Review Article

Plant therapeutic proteases: chemical aspects, applications and pharmaceutical formulations

Abstract

Background: Plants are important sources of therapeutic proteases with expressive activity, stability, specificity, and efficiency. These proteases are employed at low concentrations and produce lesser side effects. They have complex tridimensional structures whose maintenance is a challenge, requiring specific conditions to guarantee the biological and pharmacological activities of these compounds.

Aims: To conduct a literature review about plant therapeutic proteases, their principal biochemical aspects, potentials and clinical applications, and main pharmaceutical formulations.

Material and methods: The present study consisted of a bibliographic survey of the major plant therapeutic proteases. An investigation was performed in the PUBMED, SciELO, ScienceDirect and Academic Google databases using the keywords plant enzymes, therapeutic protease, immobilization, formulation.

Results: Some plant therapeutic proteases, such as papain and bromelain, are employed to treat many diseases and conditions, but the complexity of their structures is an important limitation of their uses. Thus, the structure and activities of their formulations need to be stabilized and protected against degradation, with improved pharmacokinetics, a prolonged time of action, reduced toxic effects, and proper direction towards their therapeutic target. Nanotechnology has made it possible to manufacture drug carriers such as polymeric nano- and microparticles, hydrogels, dendrimers and liposomes which are able to increase their efficacy and clinical applicability, as well as patient compliance. Sustainability initiatives that use Green Chemistry together with nanobiotechnology have managed to reduce the risks of toxicity to organisms and the environment. Green synthesis uses lower concentrations of metal ions, water-soluble, biocompatible and non-toxic compounds, as well as seeking energy efficiency and using renewable sources of raw materials.

Conclusions: Investigations about new formulations of plant therapeutic proteases using biodegradable and biocompatible polymers is of great biomedical interest because they generate less toxic new biopharmaceuticals, in addition to protecting and stabilizing the enzymatic structure.

Keywords: plant enzymes, therapeutic protease, immobilization, formulation.

1. Introduction

Plants have been used as medicine since ancient times. They are sources of a wide variety of biologically active molecules whose chemical identification and pharmacological properties have been extensively investigated [1]. Plant metabolites from secondary metabolism have been most extensively studied and characterized since they are expressed in response to variations in environmental conditions and as a defense against microorganisms, insects and predators. However, their primary metabolites, such as proteins, have been poorly studied and their pharmacological potential is underexplored, while their major function is considered to be the provision of amino acids for human and animal diets [2]. Plant proteases are the proteins most extensively employed for pharmacological purposes [3]. They catalyze protein and peptide hydrolysis reactions, regulating the physiology of organisms. Due to their selectivity and efficacy, they are of paramount importance in the treatment of numerous diseases [4]. However, their therapeutic potential and clinical applications are often affected by difficulties related to administration, biochemical instability, pharmacological activity and reaching the therapeutic targets. In recent years, different ways of encapsulating and attaching a polymer to proteases using suitable carriers have been studied in order to permit oral administration, avoiding the aforementioned problems and thus preserving their therapeutic effect [5].

Recent investigations in the nanotechnology field have developed nanostructured release systems that modulate drug release within the therapeutic interval and for a prolonged time in a single dose [6]. This review aims to draw attention to plant proteases as important therapeutic agents since they have expressive enzymatic activity and stability and are relatively easy and inexpensive to obtain from natural resources. We also comment about formulation strategies that will maintain their pharmacological activity.

2. Methodology

The present study consisted of a bibliographic survey of the types of therapeutic proteases found in plants, their biochemical aspects, applications and possible formulations as biological medicines. From 2019 to 2021, an investigation was performed in the PUBMED, SciELO, ScienceDirect and Academic Google databases using the keywords plant enzymes, therapeutic protease, immobilization, formulation.

We selected a total of 104 publications (articles and books) reporting scientific research on the enzymatic and pharmacological activity of proteases found in plants and their possible formulations involving aggregation to nanoparticles, hydrogels, liposomes or dendrimers. The approaches used involved the period from 1957 to 2021.

3. Literature Review

3.1 Proteases

Proteases, peptidases or proteolytic enzymes irreversibly cleave the peptide bonds in proteins and in peptides, originating proteins, peptides or free amino acids of smaller molecular mass [7]. They are found in all organisms, organs, and organelles, and about 2% of an organism's genome has sequences that code for proteases. They have enormous chemical, kinetic and structural diversity which adapts them to their wide range of functions and to the different environments where they catalyze [7,8,9].

