beta\text{-Ig}$) control. Thus, a positive value ranging from 0% to 100% of IgE-binding inhibition would indicate a reduction of IgE-binding to the treated proteins (a hypothetical reduction of the allergenicity), while negative values would indicate an increasing on protein IgE recognition (theoretical increase of allergenicity).

3. Results and discussion

In this work, a competitive indirect ELISA (ciELISA) has been developed, optimized and used for $\beta\text{-lactoglobulin}$ ($\beta\text{-Ig}$) recognition and to determine Immunoglobulin E (IgE) binding differences between native and treated protein in different matrix conditions.

The selection of this method was done based on the availability of specific antibodies: Sandwich ELISA implementation requires at least two different binding sites and consequently, the use of two different antibodies to detect a protein. In this case, working with one clone of a monoclonal antibody which recognizes only one epitope of the protein, the competitive method was selected. The ciELISA method developed and calculations are based on the competition between native $\beta\text{-Ig}$ bound to the plate and the native or modified (treated) protein contained in the standards or samples for binding to the anti- $\beta\text{-Ig}$ IgEs. Thus, the differences in structure and antibody binding caused by the treatments can be easily determined by means of just one anti- $\beta\text{-Ig}$ antibody.

Preliminary tests with a direct ELISA were carried out to assess the optimal $\beta\text{-Ig}$ coating, anti- $\beta\text{-Ig}$ IgE and anti-kappa-HRP antibody concentrations. This first aim was to find out the optimal reagent concentrations and conditions to be equal as the negative control (the mixture without $\beta\text{-Ig}$ in the $\beta\text{-Ig}$ -IgE competition step) for the subsequent competitive method development. For anti-human Kappa conjugate dilutions, 1/2000 and 1/16000 dilutions were discarded for leading to very high and too low absorbance, respectively, and data are not shown. The absorbance results obtained for the rest of the tested combinations of reagents are shown in Fig. 1. As expected, the highest values of absorbance are given at the lowest dilutions of IgE and at higher concentration of anti-IgE, with slight differences between the coating $\beta\text{-Ig}$ concentration. As Fig. 1 shows, conditions giving an absorbance higher than two units ($\text{OD}_{450} > 2$) presented a higher variability. That can be due to instrumental deviations from Lambert-Beer's Law [26], which means working in the non-linear range of absorbance of the spectrophotometer used. Consequently, the conditions that gave an OD_{450} over two units were discarded for following assays. Taking into account that in a competitive method the relationship between concentration and absorbance is indirect, the conditions selected here will relate, accordingly, to the sample with the highest absorbance inside the linear range of detection. Correspondingly, too low absorbance ($\text{OD} < 0.6$) could not permit a proper quantification of differences in $\beta\text{-Ig}$ concentration in the subsequent competitive method, due to insignificant differences in absorbance

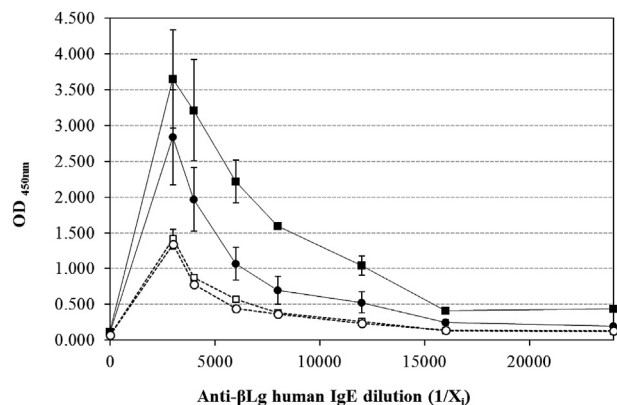


Fig. 1. Absorbance levels related to anti- β -Ig IgE dilution used for the different conditions tested with the direct method: wells coated with $5 \mu\text{g mL}^{-1}$ β -Ig and incubated with 1/4000 (■) and with 1/8000 (□) anti-IgE antibody; and coated with $10 \mu\text{g mL}^{-1}$ β -Ig and incubated with 1/4000 (●) and with 1/8000 (○) anti-IgE antibody, respectively. Vertical error bars correspond to Standard Deviation (SD).

