Chapter 3

Effect of High-Pressure Technologies on Enzymes Applied in Food Processing

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Additional information is available at the end of the chapter

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Abstract

High isostatic pressure (HIP) and high-pressure homogenization (HPH) are considered important physical technologies that able to induce changes on enzymes. HIP and HPH are emerging food processing technologies that involve the use of ultra high pressures (up to 1200 MPa for HIP and up to 400 MPa for HPH), where the first process is based on the principle that the maintenance of a product inside vessels at high pressures induces changes in the molecules conformation and, consequently, in the functionality of polysaccharides, proteins and enzymes. To the contrary, for HPH process, the high shear and sudden pressure drop are the responsible phenomena for the changes on the processed product. This chapter aims to evaluate comparatively the effects of HIP and HPH on the activity of enzymes currently applied in food industry and to identify the main structural changes induced by each process. The overall evaluation of the results shows that mild conditions of both processes were recently highlighted as able to improve the activity and the stability of several enzymes, whereas extreme process conditions (pressure, time and temperature) induce enzyme denaturation with consequent reduction of biological activity. Considering the complexity and diversity involved in the enzyme structure and its ability to react, it is not possible to determine specific conditions that each process is able to promote increase or reduction of enzyme activity, being necessary to evaluate HIP and HPH for each enzyme. Finally, in terms of molecular structure, the effect of HIP and HPH on enzymes can be explained by the alterations in the quaternary, tertiary and secondary structures of enzymes, which directly affects its active site configuration.

Keywords: emerging technologies, high pressure processing, high isostatic pressure, high pressure homogenization, food processing, food enzymes, enzymatic activity, molecular structure

1. Introduction

Enzymes are globular proteins that catalyze biochemical reactions. This occurs due to the spatial configuration of the enzymes and catalytic site, which is determined by the quantity and sequence of amino acids and the organization of these chains, with folds and twists induced by attractions and repulsions among the near amino acids, resulting in a structure with minimal energy content [1].

The enzyme reaction occurs due to the interaction of the catalytic site and substrate, forming a complex enzyme-substrate following by the product formation [2]. The maximum velocity of this reaction occurs at specific pH, temperature and salt concentration and, at non-optimum conditions, the enzyme reaction is highly affected [3]. However, in many times, the desirable industrial process conditions are different of the optimum enzyme activity, making difficult the industrial application. Additionally, high costs and low stability limits the extensive use of enzymes in many processes.

Several chemical and physical methods were studied aiming to improve the performance of enzymes, withdrawing limitations and consequently increasing the range of application. Among the physical methods proposed, high isostatic pressure (HIP) and high pressure homogenization (HPH) are considered an important way to induce interesting changes on enzymes [4, 5]. HIP and HPH are emerging food processing technologies that involve the use of pressures up to 1000 MPa for HIP and up to 400 MPa for HPH to cause desirable changes in food and other products. The HIP process is based on the principle that the maintenance of a product inside vessels at high pressures induces changes in the molecules. To the contrary, for HPH process, the high shear and sudden pressure drop are the responsible phenomena for changes on the processed product.

The overall evaluation of the results obtained for many authors shows that mild conditions of both processes were able to improve the activity and the stability of several enzymes, whereas extreme process conditions (pressure, time and temperature) induce enzyme denaturation with consequent reduction of biological activity [4–9]. Considering the complexity and diversity involved in the enzyme structure and its ability to react, it is not possible to determine specific conditions that each process is able to promote increase and reduction of enzyme activity, being necessary to evaluate HIP and HPH on each enzyme.

In terms of molecular structure, the effect of HIP and HPH on enzymes can be explained by the alterations in the quaternary, tertiary and secondary of enzymes, which directly affects the enzymes active site configuration, inducing exposure of hydrophobic amino acids, exposure of SH groups due to unfolding of the protein, a reduction in the total SH content due to new disulfide bonds formation and changes in the α-helix, β-sheet and β-turn ratio composition due to alterations of the secondary structure [4–6, 10, 11]. However, the occurrence of these phenomena—sequence of occurrence, intensity and required pressure—might be different for HIP and HPH.

The impact of each process on enzymes was evaluated by few published revisions [4, 6, 12], however, no one dedicated to compare the effect of HIP and HPH on the main enzymes used

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in the food processing, aiming to describe the differences among the process parameters and its consequences in the performance and structure of processed enzymes. Moreover, no revisions have already evaluated the impact of these processes on enzyme in different matrixes. Therefore, this chapter aimed to evaluate comparatively the effects of high isostatic pressure and high pressure homogenization in the activity of enzymes currently applied in food industry and to identify the main structural changes induced by each process pressure on the enzymes. Additionally, this work will be useful to challenge the scientific community to fulfill the information gaps in this area and for the industry, that will have access to a comparative evaluation of these two technologies, being an important way to decide which technology is better to be applied in order to have satisfactory results for different enzymes.

2. High isostatic pressure and high pressure homogenization technologies

The high isostatic pressure (HIP)—also known as high hydrostatic pressure or high pressure processing—and high pressure homogenization (HPH)—also called as ultra-high pressure homogenization or dynamic high pressure—are emerging process initially developed for food preservation by inactivation of microorganisms, with lower sensory and nutritional changes compared with the thermal process [12]. However, the studies of the consequences of these processes on food matrix highlighted that they were also able to induce changes on the food constituents, allowing the development of new applications and products/ingredients [13].