These enzymes are classified according to their cleavage sites: exopeptidases (EC 3.4.11-19), when they act on peptide bonds at the N- or C-terminal of polypeptide chains, and endopeptidases (EC 3.4.21-99), when they act inside the chains. However, proteases are mainly classified according to the catalytic amino acid of the active site involved in catalysis. The hydroxyl group of serine (EC 3.4.21) and threonine (EC 3.4.25) and the sulfhydryl group of cysteine proteases (EC 3.4.22) are nucleophilic agents, while activated water is the nucleophilic agent in aspartic (EC 3.4.23), glutamic (EC 3.4.19) and metalloproteases (EC 3.4.24), whose catalytic amino acid residues are serine, threonine, cysteine, aspartic acid, glutamic acid and an ion for enzymatic catalysis [10]. The breaking of peptide bonds is classically mediated by hydrolases (EC 3.4), but it can also be mediated by carbon-nitrogen lyases (EC 4.3.2) called asparagin peptidases, which represent the seventh group of proteases [9].

Proteases are further classified according to the pH range where the enzymatic activity is maximum because the ionization of catalytic amino acids influences the catalysis. In addition, optimum pH values also suggest the cell compartment where the protease catalyzes. Aspartic proteases preferentially act in the acidic pH range; cysteine at slightly acidic pHs and serine and metalloproteases at neutral to alkaline pHs [11].

The MEROPS database classifies proteases and protease inhibitors into clans and families according to the percentage of similarity between the amino acid sequences (primary structure) and the active site of the proteases (peptidase unit) or the inhibitory domain of proteases. Each family is identified by a letter that represents the catalytic type of each protease: aspartic (A), cysteine (C), glutamic (G), metallo (M), asparagine (N), mixed (P), serine (S) , threonine (T), and unknown (U) [9].

Proteases are primarily related to protein digestion for amino acid assimilation. However, these enzymes are also essential for physiological responses such as: blood clotting, fibrinolysis, extracellular matrix remodeling, activation and inactivation of biologically active molecules, protein folding and degradation, apoptosis, and complement cascade, among others [12,13]. Therefore, they participate in cancer invasiviness, necrosis, and tissue damage in response to pathogenic microorganisms [14].

Plant peptidases express many types of proteases that are crucial for growth, development, defense against pathogens, senescence, apoptosis, xylem formation, tissue and organ differentiation, seed maturation, mobilization of protein reserves, germination, cell division, reproduction, adaptation to environmental changes, metabolism control, and many other functions [13,15,16,17]. Their potential biotechnological and pharmacological applications have been investigated, since plant proteases have important activity and stability in response to temperature, pH and ionic strength variations of the environment, which are essential requirements for their applications [16,17]. Therefore, the identification and understanding of the action of plant proteases permits the use of these enzymes as valuable therapeutic agents for the development of new biopharmaceuticals with greater specificity and less toxicity for the treatment of various pathologies and conditions, which are intractable with small synthetic drugs [18].

3.2 Biotechnological use of proteases

Biotechnology refers to the methodology sets that use living organisms or their parts for the production or modification of products or services, and for the genetic improvement of plants and animals, applied to industry, health and the environment. It is a multidisciplinary area of knowledge that involves Biochemistry, Molecular Biology, Microbiology, Chemical Engineering, and other sciences. Biotechnology is responsible for the development and production of a wide variety of products such as foods, textiles, antibiotics, and biopharmaceuticals commonly containing proteins with functions as enzymes, hormones, antibodies, growth factors, and vaccines [19,20].

According to the Allied Market Research report, the global enzyme market was about 7,082 million dollars in 2017, and is projected to reach $10,519 million in 2024. This growth will result in gains because enzymes are very specific, fast and nontoxic, properties that minimize the cost and reduce the time of the manufacturing process [21,22]. However, restrictions related to the chemical properties of enzymes, such as low stability, have been a challenging factor for their use. These challenges have been solved with the use of enzyme-based technologies, resulting in gains associated with the production of food and beverages, animal feed, biopharmaceuticals, and diagnostics [22,23]. Proteases represent 50% of the macromolecules employed in biotechnological processes [21]. Furthermore, it is important to emphasize that they need to have essential attributes such as expressive proteolytic activity, high specificity, and important stability at high temperatures and in the presence of chemical agents [17].

