



Control of Milk Allergenicity



Sara Sofia de Aguiar^{1,2#}, Maria Inês Veloso^{1,2#}, Ana Luísa Fernando^{1*}, Ricardo Franco²

¹Departamento de Química:REQUIMTE-UCIBIO de Ciências e Tecnologia da Biomassa, Universidade NOVA de Lisboa, Portugal

²Departamento de Química:REQUIMTE-UCIBIO, Universidade NOVA de Lisboa, Portugal

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*Corresponding author: Ana Luísa Fernando, Departamento de Ciências e Tecnologia da Biomassa, Faculdade de Ciências e Tecnologia, FCT, Universidade NOVA de Lisboa, Campus de Caparica, 2829-516 Caparica, Portugal, Tel: +351 212948543, Email: ala@fct.unl.pt

Abstract

Food allergies are classified among the largest problems of human health by World Health Organization (WHO), with 2-10% of the world's population (children and adults) being confronted with it. Milk protein allergy is one of the most common types of allergies. But milk and dairy products are widely consumed and represent not only an inexpensive and easily accessible source of protein, calcium and vitamin D, but also an important share in the world food industry economy. Here, it is presented an overview of the different approaches, tested and developed to help the dairy industry in controlling the allergenicity of these products. Special emphasis is given to protein cross linking by transglutaminase, a recent technique that has attracted increasing attention in the scientific and industrial community. In fact, it does not involve the use of chemicals, it is easy to control and it is not necessary to remove the allergenic protein after the treatment, leading to a final non-allergenic product with equivalent protein content to the original product.

Keywords: Milk; Allergenicity; Control treatments

Abbreviations: BSA: Bovine Serum Albumin; DTT: Dithiothreitol; HIFU: High Intensity Focused Ultrasound; B-Me: B-Mercaptoethanol

Introduction

Milk proteins, cause of allergy

Milk has always occupied a prominent place in the human diet [1]. It is consumed not only directly in its natural, liquid form, but also used as raw material for the production of a wide range of dairy products such as cheese, yogurt, butter, desserts, among many others. It is a highly nutritious food; it provides vitamins and minerals, from which vitamin D and calcium can be highlighted, but it stands out mainly because it is an important source of protein [2,3].

Milk also represents a source of allergies. Milk allergy is an abnormal immunological reaction to cow's milk proteins. β -lactoglobulin (the main whey protein) is responsible for 66% of milk allergies [4]. Caseins, α -lactoalbumin and serum albumin also have allergenic potential, although much less than the previous one [5]. Food allergies are considered by WHO as the sixth major public health problem, with milk allergy being one of the most prevalent (10-40%). It affects around 0.3-7.5% of the world population, especially children. It is difficult to diagnose, since the symptoms are not specific, and involve skin, gastrointestinal or respiratory problems. Other symptoms include headache, anemia, irritability and anaphylactic shock, leading to death in extreme cases [6].

Nowadays, prevention and control of milk allergy relies almost exclusively on the elimination of dairy products consumption. However, such a solution becomes quite complicated since milk proteins appear very frequently in processed products, and therefore is difficult to avoid their ingestion. Furthermore, the fact that milk is one of the most important sources of protein and calcium in the diet of the general population, its elimination can lead to a nutritional deficiency and, in the case of children and juveniles; it can affect growth [7]. Also from the industry point of view, the elimination of the consumption of dairy products is not a viable alternative, as it meant the loss of potential consumers. Thus, it is very convenient for the industry to invest in the search for techniques that allow the production of hypoallergenic dairy products, enabling its consumption by a wide range of the population, broadening their market.

Treatments Applied to Milk for Allergenicity Control

Heat treatment

One of the methods that is commonly used to reduce milk protein allergy is heat treatment. Most food processing actually involves thermal processes, being the most common ones pasteurization and sterilization. In the case of milk it

is even mandatory to apply heat treatment in order to ensure microbiological quality suitable for safe consumption.

It is known that when a dairy product undergoes prolonged heating, there are important changes in the structure of its proteins, namely denaturation. Since the organized structure of the proteins is destroyed, so are their epitopes (i.e. regions that bind to the antibodies, triggering an allergic reaction in the body), which translates into a decrease in the allergenicity of the final product. There are in fact several studies that indicate that dairy products undergoing a heat treatment trigger fewer allergic reactions. However, these treatments differ greatly in temperature and time of application. This may be related to the fact that milk proteins show great differences in their resistance to heat. Whey proteins are the most sensitive to increased temperature. It is known that, specifically within this group of proteins, α -lactalbumin is the most vulnerable, followed by β -lactoglobulin, bovine serum albumin (BSA), and finally the immunoglobulins, the most resistant. In any case, it is possible to conclude that at a temperature of 80 °C, or above, a period of at least 15 minutes is always required in order to achieve a reasonable reduction of allergenicity.

