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PRETREATMENT TECHNOLOGIES (ULTRASOUND AND IONIC LIQUIDS) IN THE ENZYMATIC HYDROLYSIS OF WHEY PROTEINS: A REVIEW

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ABSTRACT

Whey protein has been explored as a substrate to produce bioactive peptides via enzymatic hydrolysis. However, the two main whey proteins, β-lactoglobulin and α-lactalbumin are considered potential allergens that can cause allergic reactions for infants and young children. Enzymatic hydrolysis can be used for reducing the antigenicity and allergenicity of milk proteins and, thus, producing hypoallergenic formulas. It is also the main method used to obtain bioactive peptides. Different protein pretreatment methods such as the use of ultrasound technology and ionic liquids have been explored to enhance the enzymatic hydrolysis process. The main challenge is to increase the degree of protein hydrolysis and obtain short-chain peptides with high biological activity, as well as to improve the functional properties of the hydrolysates.

Keywords: Food allergy. Whey protein. Bioactive peptides. Ultrasonic. Ionic liquid.

1 INTRODUCTION

The proteins that make up milk are commonly used as a dietary source for newborns and children due to their high nutritional value.[1] However, many of them are considered potential allergens.[2] Milk is identified by the Food and Agriculture Organization of the United Nations (FAO) as one of the major allergic foods.[3] Allergic reactions occur in children, usually between the ages of 0 and 3 years, and it is estimated that 2–7.5% of children have a milk allergy.[2,3] The proteins present in milk can be divided into three groups, according to their location and solubility, namely: casein, milk fat globule membrane proteins and whey proteins.[1,4] The latter group constitutes an important source of essential and branched-chain amino acids[5] and has, therefore, been explored as a substrate to produce bioactive peptides via enzymatic hydrolysis.[6-8] However, the two main whey proteins, β-lactoglobulin and α-lactalbumin are also major allergens compounds of whey.[9]

Enzymatic hydrolysis is considered as a promising alternative for reducing the antigenicity and allergenicity of cow's milk proteins and, thus, producing hypoallergenic formulas.[3] It is also the main method used to obtain bioactive peptides, which, depending on their composition and corresponding amino acid sequence, can improve different bodily functions.[10] However, the challenge in producing these molecules is to increase the degree of protein hydrolysis and obtain short-chain peptides with high biological activity, as well as to improve the functional properties of the hydrolysates, such as solubility, emulsification, foam formation, and gelling capacity.[6,7] Therefore, different protein pretreatment methods have been explored to enhance the enzymatic hydrolysis process.[11]

This paper reviews the main aspects related to the enzymatic hydrolysis of whey proteins, as well as different pretreatment technologies (ultrasound technology and the use of ionic liquids), that have been investigated to optimize the process and improve the biological and functional characteristics of the hydrolysates.

2 WHEY PROTEIN AND BIOACTIVE PEPTIDES

Whey is the aqueous portion released from the curd during the production of cheese or casein. The protein fraction of bovine whey is composed of β-lactoglobulin (50–60%), α-lactalbumin (15–25%), immunoglobulins (<10%), bovine serum albumin (6%), lactoferrin (<3%), as well as other peptides, such as lactoperoxidase and glycomacropeptide.[12,13] Whey proteins are sources of bioactive peptides, specific fragments of a given protein that exert several physiological effects on health[6], including antioxidant, antimicrobial, anticancer, immunomodulatory, and blood pressure-lowering effects.[7,14,15] Several studies have also related bioactive peptides obtained from whey proteins with the stimulation and proliferation of human lymphocytes, phagocytic activity of macrophages, antibody production and cytokine regulation, demonstrating the immunomodulatory activity of these molecules.[16]

3 ENZYMATIC HYDROLYSIS OF PROTEINS

Protein hydrolysis is one of the main methods used to produce bioactive peptides and can occur chemically or enzymatically. The chemical route, which can be acidic or alkaline, is less used because it is more difficult to control the reaction. On the other hand, enzymatic hydrolysis has been gaining prominence due to its shorter reaction time and because it does not leave traces of solvent in the product. In enzymatic hydrolysis, enzymes promote the breakdown of peptide bonds, in addition to accelerating the reaction. The extent of proteolysis is defined by the degree of hydrolysis (DH), which refers to the percentage of breakdown of these bonds.[17] The main parameters that influence this process and, consequently, the characteristics of the hydrolysate are the choice of enzyme, which determines the specificity of the cleavage of protein bonds, the enzyme/substrate ratio, pH, temperature, hydrolysis time and total solids present during the reaction.[18] Different technologies have been developed for the treatment of

proteins before or during enzymatic hydrolysis, significantly improving the degree of hydrolysis and obtaining bioactive peptides. Such processes contribute to the unfolding of proteins, favoring the access of enzymes to peptide bonds, without altering their functional and nutritional properties.[19,20]