3.3 Therapeutic proteases

Proteolytic enzymes constitute a growing class of biopharmaceuticals, with the approval of more than 30 therapeutic proteases by the Food and Drug Administration, USA (FDA), in addition to the new proteases that are still in the clinical study phase [24].

Therapeutic proteases are enzymes employed for the treatment of diseases, surgical procedures, and diagnosis. They must have purity according to the pharmaceutical form used, specificity, low antigenicity (avoidance of immunological reactions) and stability under physiological conditions. Proteases have been successfully used for the treatment of hemophilia, traumatic bleeding, thrombosis, heart attack, cerebrovascular ischemia, vitreomacular adhesion, cystic fibrosis, muscular dystrophy, celiac disease, septicemia, digestive failure (pancreatic and intestinal), debridement and wound healing, cardiovascular surgery, and catheterization [18,24]. Intravenous biopharmaceuticals are heterologous proteins involving a more suitable delivery of protein obtaining by minimizing the risks of contamination and immunological reactions in patients when compared to proteases extracted from human or animal tissues, which are generally used in topical or oral medications. Heterologous expression, although very expensive, provides greater amounts of proteins in relation to its extraction from natural sources, whose purification yield is, in general, low and variable due to their low concentrations in biological tissues and fluids [17]. However, some commercial proteases are abundantly obtained from natural resources such as collagenase (EC 3.4.24.3) from *Clostridium hystoliticum* which is secreted into the culture medium [25], trypsin (EC 3.4.21.4) obtained from bovine pancreas [26], pepsin (EC 3.4.23.1) extracted from the stomach of ruminants, and papain (EC 3.4.22.2) obtained from the latex of *Carica papaya* [27].

The use of proteases in medicine dates back to the late 19th century. Crude porcine pancreatic enzyme preparations were employed to treat gastrointestinal disorders. Before the first World War, Takamine®, produced by the fungus *Aspergillus oryzae* and containing proteases and amylases, was developed in order to manage digestive dysfunctions [28]. The use of therapeutic proteases is the only strategy for the treatment of hemostasis disorders such as haemophilia and thrombosis. Urokinase, also known as urokinase-type plasminogen activator (uPA), originally isolated from human urine, was approved by the FDA in 1978 for the treatment of thrombosis, while coagulation factor IX, originally isolated from human plasma, was approved in 1986 for the management of hemophilia B. Later, other proteases such as thrombin, obtained from bovine plasma and enzymes extracted from the pancreas, such as trypsin, chymotrypsin, elastase and carboxypeptidases, were approved for commercialization and used with great success for various purposes. Topical thrombin formulations are used in bandages to accelerate the healing of large wounds and burns, while capsules containing pancreatic proteases are administered orally for the treatment of digestive disorders. In 1987, the first recombinant protease, tissue plasminogen activator (tPa), was approved for the treatment of thrombosis and marketed as alteplase®, reteplase® and tenecteplase® [18].

3.4 Therapeutic plant proteases

Plants express various enzymes with significant protease activity on different substrates of biological interest. In addition, these proteases are stable at high temperatures and in the presence of chemical agents. As previously mentioned, such features are essential for their medicinal and biotechnological use [29]. For these reasons, their structures, physicochemical and kinetic features, as well as their potential applications, have been extensively studied [30].

Plant therapeutic proteases are not heterologous proteins, because they are obtained directly from plant organs or latex, and they can be extracted without affecting plant viability, unless they are obtained from the roots. Their preparations are not pathogenic for animals since they do not contain infectious agents that cause diseases in vertebrates. In addition, the methodology for obtaining them is relatively simple, easy and of low cost [31,32]. These proteases are widely used as therapeutic enzymes in the treatment of many diseases and conditions and they also participate in various biotechnological processes [30,33] (Table 1). It is unquestionable that these plant proteases have a wide pharmacological potential, justifying their use in different pharmaceutical formulations.

Table 1 - Examples of plant proteases and some medicinal and biotechnological uses (Adapted from Silva-López & Gonçalves, 2019) [30].