2.1. High isostatic pressure—principle and operation

The industrial application of HIP technology started in Japan in the early 1990s and has been gaining popularity and acceptance worldwide. Combining the interests of industry and consumers for these products, the development of new equipment with higher capacities helped to reduce costs and expand both the purchase intent of consumers (due to the perception of high quality products) as the search for new industries by new products, favoring the expansion of this technology [14].

The HIP is usually applied by subjecting the food, commonly vacuum sealed in flexible packaging, at a pressure up to 1000 MPa (10,000 bar), for a pre-defined time and at determined temperature to obtain the desired goal for each product. This process can be used to process liquid, semi-solid, or solid food. Equipment of higher volumes (687 L or 525 L) reaches pressures up to <310 MPa [15] or 600 MPa [16], respectively and temperatures up to <50°C, whereas equipment of lower volumes (<150 L) reaches temperatures up to 95°C and pressures up to 700 MPa [15]. The lab scale equipments reaching extreme pressures and temperature (900 MPa/110°C/chamber of 5L or 1400 MPa/110°C/chamber of 35 mL) [17]. During pressurization, the pressure is transferred instantaneously and evenly throughout the food (isostatic principle), regardless of the size and geometry of the product [18]. This is considered the main advantage of HIP processing, since there is no an equivalent to the so-called point of lower heating rate or "cold point" as in the case of heat conductive processes.

A typical system of HIP consists of a pressure chamber with closure and pressure generating system. Generally, it also have an apparatus coupled to the temperature control of the chamber. The batch process has three stages: the indirect pressurization using a liquid of low compressibility (e.g. water), the retention time at the desired pressure and depressurization. Semi-continuous processing can be obtained using multiple sequential chambers connected in series; while some cameras are under pressurization, others are being pressurized, unloaded, or loaded [14].

The pressurization is accompanied by a uniform temperature rise as consequence of the adiabatic heat of compression, being this specific to each compound [18]. For example, at 25°C, the water increases 3–5°C to every 100 MPa [18]. The adiabatic heating is completely reversed after the release of pressure. Although this temperature increase is relatively small, it can substantially contribute to the lethality of microorganisms in the overall process, resulting in significant implications when pressure is applied at elevated temperature [19]. On the other hand, this temperature increase can impact food structure, changing polysaccharides and proteins do to thermal effects. Therefore, when undesirable effects are observed due to adiabatic heating, the processes need to be carried out at lower temperature.

In molecular terms, the HIP breaks noncovalent bonds, such as ionic and hydrophobic bonds, but has little effect on covalent bonds. As a result, large biomolecules such as proteins and polysaccharides are affected by changes in its secondary, tertiary and quaternary structures (depending on the applied pressure), but small molecules are usually unaffected [6]. As the color components, flavor and vitamins are small molecules, the HIP process has little effect on these molecules in the food [6]. Furthermore, the process of pressurization followed the principle of "Le Chatelier," inducing a reduction in molecular volume and, consequently exponentially accelerating the occurrence of reactions favored by these conditions [20]. Thus, the rates of the chemical or physical reactions resulting in lower volume products are accelerated by the HIP, whereas the reactions that result in an increase in the total volume are retarded.

2.2. High pressure homogenization–Principle and operation

High pressure homogenization (HPH) is a nonthermal physical process applied for fluid foods [21]. This technology was introduced in the food field in the 1980s to improve the homogenization efficiency and emulsification of dairy products and emulsions, showing the same principle of operation of conventional homogenizers, however, using pressures around 10–15 times higher than usually applied, i.e., pressures up to 350 MPa [12].

In equipment, fluid is forced to pass through a homogenizing valve at high pressures [21]. The passage through the narrow gap (of micrometer order) and the abrupt decompression of the fluid generate an increase in speed (between 150 and 300 ms^{-1}) [22] and an increase in temperature (about 1.5–2.5°C every 10 MPa pressure increase) due to the intense friction in the homogenizing valve region [12]. In addition to shear effects, the fluid undergoes an intense drop of pressure, turbulence and cavitation, what leads to microbiological inactivation and modification of the constituents of the food [12, 13, 21, 22].

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The main changes on food constituents are related to disruption of lipids globules, reduction of molecular weight of linear polysaccharides and modification of the quaternary and tertiary structure of proteins [4, 5, 13]. For some applications, these effects are positive and, therefore, the HPH emerged as a suitable operation to improve the versatility of biomolecules (such as polysaccharides and proteins) as food ingredients [13].

An important drawback of HPH technology is the difficult to be industrially implemented to do the small flow capacity of the available equipment at high pressures. Nowadays, the industrial equipment operates at pressures up to 150 MPa with maximum flow rates of 5.000 L/h [23], while equipment that reaches higher pressures (up to 400 MPa) work at maximum flow of 240 L/h [17]. However, due to the high industrial interest, new equipment with higher capacity has been developed, allowing industrial application of this technology.

3. Effect of high isostatic pressure and high pressure homogenization technologies on the activity and molecular structure of the food enzymes

The effects of HIP and HPH on enzymes were firstly studied considering the requirement of several enzymes inactivation (pectin methylesterase, polyphenoloxidase and peroxidases) to guarantee food product stability during storage [6], since these technologies were initially proposed to replace thermal processing in industrialized food [13, 18]. The results obtained in part of these researches showed that enzymes subjected to HIP or HPH processing had a behavior different from the commonly observed for thermal treatment, with activation at low pressures [4–13, 24]. Based on these results, both processes started to be considered as an interesting tool to change the performance of enzymes with commercial interest and several authors dedicated to study these effects on different enzymes, approaching functional and structural enzyme changes induced by HIP and HPH processing [4–13].

