



Original

# Influences of different thermal processings in milk, bovine meat and frog protein structure

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## Abstract

Several studies have associated the digestibility of proteins to its immunogenic potential. Though, it was objectified to evaluate the impact of the thermal processing with high and low temperatures on the proteins structure of three types of foods, by means of the digestibility in vitro and electroforesis en gel de poliacrilamida. The pasteurize was observed in such a way, firing 95 °C during 15 minutes, how much freeze dried causes qualitative and quantitative modifications of constituent proteins of the food. The most sensible proteins to the increasing thermal processing order were beef, frog meat, and the last, cow milk.

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Key words: Food allergens. Thermal processing. Digestibility.

## INFLUENCIA DEL TRATAMIENTO TÉRMICO EN LA ESTRUCTURA PROTEÍCA DE LA LECHE, CARNE Y RANA

### Resumen

Varios estudios han asociado la digestibilidad de las proteínas para su potencial inmunogénico. En este sentido, el objetivo fue evaluar el impacto del tratamiento térmico a temperaturas altas y bajas en la estructura de la proteína de los tres alimentos a través de la digestibilidad in vitro y la electroforesis en gel de poliacrilamida. Se observó que tanto la pasteurización, la cocción a 95 °C durante 15 minutos y liofilización dio modificaciones cualitativas y cuantitativas de los constituyentes de proteínas de los alimentos. Las proteínas más sensibles al tratamiento térmico en orden ascendente fueron carne de res, carne de rana y, finalmente, la leche de vaca.

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Palabras clave: Alérgenos alimentarios. Tratamiento térmico. Digestibilidad.

## Abbreviations

PAGE: Polyacrylamide gel electrophoresis.  
IgE: Immunoglobulin type E.  
PT: Prick test.  
BPCT: Blind placebo-controlled trial.  
BSA: Bovine serum albumin.  
HT: Heat treatment.  
% D: Digestibility percentage.  
LB: Lysis buffer.  
SB: Sample buffer.  
kDa: Kilodaltons.

GMO: Genetically modified organisms  
i.n. milk: In natura milk  
HT milk: Powdered milk.  
R frog: Raw frog meat.  
HT Frog: Cooked frog meat.  
R beef: Raw beef.  
HT Beef: Cooked beef.  
OSA: Ovine serum albumin.  
MW: Molecular weight

## Introduction

Thermal processing is used to improve the quality of food microbiological safety, either by eliminating micro-organisms or toxins or by improving the nutritional value which results from digestibility increase.<sup>1</sup> Significant changes occur in the tertiary structure of proteins during heat treatment. The nature and extent of these changes depend on the temperature and duration

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of thermal processing, as well as the inherent protein characteristics and the physical-chemical conditions involved.<sup>1</sup>

Several allergens found in foods are heat-resistant and stable to digestion performed in the gastrointestinal tract, leading some researchers to correlate the allergenic potential of some foods to their stability to the action of proteolytic enzymes.<sup>1,2,3</sup> In addition to denaturation, other covalent modifications due to heat or food storage can lead to change in food allergenicity. Some examples are lipid oxidation reactions or the direct oxidation caused by oxygen-reactive intermediates.<sup>4</sup>

Food-induced allergic reactions are responsible for a variety of symptoms involving the gastrointestinal, respiratory and skin systems, and can be caused by mechanisms whether mediated or not by immunoglobulin type E (IgE).<sup>5</sup> Any type of food can cause an allergic reaction in the presence of genetic susceptibility, but in effect a small number of foods are actually responsible for most reactions. These include cow's milk, eggs, fish, seafood, peanut, soybean, wheat, beef, pork and some citrus fruits.<sup>5,6,7,8,9</sup> Studies suggest that about 2% of adults worldwide have food hypersensitivity, 1% of which is food allergy itself; figures are generally higher for children under three years old, ranging between 6% and 8%.<sup>1,2</sup>

The application of heat treatment can usually reduce fresh fruit allergenicity easily, allowing the food industry to produce allergy-safe food.<sup>10</sup> To assess the influence of heat treatment on allergy clinical reactivity, Fiocchi et al.<sup>11</sup> compared the effects of domestic cooking and industrial processing using the prick test (PT) and the blind placebo-controlled trial (BPCT) in institutionalized children. In the first test, industrially processed meat extract was dissolved in glycerol (50%) and compared with raw, cooked and freeze-dried powdered beef extracts. Purified bovine serum albumin (BSA) was used as positive control; 10 children were positive for at least 03 of the items tested. In a second test, the same individuals participated in the BPCT for industrially processed beef steamed for 5 minutes at 100° C, lyophilized raw beef and purified BSA, where turkey meat was used as a placebo. The protocol used an initial dose of , which was doubled every 30 minutes (24, 48 and of test food or placebo) for 4 hours and discontinued when the first symptoms arose, or when there was a negative response after the eighth dose. Positive responses were found only for purified BSA in 50% of individuals, who manifested rhinitis, angioedema, urticaria and asthma, thus demonstrating that heat treatment is able to reduce protein allergenicity.