Protease	Origin	Medicinal uses	Biotechnological uses
Actinidin	Kiwi *(Actinidia deliciosa)*	Diabetic foot ulcer, protein digestion and constipation	Dietary supplement and meat tenderization.
Bromelain	Pineapple (*Ananas comosus*)	Thrombosis, arthritis, wounds, cancers, asthma, bronchitis, sinusitis, analgesia, edema, vascular, cardiac and inflammatory diseases	Meat tenderization, bread production, tooth whitening.
Cardosin	Cardoon (*Cynara cardunculus*)	No investigated medicinal effect	Milk clot and cheese manufacturing.
Cucumisin	Melon (*Cucumis melo*)	Thrombosis	Meat tenderization, collagen hydrolysis to obtain gelatin, milk clot and synthesis of dipeptides.
Ficin	Fig (*Ficus* genus)	Vermifuge	Peptide synthesis and antibody fragmentation.
Oryzasin	Rice (*Oryza sativa*)	No investigated medicinal effect	Milk clot
Papain	Papaya (*Carica papaya*)	Edema, sinusitis, digestive disorders, caries removal, wound healing, infections, cancer	Detergent, leather and meat tenderization, peptide synthesis, antibody fragmentation, beverage production, reducction of food allergy
Phytesin	Barley (*Hordeum vulgare*)	No investigated medicinal effect	Milk clot
Zingipain	Ginger (*Zingiber officinale*)	Antiproliferative agent in animal models of cancer/	No biotechnological uses investigated

There are many other plant proteases that have been investigated due to their biotechnological and therapeutical potential and promising results have been observed [34,35]. However, few plant proteases are commercialized and used as therapeutic agents or for biotechnological purposes. These are papain, bromelain and ficin, and some biochemical and pharmacological aspects of these proteases will be addressed in this manuscript.

Papain

Papain is a cysteine protease (EC 3.4.22.2) mainly extracted from *Carica papaya* latex (papaya papaya) and *Vasconcellea cundinamarcensis* (sugar papaya), but it can also be found in many parts of these plants. It is a 23 kDa single polypeptide chain endopeptidase [36] and was the second protein to be crystallized (1968) and the first cysteine protease with an elucidated 3D structure (1984). Papain is a model of the cysteine protease family and, according to MEROPS [37, 38], it belongs to the papain superfamily and C1A subfamily

This enzyme is stable at high temperatures and has many medicinal uses such as: treating edema, sinusitis, leaky bowel syndrome, gluten intolerance, digestive disorders and removing cavities [39], with antibacterial [40], anthelmintic [41] and antifungal [42] activities. This protease has an anti-angiogenic effect, preventing the proliferation, invasion and migration of tumors, as well as inducing apoptosis in human tumor cell lines [43]. It has been employed in tissue debridement to stimulate the healing of ulcers since it hydrolyzes necrotic tissues, aiding tissue regeneration. In addition, it stimulates the production of cytokines that repair cells and slow down the growth of microorganisms [29].

In papaya, papain leads to latex clotting, forming a physical barrier as a primary step in the defense mechanism [44]. Although it is the most studied cysteine protease, there are few studies on its therapeutic applications and no reports on its toxicological data. The available studies, however, provide a model for the study of cysteine proteases that are used in the treatment of many diseases [45].

Bromelain

Bromelain is an aqueous extract rich in cysteine proteases obtained from the stems and fruits of Bromeliaceae family species, with pineapple (*Ananas comosus*, *A. sativus*, *Bromelia ananas*) being the species most frequently studied. This extract contains four proteases with molecular masses between 20 and 31 kDa that belong to the papain superfamily: stem bromelain (EC 3.4.22.32), comosaine and fruit bromelain (EC 3.4.22.33), and ananaine (EC 3.4.22.31). All of these enzymes have bromelain protease activity and exhibit expressive stability at high temperatures. The extract is prepared from pineapple juice by centrifugation, ultrafiltration and lyophilization and produces a yellowish powder which is applied by food, beverage, cosmetic, textile and pharmaceutical industries [46].

Pineapple (chemically known since 1876) is used as a medicinal plant in several cultures and its medicinal properties are attributed to bromelain. Due to its complex composition, this protease has many pharmacological properties and has been used to treat rheumatoid arthritis, thrombophlebitis, wounds, cancer, angina, bronchitis, sinusitis, osteoarthritis, surgical trauma, and pyelonephritis, as well as to improve the absorption of certain drugs. This extract importantly alleviates pain and edema and shortens healing time compared to conventional treatments [47,48]. Bromelain induces the reduction of inflammatory and pain mediators, acting as an anti-inflammatory agent in many conditions, attenuating asthma [49], rheumatoid arthritis and osteoarthritis [50]

The course of intestinal infections is affected by oral treatment with bromelain, which degrades the adhesion receptor of bacteria to the intestinal mucosa [51]. In addition, bromelain has anthelmintic and antifungal activities [47].