3.1. Enzymes activity

The results obtained by the authors who started to study the effect of HPH and HIP on enzymes of commercial interests corroborate the initial founds obtained with endogenous and deleterious enzymes of fruits, i.e., HIP and HPH could modify the activity of enzymes, inducing an activation at lower pressures and inactivation at higher [4–11, 24–26]. This behavior has been observed for the majority of studied enzymes, however, some of them showed high baroresistance, being not or almost not modified by HIP or HPH [27]. Moreover, although inactivation/activation be commonly observed by HPH and HIP, the level of pressure required for each process promotes that these changes are different, being the level of pressure in HPH necessary to induce inactivation lower than the normally required in HIP [4, 6]. Therefore, it is possible to suppose that the sample energy gain induced by the shear stress, cavitation and turbulence caused by the abrupt pressure dropped in HPH is higher than that induced by the maintenance of the same sample at the same pressure.

In addition to the process pressure, the effect on enzymes is also affected by the inlet temperature of the sample in the homogenizer and number of cycles applied in HPH [28] and

by time, temperature and number of compression cycles in HIP [6]. For HPH, it is normally observed that increase of inlet temperature reduces the level of pressure required to start enzyme inactivation, probably due to the association of homogenization with thermal effects in the homogenization valve, while the effects of multiple cycles of homogenization are minimum for the majority of enzymes evaluated, i.e., the higher activity alteration occurs at the first cycle of pressurization [28]. For HIP processes, the majority of the enzymes show higher enzyme inactivation as higher were the temperature and time applied, being the effect of temperature more pronounced than the time effects [6]. Furthermore, in respect to the application of pulsed pressure cycles, it was observed that activation is probably stimulated when sort cycles (pulses) are applied at activation pressures, whereas inactivation rates increase when cycles are applied at inactivation pressures [29].

Other factors that also affected the effect of HIP and HPH on enzymes are the media used for enzymes solubilization (pH, salt concentration), presence or absence of substrate and enzyme concentration [4, 6, 8, 9]. The pH and salt concentration changes the native conformation of the enzyme, i.e., the enzyme can be processed at different initial configuration (high or less exposure of structure and active sites), impacting the process final effect [8]. Commonly, the use of acid pH has a synergic effect with pressure processing, reducing the activity of processed enzymes [6, 8]. The presence the subtract allows the occurrence of enzyme reaction under pressure (for HIP) or the process of enzymes partially configured as enzyme-substrate complex for both technologies, which alters the effect of process on enzyme and on the reaction products formation [30]. In this case, the observed effects are diverse, being not possible to establish a tendency of activation/inactivation induced by the presence/absence of substrate. Finally, the enzyme concentration can alter the effect of HPH processing, since higher enzyme concentration allows the occurrence of shear stress not only between molecules and equipment wall but also between molecules [31]. Therefore, it is possible to generally say that the HPH effects (enzyme activation or inactivation) are directly proportional to the enzyme concentration.

Another important point is that the impact of HIP and HPH on products can be measured directly on enzyme activity or in the changes caused by enzyme in the products (e.g. fruit products browning or softening). When enzyme activity was measured in laboratory using enzyme activity assays, several researches showed that higher increase of enzyme activity were observed at pH and temperature different from the optimum established for native (non-processed) samples [4, 8, 9, 32], indicating that process can alter the optimum conditions of enzymes, which can be very interesting from the industrial point of view. Additionally, when the enzyme is endogenous in the processed product, high differences in activation/inactivation level caused by process are observed, highlighting: (i) the importance of matrix and consequently the reaction media characteristics and (ii) the difficulty to compare results obtained for the same enzyme in different products or using different methods of enzyme activity quantification. These factors directly impact the establishment of general rules about the effect of HIP or HPH on enzymes, being necessary the enzyme evaluation in each food matrix and specific activity measurement conditions to determine the real impact of both processes on the enzymes. **Table 1** shows the results obtained for enzyme activity of the main food processed by HIP and HPH.