between the samples. Also, a rational use of reagents was taken into account to select the optimal conditions (use of the minimum quantity of reagents to obtain the best results): At the view of the results, the selected conditions for subsequent experiments were as follows: wells coated with $5 \mu\text{g mL}^{-1}$ β -Ig, blocking step with 1% BSA, a working dilution of 1/4000 (final concentration) for anti- β -Ig IgE and a working dilution of 1/8000 for anti-kappa-HPR antibody.

Once reagents concentration and other conditions were fixed, a ciELISA was developed to know the appropriate range of β -Ig concentration where the relationship between β -Ig concentration and absorbance at 450 nm was lineal and quantifiable.

The relationship between the absorbance and the β -Ig concentration (logarithm) was inversely linear (at higher concentration of β -Ig, lower values of OD) since this is a competitive method. The linearity of the absorbance with the β -Ig concentration used in the standards is maintained in the range from $0.078 \mu\text{g mL}^{-1}$ (78 ng mL^{-1}) and $10 \mu\text{g mL}^{-1}$ ($10\,000 \text{ ng mL}^{-1}$) ($r^2 > 0.98$) (Fig. 2). The detection limit (LOD) of the assay was defined as the protein concentration corresponding to the mean absorbance of a negative control minus 3.3 times the value of its standard deviation [27] and corresponds to $0.114 \mu\text{g mL}^{-1}$ (114 ng mL^{-1}).

These results are in agreement with results obtained by Jylhä [21] for native β -Ig. In that previous work, studying decimal

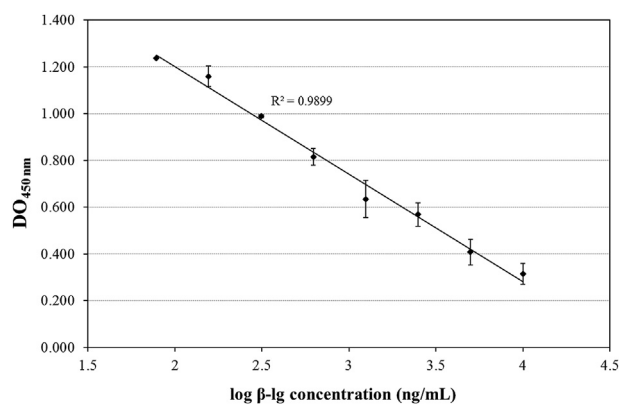


Fig. 2. Calibration curve obtained for indirect competitive ELISA for the determination of linear range of β -Ig concentration measured in standard solutions of pure β -lactoglobulin in PBS ($r^2 = 0.9899$). Vertical error bars correspond to Standard Deviation (SD).

dilutions of β -Ig (10, 1, 0.1, 0.01 and $0.001 \mu\text{M}$), apparently, the highest absorbance variation for competitive ELISA was obtained in a β -Ig concentration ranged between 0.1 and $0.01 \mu\text{M}$ β -Ig ($0.18\text{--}1.80 \mu\text{g mL}^{-1}$) [21]. However, these authors did not test more intermediate β -Ig concentrations, and the LOD and linear range could not be established. Although detection of β -Ig is not the main purpose of this work, here we have determined the detection limit of the method using the recombinant human IgE. Our results confirm that this method could be used for β -Ig detection with a similar and even lower LOD than other competitive ELISA-based methods [15,28]. Also, for further experiments, we have determined the working range of β -Ig concentrations where the relationship with absorbance is linear, for reliable quantification of protein modifications.