Debridement is the clearance of dead, infected, senescent and/or devitalized tissues from a wound that interfere with healing. This procedure converts a chronic wound to an acute one, reducing bacterial growth. Bromelain degrades necrotic tissue, regulates cell maturation and multiplication, stimulates collagen and elastin synthesis, and removes perivascular fibrin. It also hydrolyzes the damaged components of the extracellular matrix, releasing growth and angiogenic factors sequestered in this matrix and activating chemokines and cytokines. Bromelain debridement accelerates blood perfusion recovery, improves inflammation, increases fibroblast and smooth muscle cell chemotaxis, and is more efficient than painful surgical debridement. Thus, the patients are not exposed to anesthesia, bleeding and infections. Enzymatic debridement reduces wound healing time, morbidity and mortality in severely burned patients. Bromelain has very low toxicity and is not carcinogenic or teratogenic [32].

Ficin

Ficus species produce latex from laticiferous cells. Latex is a complex, sticky, milky liquid that is excreted in response to injury to protect the plant from invading pathogens, as mentioned for papain. Protease fractions from latex of Ficus species predominantly contain cysteine proteases, but serine and aspartic proteases are also found. The latex of the fig tree, *Ficus carica*, has a high activity of the cysteine protease known as ficin (EC 3.4.22.3), which consists of six isoforms, A, B, C, D1, D2 and E, with single-polypeptides chains of about 24 kDa [52]

Ficin can be used in many types of industries (Table 1). The latex of some Ficus species is traditionally used as an anthelmintic agent, although it has not been submitted to clinical or toxicological trials [53]. Ficin also has intense collagenolytic and chitinolytic activity, the latter giving the plant resistance against fungi and insects [54].

All therapeutic proteases, and every medicine, need to be biologically active to perform their pharmacological functions, and this is only possible if the chemical structure is maintained. Therefore, the development of specific formulations that maintain their structures and activities guarantees their therapeutic uses.

3.5 Pharmaceutical formulation of proteases

Pharmaceutical formulations are intended to ensure the stability, solubility and biological and pharmacological activities of a drug [55]. Historically, the first medicines date back to Galen (129-199 A.D.), who discovered and used natural medicines in their pure forms. Their descriptions include various substances of natural origin, as well as formulas and methods of manipulation, proposing preparations of plant substances by mixing or fusing the individual components. In 1948, after World War II, Alexander Fleming discovered penicillin, an antibiotic that has saved many lives [56,57]. In the 1980s, recombinant DNA technology led to an increase in the number of recombinant proteins with high therapeutic potential, especially enzymes [58].

The use of proteases as therapeutic molecules is crucial for the treatment of many diseases due to their high specificity and activity. It is important to emphasize that these enzymes are used at much lower concentrations than those of low molecular weight synthetic drugs in order to achieve similar pharmacological effects, besides, they cause fewer adverse effects. Despite the current biotechnological advances, the use of proteases as drugs is still a great challenge since they have complex and unstable structures, high molecular weights and low permeability through the biological membranes of target cells [59]. Therefore, their transport and release in the body are difficult and they may lose their activity, which directly depends on the maintenance of their structure. In addition, their absorption is limited, and they generally have a short half-life in the body due to enzymatic degradation at the administration site or during the journey to the action site [60,61]. Conventional methods of administration are designed to rapidly release biologically active molecules with therapeutic potential. Generally, water-soluble diluent systems are used to favor the drug's solubility. However, keeping plasma concentration levels within the therapeutic range is still one of the biggest challenges [62].