Sites of IgE binding to the protein allergen may consist of consecutive segments of the amino acid or different parts of the amino acid sequence held together by protein conformation, which are the so-called conformational antigenic determinants.<sup>12,13</sup> Some antigenic determinants are accessible in native proteins and are lost when they are denatured; others are

exposed when the protein unfolds; there are also some determinants arising from covalent modification caused by peptide bond breakdown.<sup>14</sup> According to some researchers, peptide action is able to influence serum albumin allergenicity by cleaving amino acid sequences and turning an allergen into a non-allergenic protein.<sup>2</sup>

Low-temperature industrial processing can also modify food protein structure since the food protein structure between proteins and water is reduced.<sup>1</sup> Freeze drying is the most commonly used method to prepare dehydrated proteins, which should have adequate stability in long storage periods at room temperature.<sup>7</sup> Freeze drying basically involves three steps: freezing, primary drying and secondary drying. Freezing stops chemical reactions and possible biological activities in the sample. The previously frozen material is dried by sublimation followed by desorption, using low-temperature drying at reduced pressure.<sup>15,16</sup>

In this regard, this study aimed to evaluate the impact of high and low-temperature heat treatment on the protein structure of three foods by means of *in vitro* digestibility and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

## Material and methods

The samples analyzed were selected in order to be compared regarding their stability during thermal processing. An analysis was made of cooked, raw, and raw lyophilized frog meat and beef samples; and *in natura*, *in natura* lyophilized, pasteurized, and industrially-processed powdered cow's milk.

Frog meat is cited in the literature as a possible replacement protein source in diets for allergic individuals, despite the scarcity of studies addressing its use.<sup>17</sup> Beef has a low incidence of allergy, whereas cow's milk has more than 25 different and potentially antigenic proteins. These include a- and b-lactoglobulins, and S1 and S2 a and k caseins, which are known to be allergenic when ingested by susceptible individuals. The incidence of cow's milk allergy in the pediatric population ranges from 0.5 to 7.5%.<sup>19,20</sup>

### Sample collection and preparation

*In natura* and pasteurized milk was obtained from the Universidade Federal de Viçosa (UFV) Dairy Cooperative; frog meat came from the Frog Farm at UFV, while powder milk and beef were purchased from local traders.

The beef and frog meat samples were processed to simulate home heat treatment (HT) at the Laboratory of Experimental Study of Food of the Nutrition and Health Department under dry heat at a temperature of 95° C for 15 minutes. Subsequently, the samples for *in*

*vitro* digestibility were submitted to dehydration in an oven at 65° C for 4 hours. For milk samples, only industrial processing was used.

### Protein value

To determine nitrogen content, the samples were analyzed by the semi-micro Kjeldahl method in accordance with regulations of the Association of Official Analytical Chemists.<sup>21</sup>

### In vitro digestibility

*In vitro* digestibility was evaluated with the method described by Hsu et al.<sup>22</sup> according to which digestibility is characterized by pH decrease in the protein solution measured in the first 15 seconds and then at every minute for 10 minutes after the enzyme solution is added.

The samples were suspended in distilled water, of protein/mL, with final pH equal to 8, stirred au bain-marie at 37° C. For the hydrolysis of the prepared samples, we used 5 mL of enzyme solution containing 2.5 mg/mL trypsin and 1.6 mg/mL pancreatin.

Digestibility percentage (% D) was calculated with the equations described by Pires et al.<sup>23</sup> originating from the correlation between values observed in *in vitro* analyses with *in vivo* experiments.

### Polyacrylamide gel electrophoresis

In this procedure, samples of the following kinds of food underwent polyacrylamide gel electrophoresis (PAGE): frog meat and beef samples that were raw, raw lyophilized and dry-heated ( for 15 min); *in natura*, *in natura* lyophilized, pasteurized and powder cow's milk obtained by industrial processing. Electrophoresis was performed according to Laemmli<sup>24</sup>.