However, it should be noted that this type of processing can only reduce allergenicity, i.e. it does not completely eliminate it. There are even some studies that point to a slight increase of allergenicity after heat treatments [8,9]. This fact is explained due to epitopes that are "hidden" inside the structure of the native protein, and that, with the beginning of the heating, are exposed due to the unfolding of the protein that occurs.

In addition to this disadvantage, it has also to be taken into account that thermal processing implies a loss of both nutritional and organoleptic quality, which makes it less attractive to the consumer. For all these reasons, simple heat treatment is not the most satisfactory technique to achieve the minimization of milk allergenicity.

Enzymatic processes

Hydrolysis: Hydrolysis is known as one of the methods to reduce the allergenicity of proteins. To reduce allergenicity, proteins are broken down by the enzymes into smaller proteins or into amino acids. The proteolytic treatment may remove the conformational epitopes as a result of the loss of tertiary structure, which, consequently, may reduce the allergic potential of the proteins [10,11]. However, the enzymatic digestion of proteins may lead to the appearance of new antigenic substances [12,13].

It is the specificity of the enzyme, and not the degree of hydrolysis or the molecular weight distribution of the hydrolysates, which determine the residual antigenicity of the whey proteins. Therefore, it is essential to choose the most appropriate enzyme, taking into account the specificity of the antigenic epitopes, in order to obtain an hydrolyzed product with reduced allergenicity.

Various enzymes have already been tried, either individually or combined. Digestive enzymes were used, like trypsin, papain, neutrase, alcalase, protease S, pepsin and chymotrypsin, to simulate the digestive processes [14]. Study show that the combination of some of these enzymes is more effective compared with a single enzyme treatment. Also used are enzymes of bacterial and fungal origin, which are more specific and easier to obtain in large quantities [15]. However, enzymatic digestion of proteins may also give rise to new antigenic substances.

The combination of enzymatic hydrolysis with heat treatment, remarkably increased tryptic and peptic hydrolysis of β -lactoglobulin. This combination of treatments exposes the cleavage sites resulting from thermal denaturation and increases susceptibility to proteolysis, thereby reducing milk allergenicity [16].

Cross linking: Cross linking between the various food proteins enhances food stability. The creation of extra chemical bonds, which may occur naturally or through processing has been tested and implemented in the food industry, such as in the cereal, dairy, meat and fish processing industries [17]. Enzymes, which are used as additives, are used as protein cross linking agents because of their high specificity, mild conditions of operation and low risk of formation of toxic products.

There are several enzymes that allow the cross linking of whey proteins, such as horseradish peroxidase, phenol oxidase, sulfhydryl oxidase, tyrosinase, laccase and transglutaminase. The most widely used is transglutaminase [18] as it is the only enzyme approved by the European Union for the purpose of food marketing [19,20].

However, the modification of protein structures, widely used by the food industry, to create new and improved functional properties may also result in an opposite effect to what is desired. Structural changes on the protein content, in addition to optimizing the product to the desired extent, carry some risks as they might increase the development of foods with a greater allergenic potential. Thus, all new cross linked proteins must be tested for their allergenicity before they are released to the market.

Cross linking with transglutaminase: Transglutaminase is routinely used in the dairy industry for both economic and functional reasons. The enzyme is used in whey, more specifically, as it increases and improves the functional properties of its proteins, such as elasticity, water holding capacity, heat stability, foaming and emulsifying activity [21].

Transglutaminase, EC 2.3.2.13, catalyzes the cross linking of proteins without the need of mediators to enhance enzymatic catalysis. The enzyme can cross-link β -lactoglobulin and α -lactoglobulin through the formation of intra- and intermolecular ϵ -(γ -glutamyl) lysine bonds. In this way, there is the formation of polymers of higher molecular weight, consequently increasing the molecular weight of whey proteins that aggregate [22,23].

However, because of the compact globular structure of whey proteins, transglutaminase might not access all protein residues, decreasing the yield of the cross linking reaction. To overcome this problem it is necessary to carry out chemical or thermal pre-treatments in order to denature the proteins, and thus facilitate the access of transglutaminase to a maximum of residues, increasing the efficiency of the cross linking reaction .

Transglutaminase-cross linked whey proteins can be added to food, improving its functional properties. In the specific case of β -lactoglobulin, the polymerization of whey by transglutaminase may reduce its allergenic potential without the need to remove this protein, and therefore without losing the inherent functional properties of whey. After the cross linking reaction, the allergenic epitopes of β -lactoglobulin are hidden, and so they are no longer available to react with the immune system.