Matrix	Enzyme	Effect of HIP on enzymatic activity	Effect of HPH on enzymatic activity
		Fruit and vegetables	
Tomato juice	PME	Reduction of 70% at 800 MPa/15 min/65°C [33]	
	PG	Reduction of 100% at 500 MPa/10 min/55°C [33]	
	LOX	Reduction of 100% at 550 MPa/12 min/20°C [34]	
	HPL	Reduction of 80% at 650 MPa/12 min/20 °C [34]	
Orange juice	PME	Reduction of 50% at 400 MPa/90 min/30°C [2] Reduction of 93% at 450 MPa/30 min/50°C [35] Reduction of 100% at 650 MPa/3 min/25°C [36]	Reduction of 20% at 170 MPa/T inlet of 25°C [37] Reduction of 50% at 250 MPa/T inlet of 45°C [38] Reduction of 75% at 150 MPa at 68°C for 15 s [39] Reduction of 90% at 150 MPa at 68°C for 10 s [40] Reduction of 96% at 300 MPa/T inlet of 20°C [41]
Strawberry pulps/ purée	BGL	Activation of 16.6% at 400 MPa/20 min/25°C and reduction of 41.4% at 600 MPa/25 min/25°C [42]	Indicative of residual enzyme activity for color change [43]
	POD	Reduction of 74.6% at 500 MPa/25 min/25°C [42]	
	PPO	Reduction of 51.5% at 600 MPa/25 min/25°C [42]	
	POD	Reduction of 50% at 500 MPa/15 min/50°C [44]	
	PPO	Reduction of 72% at 500 MPa/15 min/50°C [44]	
Wild berry pulp	POD	Activation of ~182% at 200 MPa/5 min/20°C [45]	
	PPO	Activation of ~156% at 200 MPa/5 min/20°C [45]	
Apple juice	PPO	Reduction of 91% at 450 MPa/60 min/50°C [35] Reduction of ~51% at 750 MPa/50 min/50°C [46] Activation of ~120% at 200 MPa/1 min/25°C and reduction of ~95% at 900 MPa/1 min/25°C [47]	Reduction of 100% at 300 MPa/T inlet of 4 °C [48]
	POD	Activation of ~70% at 500 MPa/1 min/25°C and reduction of ~95% at 900 MPa/1 min/25°C [47]	

Matrix	Enzyme	Effect of HIP on enzymatic activity	Effect of HPH on enzymatic activity
	Amylase	Reduction of 90% at 400 MPa/30 min/22°C [49]	
	PME	Reduction of 99.7% at 400 MPa/180 min/25°C [50] Reduction of ~81% at 750 MPa/90 min/50°C [46]	Reduction of 100% at 300 MPa/T inlet of 4°C [48]
Banana juice	Pectinase	–	Activation of ~262% at 100 MPa/T inlet of 4°C [51]
Kiwifruit juice	POD	Activation of ~10% at 200 MPa/20 min/10°C and reduction of ~70% at 600 MPa/30 min/50°C [52]	
Litchi juice	POD	Activation of ~125% at 300 MPa/1 sec/25°C and reduction of ~30% at 600 MPa/45 min/25°C [29]	
	PPO	Activation of ~30% at 300 MPa/1 sec/25°C and reduction of ~30% at 600 MPa/45 min/25°C [29]	
Litchi-based mixed fruit beverage	POD	Reduction of ~80% at 480 MPa/10 min/70°C [53]	
	PPO	Reduction of ~80% at 550 MPa/10 min/70°C [53]	
	PME	Reduction of ~80% at 370 MPa/10 min/70°C [53]	
Pear	POD	Activation of ~23% at 600 MPa/3 min/40°C and reduction of ~92% at 600 MPa/5 min/100°C [54]	
	PPO	Reduction of ~90% at 600 MPa/3 min/100°C [54]	Activation of ~83% at 180 MPa/T inlet of 25°C [10]
	PME	Reduction of ~83% at 600 MPa/1 min/100°C [54]	
Mushroom	PPO		Activation of ~11% at 110 MPa/T inlet of 25°C [11]
Cocoyam	POD	Reduction of ~35% at 600 MPa/5 min/25°C [30]	
	PPO	Reduction of ~10% at 600 MPa/5 min/25°C [30]	
Peruvian carrot	POD	Reduction of ~40% at 600 MPa/5 min/25°C [30]	
	PPO	Reduction of ~70% at 600 MPa/5 min/25°C [30]	
Sweet potato	POD	Activation of ~15% at 600 MPa/5 min/25°C [30]	

Matrix	Enzyme	Effect of HIP on enzymatic activity	Effect of HPH on enzymatic activity
	PPO	Activation of ~48% at 600 MPa/5 min/25°C [30]	
		Meat and fish	
Rabbit muscles	m-Calpain	Reduction of 92% at 300 MPa/5 min/2°C [55]	
	μ-Calpain	Reduction of 98.4% at 300 MPa/5 min/2°C [55]	
	Calpastatin	Reduction of 95.7% at 300 MPa/5 min/2°C [55]	
Pork	Acid lipase	Activation of ~40% at 150 MPa/20 min/40°C [56] Reduction of ~100% at 600 MPa/20 min/50°C [56]	
	Neutral Lipase	Activation of ~10% at 150 MPa/20 min/<40°C [56] Reduction of ~100% at 450 MPa/20 min/55°C [56]	
	Phospholipase	Activation of ~10% at 300 MPa/20 min/40°C [56] Reduction of ~100% at 750 MPa/20 min/50°C [56]	
	Lipoxygenase	Activation of ~30% at 300 MPa/20 min/30°C [56] Reduction of ~100% at 450 MPa/20 min/55°C [56]	
Atlantic salmon	Acid phosphatase	Reduction of ~40% at 500 MPa/2 min/8-9 °C [57]	
Atlantic cod		Reduction of ~30% at 500 MPa/2 min/8–9°C [57]	
Mackerel		Reduction of ~20% at 500 MPa/2 min/8–9°C [57]	
Lean meat	Cathepsin D	Reduction of ~75% at 500 MPa/5 min/2°C [58]	
	Acid phosphatase	Reduction of ~15% at 500 MPa/5 min/2°C [58]	
	Cathepsin B	Reduction of ~17% at 500 MPa/5 min/2°C [58]	
	Cathepsin H	Reduction of ~85% at 500 MPa/5 min/2°C [58]	
	Cathepsin L	Reduction of ~20% at 500 MPa/5 min/2°C [58]	
	Aminopeptidase B	Reduction of ~78% at 500 MPa/5 min/2°C [58]	