Solid samples were macerated in 200 mL of lysis buffer (LB) until completely dissolved, except for the lyophilized sample, which was suspended in 100 mL distilled and deionized water before maceration. The samples were then centrifuged (Centrifuge - Eppendorf) for 2 minutes at 14,000 rpm; the supernatant was removed for later use, while the liquid samples were added to distilled and deionized water.

Subsequently, an aliquot of 100 mL was taken from each previously prepared sample and 100 mL of twice-concentrated sample buffer (SB) was added. After a short homogenization treatment, the samples were boiled au bain-marie for 2 minutes, 10 mL of sample was applied in each "slott", and electrophoresis occurred at 10 mA for 17 hours. A standard marker for low molecular weight proteins (Mobitec®) was used, with extreme values of 116 kDa and 14 kDa.

### Statistical analysis

The data were statistically analyzed with the Statistics software by analysis of variance using the Duncan means test or Student's t test, where appropriate, with a significance level of 5%.

### Results and discussion

When protein content was compared in the studied samples (table I), statistically significant difference was found between *in natura* samples and samples heat-treated by means of cooking and dehydration. However, no statistically significant difference was found for protein content when the samples were separated into two groups: heat-treated and unheated samples.

**Table I**  
Protein content in the analyzed samples

Source	g/100 g
Cooked and dehydrated beef	88.26
Cooked and dehydrated frog meat	87.32
Lyophilized raw beef	86.30
Lyophilized raw frog meat	83.70
Powdered milk	33.12
Lyophilized in natura milk	25.09
Raw beef	21.09
Raw frog meat	17.09

Results are expressed as means of three repetitions.

Digestibility is defined as the calculation of the percentage of proteins that are hydrolyzed by digestive enzymes and absorbed as amino acids, or any other compound nitrogenated by the human organism. Digestibility also determines the protein quality of a diet.<sup>23</sup> Methods to determine *in vitro* digestibility are based on the digestion of a sample with proteolytic enzymes in standardized conditions. Protein digestibility has been routinely assessed in procedures aiming to investigate the safety of new proteins from genetically modified organisms (GMO). It is also crucial for research on the influence of heat treatment on the allergenic potential of several foods, among other applications.<sup>25</sup>

Figure 1 shows a more dramatic pH decrease until the second minute for all the samples, and then a slower drop until the tenth minute, which results from the fact that denatured proteins are more sensitive to the action of proteolytic enzymes. Thus, the breakdown of peptide bonds and hydrogen bonds tends to modify the pH of the medium because the load of acid amino acids is exposed. A cascade reaction is then initiated, since the proteins are sensitive to the pH of the solution where they are dissolved.

The results found for the *in vitro* digestion of the lyophilized and heat-treated *in natura* samples (fig. 2)

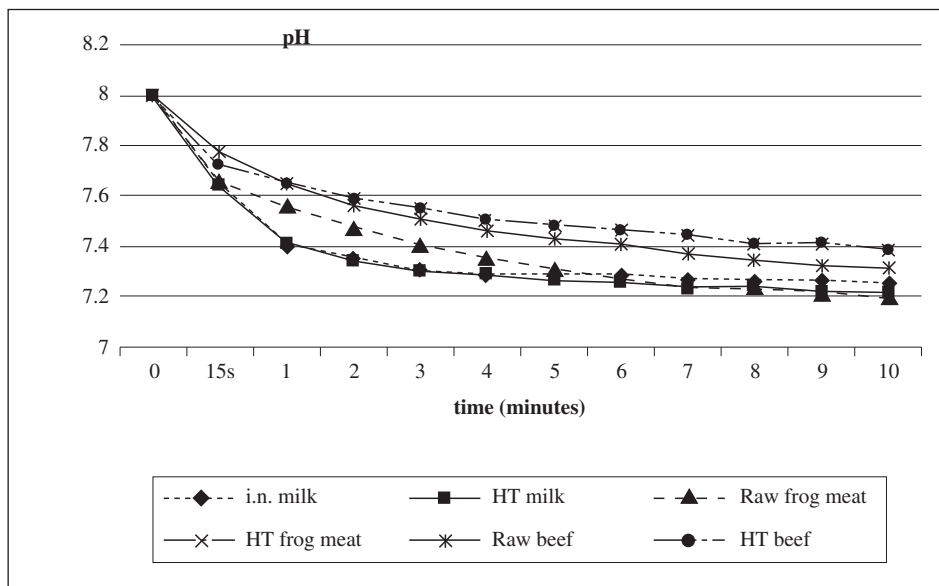


Fig. 1.—Result of the analysis by the pH decrease method after the enzyme solution was added to lyophilized samples containing in natura milk (i.n. milk), powdered milk (HT milk), raw frog meat (R Frog), cooked frog meat (HT Frog), raw beef (R Beef) and cooked beef (HT Beef).