Therapeutic proteases are usually administered in the form of aqueous solutions or suspensions via the parenteral route (subcutaneous or intravenous) which provide greater bioavailability [63,64]. However, the parenteral route has some disadvantages such as the risk of contamination, pain and discomfort for the patient during application, the need for sterile preparations, and difficulties in self-administration. These limitations of drug administration have inspired the investigation of several alternative routes for the delivery of biopharmaceuticals, such as pulmonary, nasal, oral, transdermal, vaginal, rectal and ocular routes, which have been explored in order to increase patient adherence to treatments. Most of the studies about the pulmonary route of administration have used aerosol formulations that have been very effective for the treatment of respiratory inflammation and other lung disorders [65]. This pathway represents a possibility for systemic and non-invasive release of proteases [66]. Another therapeutic route is the transdermal one, which uses adhesives and is a painless alternative to injections. However, this route is still little used and only delivers hydrophobic and low molecular weight drugs, which is not the case for enzymes [67]. The oral administration of drugs is easier, cheaper and better accepted by the patients. However, the oral administration of therapeutic proteases is very limited due to the rapid degradation of these enzymes caused by the wide variation in pH of digestive proteases of the gastrointestinal tract. Furthermore, these therapeutic proteases cannot permeate the intestinal membrane because a receptor coupled to a transporter or carrier is required for absorption. Each of these routes offer advantages and limitations, and formulations have been developed to minimize these limitations [68].

Some formulation strategies can increase the bioavailability of these drugs without a drastic change in their structure and activity, thus improving stability, efficacy and specificity, decreasing immunogenicity, and ensuring good pharmacokinetics [69]. Pegylation, which is a chemical conjugation with polyethylene glycol (PEG), is widely used to prolong the residence time of enzymes in the blood, in addition to promoting their site-specific release [70]. Although it can decrease the protein immunogenicity and increase its solubility, the main benefit of pegylation is the reduction in the frequency of doses due to their longer half-life in the body's circulation [67].

3.6 Polymeric drug delivery systems

The development of a drug delivery system must take into account its incorporation capacity, the possibility of site-specific release, the interaction with biological molecules, the degradation rate, the accumulation of the drug in organs, its toxicity and the possibility of production on a large scale. The physicochemical stability of enzymes also needs to be evaluated when choosing the method of formulation preparation, since it can be affected by environmental factors that are part of the production process, such as pH, temperature, high pressure, organic solvents, metal ions, and agitation, among other factors that can lead to loss of protein structure and activity [6].

Polymeric vehicles, besides having specific degradation characteristics, should also protect the therapeutic enzyme from proteolysis. This can be achieved by incorporating polymers, specifically cross-linked acrylic polymers such as Carbopol® (carbomer) and polycarbophil. Due to the rapid and high swelling and dispersion of these polymers in aqueous solutions, they should be incorporated into other polymers of a hydrophobic nature in order to control the erosion rate and minimize their effect on the diffusion barrier [71,72]. Different polymeric systems have been extensively studied for enzyme transport, the most important being micro- or nanocapsules, micro- or nanospheres, hydrogels, dendrimers, and liposomes (Figure 1). They are characterized by a high degree of innovation and versatility and can improve pharmacokinetics by offering site-specific prolonged release, reduction of adverse effects and increased bioavailability of biopharmaceuticals [58]. The process for obtaining these particles depends on the physicochemical characteristics of an enzyme such as size, distribution and morphology, which, in turn, determine their behavior regarding the encapsulation and release of the drug [73].

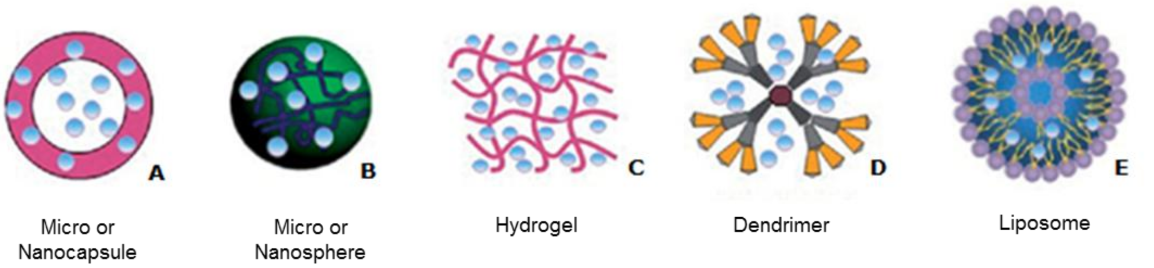

Figure 1 – Schematic presentation of the structure of some polymeric drug delivery systems: micro or nanocapsule (A), micro or nanosphere (B), hydrogel (C), dendrimer, (D) and liposome (E). (Adapted from Zhang et al, 2013) [74].