Zheng et al.[5] used a combination of the enzymes alcalase and neutrase (1:1 w/w) to promote the hydrolysis of whey proteins (whey protein concentrate - WPC 80). The researchers observed that DH increased significantly over time, with a higher reaction rate in the first 60 minutes. DH also increased with increasing enzyme concentration, however, DH decreased considerably with increasing substrate concentration. According to the authors, this reduction was due to the greater number of intact peptide bonds in the proteins. Furthermore, there was an increase in the viscosity of the substrate, which contacted the enzyme difficult.[5] Similar results were presented by Dermiki and Fitzgerald[21] who found a decrease in the activity of the enzymes alcalase and neutrase with increasing total solids content in the reaction, and by Lorenzetti et al.[8], who reported a greater interaction of the enzymes papain and pepsin with the substrate with increasing enzyme/substrate (E/S) ratio. Regarding temperature and pH, according to Kleekayai et al.[18] these are parameters that have a great influence on the denaturation of whey proteins, especially at temperatures above 60 °C and higher protein concentrations. Ballatore et al.[10] found a positive influence of increased temperature and E/S ratio on the enzymatic hydrolysis of whey proteins by trypsin, since this is an endothermic reaction. However, at high temperatures, the proteins began to denature.

4 ULTRASOUND TECHNOLOGY

Ultrasound (US) is a technology based on the propagation of mechanical waves with a frequency of 20 kHz or higher, which induces the phenomenon of acoustic cavitation. This involves the formation, expansion, and continuous collapse of microbubbles in the liquid medium, which contributes to greater mass transfer in the system and alters the structure of proteins, favoring the access of enzymes to peptide bonds.[3,11] Lorenzetti et al.[8], for example, promoted the hydrolysis of whey protein isolate (92.7% crude protein) using the enzymes papain or pepsin and applying pretreatment with US. The ultrasonic frequency used was 20 kHz and sonication occurred for 4 min at 400 W for pepsin and 2 min at 300 W for papain. In hydrolysis with papain, the researchers obtained a DH equal to 20.3% in 2 hours, a value like that obtained in 8 hours without ultrasonic treatment, thus achieving a reduction of 6 hours in the process. In hydrolysis with pepsin, pretreatment with US did not modify the DH for the same process and enzyme conditions, which was attributed to the high specificity of this enzyme for the protein isolate or to its greater enzymatic activity when compared to papain.[8]

Wu et al.[22] analyzed the effect of ultrasound power and treatment time on the DH of whey proteins. For this, sonification was performed at a frequency of 20 kHz and different power levels. The optimal power was then determined the proteins were subsequently hydrolyzed by alcalase. DH increased with increasing power up to 300 W and with increasing sonication time up to 15 min. Above these values, however, there was a decrease in this parameter. According to the authors, sonication caused changes in the secondary structure of the proteins, causing more cleavage sites to be exposed, thus allowing better contact with the enzyme. The authors also found that the surface hydrophobicity of whey proteins increased by 62.6% after pretreatment with US, indicating the unfolding of the molecules. Another indication of the unfolding of the protein molecules promoted by pretreatment was the increase in the free sulfhydryl (SH) content on the surface. The total free SH content, however, was not modified, demonstrating that the disulfide bonds of the whey proteins were not affected. In addition, Wu et al.[22] reported that the α-helix was the main secondary structure in proteins and that its content decreased with sonication, which contributed to molecular unfolding. According to Zhang et al.[3], the conformational changes promoted by ultrasonic pretreatment make the protein more susceptible to hydrolysis. Abadía-García et al.[6] investigated the production of ACE inhibitor peptides using ultrasonic pretreatment. According to the researchers, US pretreatment decreased the fractions of peptides with molecular mass greater than 10 kDa and between 5 and 10 kDa, while increasing the fractions with lower molecular mass. The results demonstrated that the peptides released using sonication before hydrolysis presented greater.