showed no statistically significant difference ( $p > 0.05$ ). This result may be a consequence of the modifications made to proteins by both freeze drying and cooking. Proteins are known to denature, sometimes irreversibly, because of several events that affect their stability, such as heating, agitation, freezing, pH changes and beyond exposure to interfaces or denaturants.<sup>26</sup> On the whole, the values obtained for digestibility percentage (fig. 2) ranged between 80%, for powdered milk, and 69%, for heat-treated beef.

Food processing can improve food taste and texture as well as inactivate antinutritional factors. However, it can also change the primary structure of proteins leading to the oxidation of sulfur-containing amino

acids and cross-linking between peptides, which decreases the bioavailability of essential amino acids.<sup>1,4,27</sup>

Both heat treatment and long-term food storage can produce harmful effects on the nutritional quality of proteins. Changes in the nutritional value include a decrease in protein digestibility, a reduction in the bioavailability of lysine and other essential amino acids, and perhaps foster the production of substances which may be growth-inhibiting or toxic, for example, lysinoalanine. At least two mechanisms are involved in the decrease in protein quality: one of the amino acid side chains is blocked, and cross-linking occurs between peptide chains by means of condensation reactions.<sup>1,28,29</sup>

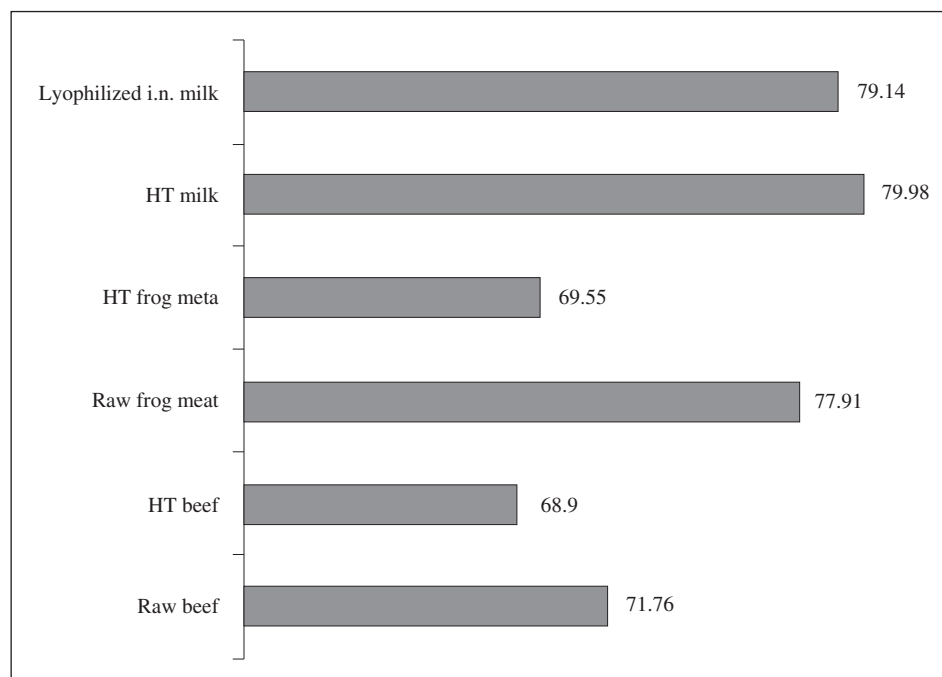


Fig. 2.—In vitro digestibility values obtained by means of polynomial equations.  $\%D = -32.841 pH^2 + 434.01 pH - 1337.7$  for milk samples and  $\%D = -230 pH^2 + 3,270.9 pH - 11,505$  for beef samples.