Matrix	Enzyme	Effect of HIP on enzymatic activity	Effect of HPH on enzymatic activity
		Milk and dairy	
Milk	Alkaline phosphatase	Reduction of 100% at 800 MPa/8 min [59]	Activation from 100 to 150 MPa and inactivation above 175 MPa [60]
	Plasmin	Resistant at 400 MPa/30 min/25°C and reduction of 87% inactivation of plasmin at 400 MPa/15 min/60° C [61, 62]	Resistant up to 200 MPa [63]
	Lactoperoxidase	50% reduction after 800 MPa/4 hours/60°C [59]	Increased activity at 75 MPa [64]
	Lysozyme	Very resistant [65]	Activity increased at 75 MPa [64] Increase activity at 100 MPa [66]
	Lipases	350 and 400 MPa up to 100 min presented an increase up to 140% in the activity [67]	Increase in lipolysis during ripening (200 MPa, <58°C) or no effects (200 MPa, >71°C) [68, 69]
	Lactoferrin		Increase activity at 100 MPa [66]
Cheese	Proteases and lipases	Accelerating or delaying the ripening process depending on the conditions and microbial cultures [70, 71]	–
		Cereals and legumes	
Green beans (crude extract)	LOX	Reduction of ~50% of activity after 500 MPa/~20°C/10 min [72]	
Citrate and TRIS-HCl buffer (pH 4 -9)	LOX from soybeans	Reduction of >90% of activity after 600 MPa/45°C/2.5 min in all evaluated pH [73]	
Barley flours	Amylase	Increase of activity between 300 and 600 MPa/10–20 min (starch gelatinization) Inactivation at pressure > 600 MPa/10 min/room temperature [74]	
Wheat flour	Amylase	Increase of activity between 300 and 600 MPa/10–20 min (starch gelatinization) Inactivation at pressure >600 MPa/10 min/room temperature [74]	

PME, pectin methylesterase; PG, polygalacturonase; POD, peroxidase; PPO, polyphenoloxidase; BGL, β-glucosidase; LOX, lipoxygenase; HPL, hydroperoxide lyase.

Table 1. Enzymatic activity of the enzymes present in foods processed by HIP and HPH.

3.2. Enzymes structure

Relatively few works focused on the evaluation of the impact of HIP and HPH on enzymes structure, aiming to explain the alterations induced by these non-thermal processes on enzyme activity [4, 5], which includes activation, inactivation, or no change. These changes can be affected by multifactorial effects, including enzyme, substrate, media characteristics and process conditions,

as described in item 3.1. Therefore, considering that enzymes alteration by HIP and HPH is an emerging field of research and the complexity of the question (due to the phenomena be governed by multifactorial effects), there is not a conclusive theory about the relationship between structural changes induced by HIP and its consequent activity changes.

The hypotheses, which discuss the impact of HIP or HPH on enzymes, consider two possible alterations: (i) changes on enzyme structure—mainly on quaternary and tertiary ones, with consequent exposure or entrapment of active site [75] and (ii) split of latent isoenzymes due to pressurization [6]. Additionally, HIP processing is necessary to consider the increase of enzyme reactivity under pressure, when enzyme and substrate are processed together [6].

The change on enzyme structure was evidenced by several studies [4–6, 10, 11, 76] that showed that pressure processing: (i) increase the exposure of hydrophobic amino acids, increasing the hydrophobicity of enzymes surface [11, 66, 76]; (ii) increase the exposure of SH groups due to unfolding of the protein and reduced the total SH content, due to new disulfide bonds formation [11, 76]; and (iii) slightly changes the content of α-helix, β-turn, β-sheet and random coil of processed enzymes, indicating that in some cases the processes can alter the secondary structure of enzymes [11]. On the other hand, no changes on primary structure were evidenced, which was expected since HIP is not considered to break covalent bounds.

At lower pressures (50–400 MPa), these slight molecular alterations were commonly linked with increase of enzyme conformational flexibility due to increase of its polar groups hydration [4], which facilitates the enzyme activity. Additionally, for enzymes processed at lower pressure, an increase of stability was attributed to higher enzyme intramolecular interactions and hydration of charged groups [4]. Conversely, the majority of work showed that there is a limit pressure/temperature/time conditions (specific for each enzyme/product) in which the enzyme energy gain is higher enough to induce sufficient structural changes that negatively affects the enzyme activity, being these effect reversible or not, depending on the applied pressure.

The split of latent isoenzymes is also an important factor to be considered for HIP and HPH applications in products that have endogenous enzymes, especially fruits and vegetables as carrots, peach, apples and others [6, 10, 11, 54]. Many reports showed that latent enzymes probably had activity induced by pressurization [6] and these enzymes normally have higher stability under pressure, being inactivated just at extreme conditions of HIP and just inactivated by HPH when the process is associated with mild heating [39]. This effect is particularly important for samples that contain polyphenoloxidase and peroxidases because of the occurrence of native resistance and occurrence of latent isoenzymes with consequent difficulty of inactivation [6, 46, 48].