In addition, the developed method in this work allows the protein recognition by means of an antibody that recognize a real IgE epitope of β -Ig [21], which are the first responsible of a type I reaction. Contrarily, most of conventional commercial detection methods are based on polyclonal IgG interactions with allergens [29]. The detection of a protein is not always related to its allergenic potential, and allergenic epitopes tend to escape the most commonly utilized sandwich ELISA-based test [30]. With the method developed in this work, now it is possible to acquire a better relationship between changes observed in OD and potential changes in human IgE immunoreactivity of the β -Ig due to processing.

3.1. Application of ciELISA in food matrices naturally containing β -Ig

Although the recombinant IgE antibody used has a high specificity [21], in order to develop a reliable immunoassay, it is necessary to check if the presence of other milk proteins and other food components do not alter or mask the recognition of the target protein (β -Ig). Also, it is known that ELISA methods are sometimes sensitive to the effect of matrix (matrix interferences) [31]. Thus, the ciELISA developed was also checked, in this preliminary stage, against complex food matrices where β -Ig can be naturally found, such in whey or milk, as main sources of β -Ig. The same experimental approach and conditions followed before for isolated β -Ig were applied for raw milk and whey, and results are shown in Fig. 3. The slight differences in concentration detected between β -Ig standards (isolated β -Ig) and milk and whey standard curves may be due to the fact that the concentration in milk and whey was calculated theoretically from a usual concentration of $3000 \mu\text{g mL}^{-1}$ [4]. Thus, the β -Ig concentration in these products

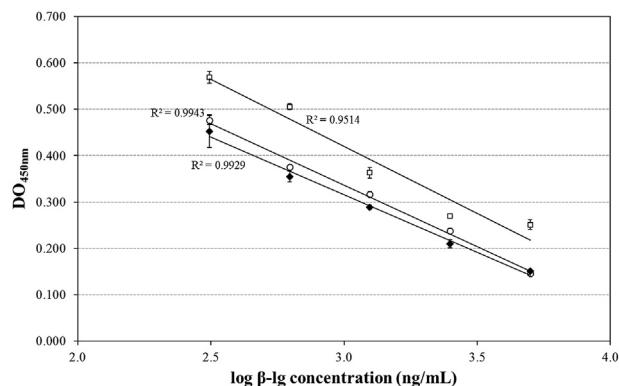


Fig. 3. Relationship between absorbance and β -Ig concentration in β -Ig standards in PBS (◆, $r^2 = 0.9929$), in skimmed milk (○, $r^2 = 0.9943$) and in acid whey (□, $r^2 = 0.9514$).

can naturally vary and may not exactly coincide. Despite these differences, as it can be seen, β -lg recognition is not affected by the presence of other proteins and food components, since the linearity is maintained ($r^2 > 0.95$ in all cases) and the relationship between detected β -lg concentration and absorbance keeps the same proportionality for milk and whey than for the isolated protein (Fig. 3).

Finally, the recognition of allergens by immunochemical methods can be affected not only by matrix composition, but also by structural changes in proteins provoked to technological processes [11]. Keeping in mind that one of our aims is to apply the developed method for demonstrating allergen and allergenic potential modifications, the next step was to check if this method is valid for the identification and measurement of the mentioned changes.

3.2. Validation of ciELISA based on recombinant human-IgE antibodies for prediction of β -lg allergenicity changes in processed samples

Processing can alter proteins by inducing denaturation (loss in tertiary and/or secondary interactions), formation of new intra- or inter-molecular bonds, aggregation, and/or rearrangements of disulfide bonds, as well as other conformational modifications, which can ultimately lead to changes in allergen reactivity [13,32,33]. To properly determine the modification of the allergenic proteins in real food products, it is essential to understand how changes produced by processing affect the antibody reactivity, more especially when the method is desired to be used for related allergenic potential.