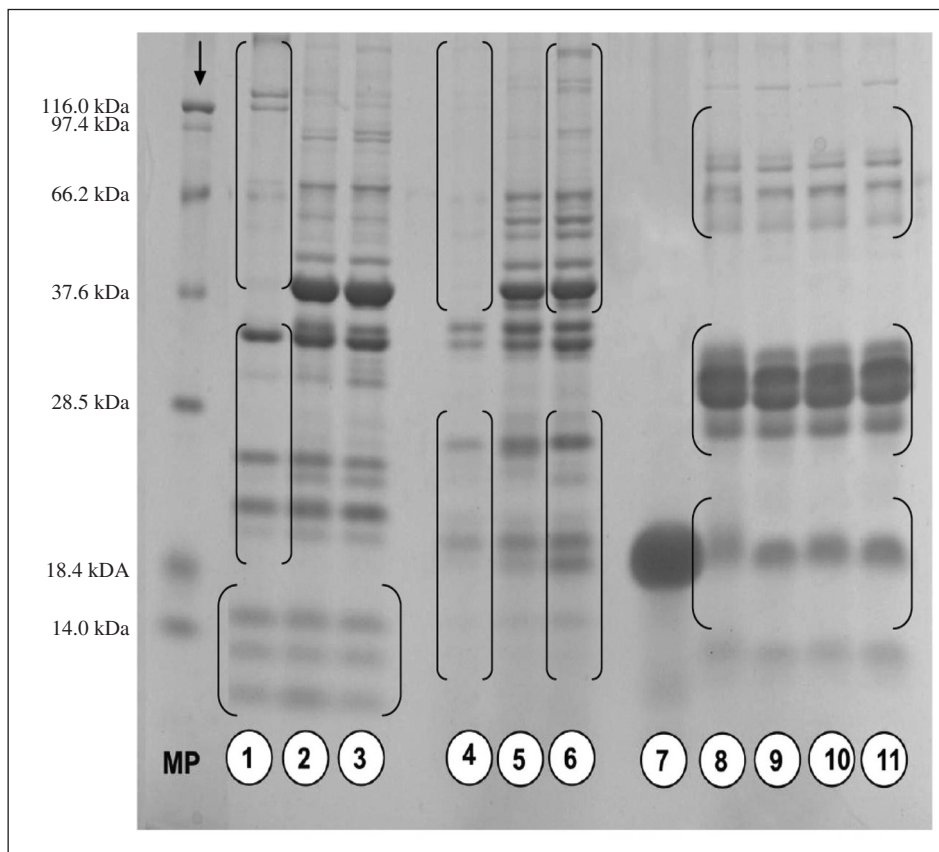


Fig. 3.—Separation of protein fractions by SDS-PAGE. MW - molecular weight marker, line 1 - heat-treated frog meat, line 2 - lyophilized raw frog meat, line 3 - raw frog meat, line 4 - heat-treated beef, line 5 - lyophilized raw beef, line 6 - raw beef, line 7 - b-lactoglobulin, line 8 - skimmed powdered milk, line 9 - pasteurized milk, line 10 - lyophilized in natura milk, line 11 - in natura milk.

Still, the values obtained for *in vitro* digestibility (fig. 2) conform with the expectations for animal proteins, since the values obtained for *in vitro* digestibility analyses are usually lower than the ones found for protein quality analyses performed in experiments with animals.<sup>30</sup>

Restani et al.<sup>31</sup> investigated different standards related to *in vitro* digestion of albumins in their allergenic potential, and discovered that 5 minutes after protease activity, there was a statistically significant reduction in the number of positive prick tests performed for BSA and ovine serum albumin (OSA), when compared to the same test performed with proteins in their native state.

To perform an approximate calculation for molecular weight (MW) values of protein bands, the correlation between MW and the distance covered by the proteins of the marker was used by means of the equation  $y = -0.0699x + 2.1663$ .<sup>32</sup>

Figure 3 shows the electroforetic behavior of proteins in the beef and milk samples analyzed by PAGE according to the different heat treatments applied.

When frog meat and the different treatments applied to it are taken into account (lines 1, 2 and 3), it is observed that the low molecular weight proteins were apparently not cleaved and seem to have remained intact when cooked or lyophilized. All proteins smaller than 28 kDa remained stable either when cooked at 95°

C for 15 minutes or lyophilized, and also when untreated.

Freeze drying can cause several structural changes in the protein spectrum. Recent studies with infrared spectroscopy have reported that problems related to lyophilization-induced freezing and dehydration can lead to the molecular unfolding of proteins.<sup>33</sup> Protein drying during lyophilization usually causes  $\alpha$ -helical structures to decrease and  $\beta$ -sheet structures to decrease and have an unstructured order.<sup>4</sup>

As regards frog meat, parvalbumins are the proteins with the greatest antigenic relevance. They have low MW (around 12 kDa) and are acid, hydrophilic and highly resistant to enzyme degradation. Parvalbumins are found in fish and amphibian muscles, and are considered to be the main allergens of such foods. Hilger et al.<sup>34</sup> reported on the implication of a-parvalbumin in a case of anaphylactic shock triggered by the ingestion of thermally-processed frog meat.