Pre-treatments before the cross linking reaction: Enzymatic cross linking when applied to β -lactoglobulin has a low reaction efficiency, since the protein has an extremely compact secondary and tertiary structure that prevent the enzyme from accessing all allergenic epitopes. In order to solve this problem, pre-treatments are applied in order to denature the protein, making the enzyme target sites more available so that the reaction can occur more extensively [24,25]. Denaturation may occur chemically or thermally [24]. Chemical denaturation occurs by the addition of chemical compounds, such as Dithiothreitol (DTT) or β -mercaptoethanol (β -Me), which cleave protein disulfide bonds. In this case, there is an imbalance in the monomer-dimer conversion, without any alteration in α -helices, in order to increase the concentration of monomers in solution. In this way the protein becomes more accessible to the action of transglutaminase.

In the thermal denaturation pre-treatment, whey is subjected to a temperature above 70 °C, promoting the dissociation of β -sheets in the native structure of the protein, leading to the formation of new intermolecular β -sheets. The structure of β -lactoglobulin becomes less compacted and therefore more available for the action of transglutaminase. It is also necessary to consider the pH of the pre-treatment that must be adjusted to 7, the optimum operating pH of transglutaminase.

Chemical treatment

Glycation and maillard reaction: Another promising method for reducing the allergenicity of milk and improve its functional properties, is conjugation of its proteins with reducing sugars by the Maillard reaction [26] have demonstrated, for example, that the conjugation of β -lactoglobulin with the oligosaccharide carboxymethyl dextran (CMD) improved the emulsifying capacity of this protein while decreasing its allergenic potential [27]. The temperatures that are usually used are between 40 °C and 60 °C [28-30]. As for the mechanisms involved in this process, it has been suggested, for example, that CMD creates a barrier that surrounds the epitopes, thus preventing their reaction with the

immune system. The effectiveness of this method depends on the quantity of conjugated saccharides, their molecular weights, and is also related to both the conditions and the extent of the Maillard reaction. However, this technique is too recent and more research is still needed in order to clarify the mechanism that leads to the reduction of protein allergenicity. Optimization of the reaction conditions is also needed.

High pressure treatment digestion: At present there are in the market hypoallergenic dairy products which were produced by chemical digestion, or hydrolysis, of the proteins. Hydrolysis can be achieved through chemical processing in which both bases and acids can be used. Acids are more effective and more traditionally used by the food industry [31].

However, the chemical digestion method presents several disadvantages, namely, hydrolysis of essential amino acids compromises the biological value of the final product. Moreover, the chemical processing also contributes to an unpleasant, saltier taste, when sodium hydroxide is added to counterbalance the effect of hydrochloric acid, after the denaturation of proteins. Nevertheless, the main reason why this method is unattractive for industrial applications is related to being less safe to the consumer than alternative methods. Additionally, the current consumer, who is much better informed, does not welcome the use of synthetic chemical compounds in their food.

Lactic acid fermentation: The hydrolysis of milk proteins by lactic acid fermentation has important effects on milk digestibility and the production of bioactive peptides. The proteolytic system of fermentation is a complex process composed of proteinases, peptides and transport systems. Proteolytic enzymes are produced during lactic acid fermentation. Proteolysis will break down some of the allergenic epitopes of proteins and will consequently decrease the allergenicity of milk.

The antigenic properties of milk proteins are reduced by various types of lactic acid bacteria in which the changes in the antigenicity and allergenicity of milk proteins depend on the bacterial species as well as on the fermentation conditions. Acid-lactic fermentation lower the antigenicity of β -lactoglobulin up to 90% in skim milk and up to 70% in whey. In a study with skim milk [32], the combination of *L. helveticus* and *S. thermophilus* strains is most efficient in reducing the antigenicity of β -lactoglobulin, with a 95% inhibition ratio, and of α -lactalbumin, with an inhibition ratio of 87%. Another study, with meso- and thermophilic lactic acid bacteria, has shown that the milk to which these bacteria were added had a reduction of about 99% in antigenicity compared to a raw milk control [33].

High pressure treatment

High-pressure treatment is a technique that was recently introduced in the food production process. High pressures are usually applied in the range of 300-1000 MPa at room temperature or slightly above it [34]. This technique was firstly

applied for the elimination of microorganisms. However, this type of treatment can also lead to structural alterations, such as denaturation and the formation of aggregates of milk proteins, which may influence their allergenic potential [35-37].

By subjecting the milk to a high pressure, 200 to 600MPa, at different temperatures in the 30 to 68 °C range, the antigenicity of β -lactoglobulin in the whey can be lowered [38]. The increased antigenicity recorded with this treatment may be associated with exposure of allergenic epitopes that are hidden in the protein molecule and that, after the high pressure treatment, become available to bind to the antibodies.