Taking into account that HIP processing depends on a residence time at higher pressure, it is necessary to consider the reactivity of enzymes under pressure for samples processed (food) with endogenous enzymes and when enzyme is intentionally processed with the substrate. The changes on enzyme reactivity can be induced by several factors: (i) impact of the *Le Chatelier* principle (that postulates that enzyme reaction will be favored if the product formed

by enzyme reaction had lower volume then the substrate or inhibited if the formed product had higher volume) [6], (ii) relative increase of substrate concentration due to solvent compression upon high pressure application [4], (iii) higher physical interaction between enzyme and substrate, with consequent increase of reactivity rate [30], (iv) higher concentration polar charged groups in the Michaelis complex and in the transitional state [77], favoring the equilibrium toward enzyme release against inhibition from their conjugated complex [4] and (v) changes induced on the substrate or media of reaction, modifying the substrates availability or enzyme:substrate contact [55].

Therefore, it can be concluded that both processes are able to transform the structure of enzymes and that the observed effects are similar for HIP and HPH, altering mainly the hydrophobicity and disulfide bonds. However, from the data available in literature, it is not possible to establish a sequence of transformations and the level of pressure required in each process to the changes occur, because these data are scarce and the effects observed can be attributed to a multiple reason. Further researches need to be performed using the same enzyme and matrix to allow the adequate comparison between HIP and HPH effects on enzyme structure.

3.2.1. Effect of HIP and HPH on fruit and vegetable enzymes

Endogenous enzymes can deteriorate the color, flavor, structure and nutritional value of fruits and vegetables [6]. Therefore, considering the intention of using HIP and HPH as emerging processes to be commercially used for stabilization of fruits and vegetables products, the inactivation of these degrading enzymes is very important and must be reached with or without the assistance of mild temperature. The effect of HIP and HPH has been studied for enzymes present in fruit and vegetable purees/juices like tomato, strawberry, apple, litchi, pear, coconut water and orange and different tubers.

The fruits and vegetables processed by HIP showed different changes on enzyme activity, being affected by the kind and source of enzyme, presence of isoenzymes, integrity of vegetable (cubes, purees/pulps and juices), processing conditions and activity measurement. For HIP, the enzymes activity normally increase or did not change up to 400 MPa [4] and, above to this pressure, enzyme inactivation occurs, being the effect enhanced by increase of the time (1–180 min) or temperature (20–100°C) (**Table 1**). Among the evaluated enzymes, drastic reduction/inactivation for polygalacturonase (PG), lipoxygenase (LOX) and hydroperoxide lyase (HPL) in tomato juice [33, 34], for pectin methylesterase (PME) in pear [54], orange [35, 36, 78], apple [46, 50] and tomato juice [33], for amylase in apple juice [49] and for peroxidase (POD) and polyphenoloxidase (PPO) in strawberry pulps/purée [42, 44] and pear [54] was highlighted. On the other hand, POD and PPO showed high resistance depending on the sample, being reduced just at extreme conditions (600 MPa associated with high temperature and/or long time) in cocoyam, Peruvian carrot, sweet potatoes [32], pear [54], apple [47], litchi [53] and strawberry juices [42]. Therefore, in general, it was observed that heat-resistant enzymes could also be baroresistant. Additionally, enzyme activation was also observed in sweet potato (up to 368% for PPO and 27% for POD in puree) [32] and in *Lonicera caerulea berry* [45], kiwi fruit [52] and banana juice [51], being associated to the split of isoenzymes and/or

enzymes stabilization caused by pressure processing. The dependency of vegetable matrix could be observed for several works, however, it was not possible to establish a general rule to describe the effects, since the activation/inactivation was higher for cubes or puree in several matrix and in juices in other ones.

The impact of HPH on the inactivation of PME, POD and PPO was observed for process above 150 MPa [37–41], whereas in other ones, no significant inactivation was observed, causing negative effects (mainly browning and phase separation) in vegetable juices [43]. Comparing both processes, it was observed that the pressure required for enzyme inactivation in HPH is lower than HIP, indicating that high shear and the impact of abrupt pressure reduction in flow system are more important than the maintenance of the product at high pressure. In addition, the higher temperatures reached in HPH (increase of ~18°C for each 100 MPa) compared with temperature increase in HIP (3 °C for each 100 MPa) can partially explain the observed effect in homogenizers, indicating an association of homogenization and thermal effects.

Regarding to commercial application of HPH and HIP to process vegetable products with baroresistant enzymes, three possibilities are available: (i) the use of higher pressures (>600–1000 MPa for HIP and 400 MPa for HPH) associated with mild temperatures (50–80°C) aiming to inactivate the enzymes with better nutritional and sensory retention when compared with thermal processing alone [6], (ii) the use of mild pressure condition (up to 600 MPa for HIP or 300 MPa for HPH) and temperatures for inactivation of sensible fractions of enzymes, reducing the initial activity associated with other technological artifice to control the residual activity during product shelf life (e.g. package with good barrier to oxygen, use of gums to avoid phase separation and antioxidants) [46] and (iii) consumers education about the alterations induced by enzymes, explaining that these changes naturally occur and did not represent an unsafe product. A coconut water manufacturer adopted the strategy of explaining that the pink color occurs due to the activity of PPO that is not inactivated by mild process condition [79]. This seems to be a good alternative considering the growing consumer demand for unprocessed or low processed food and the comprehension that the use of mild processes impact the product's characteristics.