The changes in the epitopes and allergenic potential of β -lg by thermal treatments are well known [34] and have been already quantified, so the validation of the capability of this method to measure allergenicity modifications was done in thermally processed samples. Accordingly, the effect of heating on the recognition of β -lg in buffer, milk and whey by recombinant IgE has been studied. For buffer solutions, isolated β -lg was diluted in 0.1 M phosphate buffer pH 6.8 being as is the milk pH, and 0.1 M acetate buffer pH 4.6, the acid whey pH, in order to visualize the variability of the relation pH and temperature with the immunoreactivity. Also, it must be taken into account that, after recombinant IgE selection, the reduction of the binding of D1 Fab fragment used in this work to β -lg was already shown at 95 °C [21], but not at lower or higher temperatures. So, for a complete validation of the method to be applied for allergenicity-change measurements, samples were subjected to 75 °C, near the denaturation temperature of β -lg, established at 70 °C [34,35], 85 °C, 95 °C and 105 °C.

Results are shown in Fig. 4, as the inhibition percentage of IgE binding compared to the untreated sample (0% inhibition binding). As it can be seen, there is a clear influence of the heat treatment in the ability of IgE for recognition of β -lg: The inhibition of the IgE- β -lg binding increased with temperature and time of treatment in all the studied matrices. That means that the IgE-immunoreactivity of β -lg is reduced after heat treatment.

The milk whey protein β -lg, like many other proteins, is sensitive to heating over 60 °C. During heating, intramolecular and intermolecular changes and reactions occur, which are generally denoted as denaturation and aggregation [36,37]. This heat-induced conformational modification of proteins may affect their antigenic and allergenic properties.

In the case of the β -lg, our results agree with previous studies using IgE from sera from allergic patients [34,38,39]. As these studies reported, a decrease in β -lg recognition by IgE was also detected when heating at temperatures between 80 and 100 °C. Taheri-Kafrani et al. [39] reported changes in β -lg molecular weight following thermally induced denaturation and subsequent

aggregation and showed that heat-induced denaturation of β -lg was associated with weaker binding of IgE from cow's milk allergy (CMA) patients. That reorganization of β -lg is thought to mask the conformational or lineal epitopes present on the major allergen, thus reducing overall IgE binding. The additional increase in IgE- β -lg binding inhibition at 105 °C is in the same way as that observed by Bu et al. [23], who revealed a decreased antigenicity of purified β -lg to rabbit antibodies due to treatments for 20 min at temperatures between 80 and 120 °C. Correspondingly, other authors studying more intense treatments (177 °C/30 min and 260 °C/3 min) also found a total decrease in the allergenicity (measured by skin prick tests) of β -lg in dairy products [40,41]. These authors suggested that β -lg forms disulfide bonds with other proteins in the food matrix resulting in decreased epitopes availability and decreased allergenicity, especially in intensively heated milk products, which would also explain, the higher inhibition of IgE binding to β -lg when this protein is treated at higher temperatures.

Apart from these results, small and non-significant differences in the β -lg immunoreactivity in the different matrices have been observed at the different temperatures. These results emphasize the capability of this developed method to distinguish the immunoreactivity of the β -lg independently of the complexity of the analyzed dairy matrix and is in accordance to our previous results (section 3.1., Fig. 3). This fact can be due to the high specificity of the recombinant IgE used, that would recognize an epitope that is affected directly by thermal treatment [21] but not by the possible interactions between matrix components during food processing [40,42].

To summarize, regarding some previous works done with allergic patient serum, similarities to our results obtained with recombinant IgE can be found. At the view of this correspondence, the method developed in this work could be used to preliminary screening for β -lg structure modifications due to heat treatments and other processing technologies and for prediction of allergenicity without using CMA patients' sera. The use of the human IgE antibody provides an element of specificity to the assay since it detects clinically relevant allergenic proteins that can elicit allergic symptoms in sensitized individuals [20,43]. However, the variable results established by other researchers regarding processing effects on milk proteins allergenicity [28] advise that, if an important reduction of immunoaffinity is found with this method, before considering a product as “hypoallergenic”, confirmation tests with real sera must be carried out to consider other milk proteins and the variable and heterogenous human IgE response [44].