The changes caused in proteins by high pressure facilitate also their further enzyme digestion. β -lactoglobulin can be efficiently hydrolyzed by various proteins under elevated pressure, exhibiting a reduced antigenicity. Depending on the enzyme used in the treatment, when the milk is subjected to a high pressure, the hydrolysis of the whey proteins increases and consequently there is a decrease in the allergenicity of the residual hydrolysates. This reduction in allergenicity occurs because of the increased accessibility of the hydrophobic regions potentially immunogenic to the enzyme resulting in an enhancement of the hydrolysis.

Nevertheless, this procedure has disadvantages such as the formation of aggregates between denaturated proteins which precipitate and give an appearance to the final product that is not pleasant to the consumer.

Microwave

The microwave treatment applied to the food industry is a recently implemented treatment, that has been attracting growing attention over the last few years. The application of microwaves is indicated as an alternative to the thermal treatments used by the dairy industries [39-40]. Microwaves are electromagnetic waves that can heat the proteins upon interaction with them. The heating derives from the dissipation of the rotation energy states of water molecules and by movements of the ionic components of the proteins.

In this way, the influence that microwave radiation has on the structure of the proteins becomes predictable. Thus, microwave radiation is used to accelerate the hydrolysis of proteins and has been shown to be effective for mapping them through trypsin digestion. This type of radiation is used to accelerate the process of protein digestion, where traditional methods take hours, whereas microwave treatments reduce the reaction time to minutes. Microwave treatment has been used in the dairy industry since it accelerates the hydrolysis process of β -lactoglobulin thus helping to reduce its allergenic potential.

Ultrasounds

Ultrasonic treatment is an inexpensive and environmentally friendly process providing a final product that can be used in various types of foods and beverages [41]. This type of treatment, when applied on an industrial scale, allows the use

of higher powers of ultrasounds that lead to an optimization of liquid processes, disintegration of organic material or reduction of particle size. The application of ultrasounds can be done through a bath or an ultrasounds probe [42]. The ultrasonic bath, although it is a widely used method, has some drawbacks, as it uses low irradiance values, in 25 to 45kHz or 35 to 130kHz ranges, and has a heterogeneous ultrasonic intensity distribution. It is also necessary, before application of the treatment, to take into account some variables, namely,

- (i) Particle size
- (ii) Sonication time
- (iii) Frequency of sonication energy
- (iv) Temperature
- (v) The use of detergents in the water bath, which can enhance the transmission of the ultrasounds through the liquid.

The ultrasound probe is immersed directly into the solution where sonication occurs. The energy supplied by the probe is at least 100 times greater than that supplied by the ultrasonic bath. It can also be used, in conjunction with other methodologies, to decrease the allergenic potential of milk proteins.

The application of High Intensity Focused Ultrasound (HIFU) allows accelerated digestion of proteins. Here it is important to take into account (i) the diameter of the probe, (ii) enzyme concentration and (iii) the temperature of the solution where the protein digestion takes place. The application of ultrasound with the purpose of reducing the presence of allergenic proteins in milk is possible and can be advantageously extended to the food industry. This technique allows, by regulating the amplitude of the ultrasound, to control the degree of hydrolysis, taking into account the specific device used.

With the application of this treatment and the different degrees of hydrolysis, two types of products can be obtained: a complete hydrolyzate with zero allergenicity and a partial hydrolyzate with reduced or no allergenicity. This methodology also allows isolating and concentrating bioactive peptides with diverse functional applications that can range from medicinal to food industry uses.

However, this technique leads to the formation of free radicals that are harmful to health. The industry often uses food additives to reduce the formation of these radicals. Still, many of these additives can also pose a danger to the health of the consumer, and as such the utilization of this technique for allergenicity reduction of milk should be considered with great care.

Conclusion

In order to suppress, or at least reduce, the allergenicity caused by milk proteins, several methods are being developed and optimized. The treatments used are generally based on the modification of the structure of the proteins in order to mask

the allergenic epitopes present in the proteins. However, most of the studies underscore the need for combined treatments in order to optimize the results. Moreover, all the methods studied still present some limitations that might hinder their application by food industries. Several fast methods have emerged, such as the use of ultrasounds, microwaves, or high- pressure treatment, with which results can be achieved in a short time period.

Protein cross linking with transglutaminase is the technique that has received more attention lately. The use of an enzyme already accepted in the European Union for the purpose of food marketing, contributes to this fact. However, despite encouraging results from various studies, a consensus has not yet been reached on its application by the food industry, as optimal conditions for its use have yet to be defined. So far, most of the studies highlighted the need of a pretreatment that unfolds the structure of the protein so that transglutaminase functions more effectively as a cross linking agent.

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