3.2.2. Effect of HIP and HPH on cereals and legumes enzymes

Enzymes are endogenously found in beans and cereals, being the lipases, lipoxygenases (LOX) and amylases are the classes of enzymes with major relevance. Amylases are able to differently hydrolyse starch, reducing the viscosity of products and it can be undesirable in several products due to the structural loss induced by the enzyme activity or desirable, when starch is used as raw material to obtain fermentable sugar by enzymatic saccharification process. On the other hand, lipoxygenases activity is always deleterious, due to the action as an antinutritional factor that affects in nutritional and sensory perception of the products, reducing its shelf life.

Few studies evaluated the impact of HIP on native cereals and legume enzymes [80]. Additionally, no work has evaluated the effect of HPH on crude extracts of cereals and legumes. Although several work focused on the impact of HPH on legume and cereals extract,

it was previously thermally treated, inactivating the enzymes. In general, cereal and legume enzymes exposed to HIP at mild conditions demonstrated an increase in activity; however, when pressure is above to a critical energy limit (specific for each enzyme and influenced by matrix and processes conditions), enzymes lose their activity as consequence of modifications on its structure and active sites [80].

LOX is considered a relative-resistant enzyme in cereals due to the existence of isoenzymes present in this kind of food (types I, II, III). Studies showed that pressure around 600 MPa is interesting to inactivate all the isoenzymes [73], being this resistance lower than the observed for thermal processing. Therefore, HIP splits as an interesting alternative to promote LOX inactivation with minimum damage of the processed vegetable [72]. For amylases, a huge increase of the activity of starch-degrading enzymes α and β-amylases was observed at pressures of 400–600 MPa due to starch gelatinization that starts at 400 MPa and is almost complete at 600 MPa. Above this pressure, the higher intensity of the process induces negative and permanent unfolding and/or modifications of the enzymes activity site [74]. No studies evaluated the amylases enzyme activity after HIP process, but it is possible that pressures lower than 400 MPa are able to activate the amylases (as normally occurs with other enzymes). Future studies need to be performed to better explain the impact of both technologies in enzymes found in cereals and legumes.

3.2.3. Effect of HIP and HPH on milk enzymes

Enzymes present in dairy products include those endogenous of milk (as phosphatase, lactoperoxidase, lysozyme, latoferrin and plasmin), proteases and lipases from microbial origin, being these microorganisms from a contamination (especially present in milk stored for long time before processing) or intentionally added in dairy products, such as yogurt, fermented milk and cheese [59–71].

Enzymes have low ability to change the characteristics of raw or pasteurized milk due to the relative short shelf life and low storage temperature of milk, which decreases enzyme activity. To the contrary, commercial sterile milk (obtained by ultra high temperature—UHT—processing) are degraded by enzyme activity (endogenous proteases and especially from contaminants), which induces the age gelation phenomena, responsible for the end of UHT milk shelf life [61]. In dairy products, the action of these enzymes can be mostly desirable (enzymes that act as antimicrobials, preventing dairy product contamination) or eventually desirable depending on the product (e.g. lipase and protease are important for cheese maturation, but elevated activity can produce bitter peptides and rancid flavor) [70, 71]. Therefore, enzyme activation or inactivation can be desirable in different dairy products and in different time of product shelf life.

Considering the HIP effects, generally it is possible to say that enzymes are more resistant to the HIP or HPH processes than to thermal processing with similar microbial inactivation [65]. This is interesting considering the higher retention of enzymes, such as lactoperoxidase, lactoferrin and lysozyme and its antimicrobial activity [65]. On the other hand, the high stability of proteases and lipases can negatively affect the products obtained by using milk processed by HPH or HIP, since the product shelf life can be reduced especially due to changes on texture and flavor resulting from enzymes activity [67]. Therefore, the use of HPH and

HIP need to be evaluated carefully to substitute the pasteurization in milk process prior to the manufacturing of dairy products not only due to the enzyme action but also due to the effect of both processes on milk constituents.

Conversely, several works highlight the use of pressure processing as unitary operation to induce enzyme production of microorganisms due to baric stress and to lyse microbial cells, allowing the extravasation of cellular content with high amount of enzymes [81, 82]. These uses are normally interesting aiming to increase the enzyme activity on cheeses during ripening (lower pressures) and reducing the time to reach the desirable flavor and texture [82, 83]. In addition, higher pressures are highlighted as an interesting tool to prevent over ripening of cheeses due to partial enzymes inactivation [70], with consequent increment on the product shelf life. Additionally, when enzymes inactivation is desirable, mild/high temperature process can be associated with HIP (temperature during pressurization) or with HPH (high inlet temperature or temperature retention after homogenization valve) to improve the effectiveness of the process [61, 62].

3.2.4. Effect of HIP on meat enzymes

The tenderization is one of the most important effect in respect to meat quality. It occurs due to meat tissue changes induced by enzyme and biochemical reactions, being enzymes such as cathepsin (B, H, L e D), calpastatin, m-calpain and μ-calpain involved in this process. In this context, the HIP process can be a useful tool either by increasing the enzyme activity, greater exposure of enzyme/substrate, or by changes in proteins [84]. The phenomena specifically involved in these processes are as follows: (i) release of cathepsin present in lysosomes [58]; (ii) activation of calpains and cathepsin up to 150 MPa [85]; and (iii) inactivation of calpastatin, which are inhibitors of calpains [84]. Furthermore, as the calpains are calcium dependent, the HIP process can promote the increase of the calcium release and thereby increase the reaction efficiency [86]. Moreover, these enzymes can be inactivated at higher pressures probably due to denaturation [57, 84]. In this case, cathepsins are more resistant (pressures above 500 MPa [87]) compared with the calpains (activity reduced at 250 MPa for 10 min at room temperature [88]).