Finally, although it has not been the objective of this work, the developed assay could be also useful to identify the presence of β -lg in foods. With this future perspective, this method based on human recombinant antibodies could be even exploited as an integration into a new generation of innovative immunoenzymatic tests (e.g. lab-on-chip devices), allowing a direct and on-field detection of allergens [45–47]. However, as a reduction of the immunoreactivity is evidenced, other complementary IgE would be recommended to use to cover more allergenic epitopes, both thermally sensitive and heat resistant. Also, for detection purposes a more complete validation should be performed according to international guidelines.

4. Conclusions

In this work, a novel immunochemical method for the recognition of modifications in β -lactoglobulin (β -lg) allergen immunoreactivity has been developed. As it is based on a human immunoglobulin E (IgE) recombinant antibody, the use of this method and their results could help to preliminary assess and predict potential allergenicity modifications of β -lg caused by

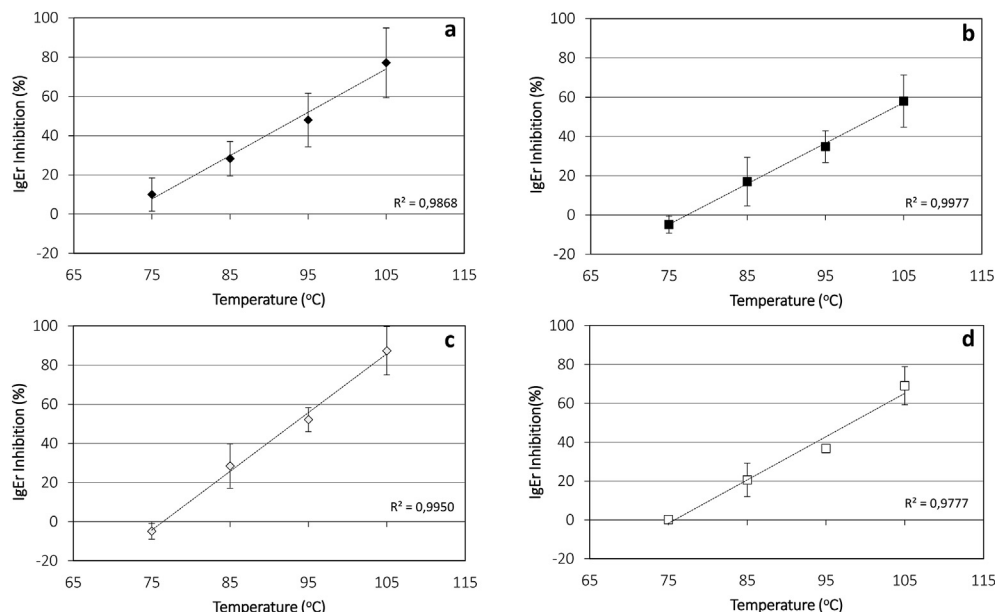


Fig. 4. Modifications (measured as percentage of IgE inhibition) in recognition of β -lg by recombinant IgE due to thermal treatments for 20 min at 75 °C, 85 °C, 95 °C and 105 °C. Different treatment media were treated and analyzed by developed competitive ELISA: Isolated β -lg in pH 4.6 buffer (4.a.); isolated β -lg in pH 6.6 buffer (4.b.); acid whey (4.c.) and; skimmed milk (4.d.).

protein changes due to food processing in different food matrices. By this way, this method would be simpler and faster than using human sera samples (more variable, non-standardized and difficult to obtain). However, confirmation tests must be carried out to consider the more variable real human serum IgE and *in vivo* responses in those samples resulting in a high IgE-binding reduction, to assure allergic consumer safety.

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