5 IONIC LIQUID PRETREATMENT

Ionic liquids (ILs) belong to a class of salts consisting of large and asymmetric structural organic cations and small inorganic anions.[3] These compounds have a melting point below 100 °C and often close to room temperature. In addition, they have environmentally friendly characteristics, among which the following stand out: non-flammability, thermal and chemical stability, low vapor pressure and recycling potential. When used in the pretreatment of proteins, ILs cause the structure of the molecule to be stretched and repolymerized, and thus the protein aggregate is released. In this way, the rate of binding of the substrate to the enzyme during hydrolysis is increased.[3] Furthermore, these solvents can be used to modify enzymes, presenting different advantages, such as high reaction conversion rates, greater selectivity, better recovery, and increased enzyme activity and stability.[23]

Zhang et al.[3] used ionic liquid pretreatment together with ultrasound to optimize the enzymatic hydrolysis of whey proteins in order to reduce the antigenicity of the hydrolysate. The researchers prepared a 10% (mass/volume) whey protein solution (WPC 80) and added the same volume of IL 1-butyl-3-methylimidazole hexafluorophosphate ([BMIM]PF6) solution. The sample was then sonicated at 300 W for 15 min, and then the substrate was hydrolyzed using the enzymes papain and alcalase.The researchers reported that there was a significant increase in the initial rate of hydrolysis after pretreatment. The DH reached 18.52% for hydrolysis with alcalase and 23.67% for hydrolysis with papain, being, respectively, 36.80% and 32.60% higher than the DH of whey proteins hydrolyzed without pretreatment. In addition, there was a significant reduction in the antigenicity of the proteins after hydrolysis. The antigenicity of α-lactalbumin and β-lactoglobulin decreased by 68.54% and 66.58%, respectively, after

hydrolysis with alcalase, and by 52.83% and 56.54%, respectively, after hydrolysis with papain. Compared with the samples hydrolyzed without pretreatment, there was a significant increase in the decline rate of protein antigenicity. According to the authors, this reduction in antigenicity can be attributed mainly to the reduced size of the peptides after hydrolysis, since the probability of a short peptide containing complete antigen epitopes decreases considerably. In fact, the hydrolyzed samples presented a higher content of short peptides, with the fraction of peptides with molecular weight less than 1500 Da representing 68.33% of the hydrolysate after reaction with alcalase and 97.17% after hydrolysis with papain.[3]

Although it presents promising effects, the use of ionic liquids to improve the protein hydrolysis process, particularly whey proteins, is still little explored. However, other protein substrates have already been hydrolyzed in the presence of ILs.[23,24] Fan et al.[24], for example, evaluated the hydrolysis of casein using the enzyme lumbrokinase and different ionic liquids. The results indicated that the effects of ILs on hydrolysis depend on the concentrations used. The presence of IL at low concentrations considerably increased the activity of lumbrokinase due to the increase in the hydrophilicity of casein by the formation of the IL-casein complex, which made the substrate more accessible to the enzyme. However, at high IL concentrations, the authors found a reduction in the activity of the enzyme, resulting from the disturbance of the hydrogen bond network in the active site of lumbrokinase. In addition, there was formation of micelles, which possibly incorporated casein, reducing its free concentration.

6 CONCLUSION

Whey is rich in proteins with high nutritional value and has been shown to be an excellent source to produce bioactive peptides through enzymatic hydrolysis. Bioactive peptides have shown antioxidant, antidiabetic, and blood pressure-lowering effects, among others, and can act directly on the human body, controlling and reducing the risk of diseases. Different hydrolysis parameters, such as time, enzyme/substrate ratio, temperature, and pH, can be optimized in order to favor the breaking of peptide bonds and the release of the peptides of interest. However, despite being an already consolidated method, enzymatic hydrolysis can also be improved by combining it with different protein pretreatment technologies. Among these, the use of ultrasound and ionic liquids pretreatment improves the degree of protein hydrolysis, as it promotes the unfolding of the protein structure, thus facilitating the access of enzymes to the substrate. Consequently, it favors the production of bioactive peptides with low molecular weight, an important characteristic for the application of hydrolysates with therapeutic and nutritional functions. The discussion on the different methods for protein pretreatment opens space for a wide range of studies, to optimize and make viable the industrial application of these technologies.

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