In contrast, the HIP process can increase lipid rancidity in meat. For example, in pork, lipases and lipoxygenases are activated up to 300 MPa and this increase is related to the level of release of these enzymes present in the lysosome [56]. This demonstrates that there is a greater influence of the increase of contact enzyme/substrate compared to the increase in enzyme activity. For HPH, there are no studies with meat because the process is carried out only for fluid.

3.2.5. Effect of HIP and HPH on commercial enzymes

The use of HIP and HPH technologies in the processing of commercial enzymes is growing over time aiming to improve its performance. Among the advantages, it highlights the increase of the activity in optimal and nonoptimal conditions and improvement/modification of the specificity.

The results obtained for enzymes processed by HIP showed that the aminopeptidases such as PepN, PepX and PepA from *Lactobacillus delbrueckii* ssp. bulgaricus ACA-DC 0105 (used as a starter culture in cheese production for ripening acceleration) were activated at pressures

of up to 200 MPa and at temperatures of up to 40°C [27]. On the other hand, PepY and PepC were more sensitive to pressure and temperature, resulting in inactivation at pressures above 100 and 200 MPa, respectively [27]. For the glycolytic enzymes, a slight increase in activity was observed at pressures of 100 and 200 MPa, but no further changes were observed in the activity of these enzymes above 300 MPa [27]. The HIP process on commercial milk-clotting enzymes (recombinant Camel chymosin, calf rennet, bovine rennet, porcine pepsin, protease from *R. miehei*) was able to increase up to 25% the proteolytic activity (up to 300 MPa) or completely inactivate at high pressures (above 550 MPa) [26]. In other study, the leucine aminopeptidase obtained from *Aspergillus* (*A.*) *oryzae* used in the reduction of soy immunoreactivity was activated at 100–200 MPa/15 min/50°C [89].

The results obtained for HPH showed that this process was able to change the optimum temperature of neutral protease from 55 to 20°C after HPH at 200 MPa [28], improve glucose oxidase activity at 75°C after HPH at 150 MPa and increase between 100% and 400% its stability under storage [9]. In addition, it was observed an increase of amyloglucosidase activity at 80°C after HPH at 100 MPa [8] and an increase in the milk-clotting activity of coagulant enzymes, especially those that have chymosin in its constituents.

4. Future challenges

The studies of applying HIP and especially HPH, are recent. Therefore, there are some gaps of knowledge that must be fulfilled, allowing complete understanding about the effect and potential of both technologies. Firstly, from the data available in the literature, it is not possible to differentiate the effects of HIP and HPH since few enzymes were evaluated in both processes and, when evaluated, normally is not using the same diluting media, concentration, or method for activity measurement. Therefore, new studies need to be performed in a comparative way (varying just process conditions) to better establish the differences and equivalences of both processes. Ideally, a refined evaluation of molecular structure of enzymes must be determined to quantify the intensity of observed alterations and to describe the sequence of alterations caused specifically by HIP and HPH, helping to explain the enzymes transformation induced and consequently try to establish a mechanistic explanation of the alteration level that induces activation and inactivation of enzymes. From these explanations, it might be possible to predict the type of alteration expected for different enzymes, making these physical methods more interesting for industrial applications. Obtaining these results is mandatory to stimulate application of HIP and HPH, considering the industrial purpose of enzymes activation/modification/stabilization (mainly for enzyme manufactures) or the requirement of deleterious enzymes inactivation (especially for food processors).

5. Conclusions

High isostatic pressure and high pressure homogenization can be considered interesting unitary operations to be applied for inducing enzyme changes. Both processes are able to

activate and/or stabilize the majority of enzymes when mild process conditions (low pressure and temperature) are applied and this is highly desirable since the use of physical methods are considered cleaner way to change molecules, minimizing the risks of transformed ones. The changes induced by HPH and HIP allow some enzymes overcome usage limitations, as inability to react at desirable process conditions (low or high temperature, pH and salt concentration) and as high costs since the increase of activity induced by the process reduces the amount of required enzyme in industrial reactions and, consequently, the costs of enzyme usage. Considering the multifactorial effects that governed the physical transformations induced by HIP and HPH, it is not possible to predict if is HIP or HPH the better method to obtain the desired activation and, possibly, for several enzymes, different conditions of each process can induce similar activation.

In respect to inactivation, a similar behavior with thermal treatment was observed, being the heat resistant also baroresistant enzymes (e.g. peroxidase, polyphenoloxidase), making necessary the association of pressure and mild temperatures to reach adequate levels of inactivation. In this case, HIP tends to be more effective than HPH to induce irreversible changes on enzymes.

The activation and inactivation effects can be related to the molecular energy input caused by HPH or HIP processing. Differently from the thermal effects, both processes involving pressurization can deliver energy enough to induce changes (enhancement of hydrophobic surface and disulfide bonds formation) that positively affect the enzymes activity due to higher flexibility and exposure sites. However, at extreme conditions, the higher energy delivered induces to drastic changes, with irreversible enzyme changes and consequent permanent activity loss. Therefore, it is concluded that HIP and HPH are versatile technologies to alter enzymes, being possible to reach activation or inactivation, depending on the process conditions chosen.

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