Hilger et al.<sup>35</sup> conducted another study where they tested the likelihood of cross reactivity between fish and amphibians in codfish-allergic individuals. The blood samples of the researched patients were analyzed by means of *in vitro* tests. Three out of thirteen samples reacted positively with a-parvalbumin and eleven out of twelve reacted with b-parvalbumin from *Rana esculenta*. Prick tests were also performed with recombinant parvalbumin in 5 individuals (three were fish-allergic and two were non-allergic). Positive results

were obtained for the allergic individuals, attesting the high likelihood of cross-reactivity.

Moreover, figure 3 (lines 1 and 2) shows that low MW proteins remained nearly unaltered when submitted to the treatments. However, proteins whose MW is approximately 56 and 50 kDa were apparently susceptible to cleavage when cooked, if compared to when they were lyophilized or even untreated.

Bernhisel-Broadbent et al.<sup>36</sup> investigated salmon and tuna extracts with SDS-PAGE. The result showed a remarkable loss of protein fractions when industrially processed salmon and tuna samples were compared to raw or conventionally cooked extracts. Moreover, the blind placebo-controlled trial (BPCT) confirmed a decrease in allergenicity in two salmon-allergic patients.

The fact that low MW proteins of frog meat are resistant to heat treatment suggests that ingesting cooked, lyophilized or raw frog meat may trigger allergic reactions in genetically predisposed individuals.

The analysis of the beef samples by the same method and submitted to the same treatments has evidenced protein sensitivity to cooking-induced cleavage (line 4) for both high and low molecular weight proteins, while the intermediate proteins remained unaltered. In spite of freeze drying, (line 5) protein bands whose MW is above 116 kDa and have approximately 125, 111 and 108 kDa were observed to be absent, when compared to the *in natura* sample.

The most important protein in diagnosed beef allergy cases is bovine serum albumin (BSA), whose molecular weight is 66 kDa.<sup>37,38</sup> According to Beretta et al.,<sup>32</sup> BSA and other serum albumins are also involved in cases of cross reactivity with cow milk.

Sampson<sup>39</sup> investigated beef allergy in children with atopic dermatitis, 15.9% of whom tested positive after PT was conducted. However, only 1.8% of the cases were confirmed after BPCT. Werfel et al.<sup>41</sup> obtained positive results for cow's milk allergy in 84% of the children tested through PT, but only 20% of the cases were confirmed by BPCT. Many children with positive PT results for beef are clinically tolerant of several kinds of meat because of enzyme digestion, which can modify the structural features of some food allergens.

Greater resistance to either high or low temperature thermal processing is observed when milk samples are compared to other sources of protein. Several studies have investigated the conformational and linear epitopes that constitute  $\beta$ -lactoglobulin and claim that its tertiary structure is probably of crucial importance in the immunoreactivity of the native form of this protein fraction.<sup>20,40,41</sup>

Host & Samuelson<sup>43</sup> investigated allergenic potential of milk in three different preparations: *in natura*, pasteurized at 75°C for 15 seconds and pasteurized and homogenized at 60°C (175 kg/cm<sup>2</sup>). PT and BPCT were positive in all the children tested, who were highly prone to allergenicity even for thermally processed samples.

The findings of this experiment corroborate epidemiological data on food allergy worldwide, since cow's milk allergy is much more prevalent than beef allergy in the world's population.<sup>19,39,40,43,44</sup>

## Conclusions

Heat treatment was found to be an efficient denaturant because it fosters the cleavage of proteins from food sources and can often reduce their allergenic potential. Another finding is that some proteins are more resistant to denaturing than others. Cow's milk proteins, for example, are less susceptible to thermal processing. Although frog meat ranked in between milk and beef as regards the thermal resistance of its constituent proteins, there is still much controversy in the literature as to whether or not it can be safely eaten by allergic patients. As a source of protein, beef showed the most sensitivity to the different thermal treatments applied, and hence appears to have low allergenicity. The consumption of other kinds of meat by genetically predisposed individuals has to be carefully handled and evaluated on an individual basis because no meat or milk can be considered hypoallergenic, and that cross reactivity among sources of protein poses a serious nutritional problem to children with food allergy, especially multi-allergenic ones.

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