These polymeric drug delivery systems can be classified according to their size and dispersion. Particles 1 to 1000 µm in diameter are classified as microcapsules or microspheres. Colloidal particles 10 and 1000 nm in diameter, in which the drug can be dissolved, encapsulated or dispersed, are nanocapsules or nanospheres (Figure 1A and 1B) [74]. Micro or nanocapsules are spherical structures with a well-defined core where the drug is located inside an aqueous or oily cavity surrounded by a polymeric membrane (Figure 2A). On the other hand, the structure of micro or nanospheres consists of a single matrix in which the drug is dispersed and encapsulated by a biodegradable polymer, forming a homogeneous mixture (Figure 2B) [75]. The small diameter of nanoparticles offers advantages over microparticles such as greater ease in crossing the intestinal epithelium compared to microparticles [76].

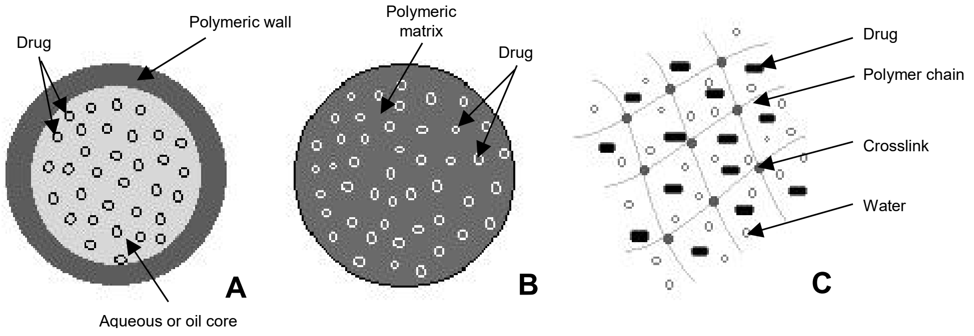

Figure 2 – Schematic presentation of the structure of micro- or nanocapsules (A), micro- or nanospheres (B) and a polymeric hydrogel (C) (Adapted from Melo, Cunha & Fialho, 2012) [58].

All of these structures are composed of biodegradable polymers such as polyesters, polyanhydrides and polysaccharides that normally are not toxic and and are easily eliminated from the body. Polymers used in nanoparticle formulations are the same as those for microparticle preparations, and are widely used in the controlled administration of drugs with extended release, including synthetic polyesters such as polylactic acid, copolymers of lactic and glycolic acids and poly(ε-caprolactone). Natural polymers, on the other hand, include some proteins such as albumin, collagen and gelatin and polysaccharides such as chitosan [77,78].

Hydrogel is an important system employed for drug delivery (Figure 1C) defined as a three-dimensional structure of highly porous polymer chains, which can be easily modeled by controlling the number of cross-links, and can absorb large amounts of water or biological fluid (Figure 2C). It is sensitive to environmental variations such as pH, electric field, ionic strength, and the presence of certain molecules, which can induc structural changes in the hydrogel. Its porosity allows the release of drugs at a rate that is dependent on the diffusion coefficient of molecules from the polymeric system to the therapeutic target [79,80]. These systems can be prepared in a wide variety of physical forms, including deposit formulations, microparticles, nanoparticles, coatings, and films [81]. Kashyap et al. (2007) developed a biodegradable hydrogel consisting of glucose linked to chitosan chains with high sensitivity to pH variation, which induces insulin release in response to hyperglycemia [82].

Dendrimer is a nanosystem used in drug transport (Figure 2D) consisting of highly branched molecules of: nanometric size; specific shape and structure; layers or generations composed of repeating units and radially connected to the starter core and functionalized end groups; hydrophobic core with hydrophilic periphery; and low polydispersity. They are closely similar to human proteins such as insulin, hemoglobin and cytochrome C, and are inert in the human body, with low toxicity and immunogenicity [83,84]. Drug payloads can be trapped in dendrimer layers through the generation of non-covalent complexes or bonded to their surface through covalent bonds. Covalently constructed dendritic macromolecules have the advantage of having more control of drug release and can be designed to limit drug release into the systemic circulation, triggering release under specific conditions. The type of bond depends on the physicochemical characteristics of a protein and the functional groups present in dendrimers. For example, hydrophobic molecules can bind to the nucleus or polyamidoamine branches, facilitating their transport through tissues and cells due to their large number of surface groups that can covalently bind to a wide variety of molecules [84,85].

Liposome is another type of polymeric system used for drug transport and delivery (Figure 1E). It consists of lipid bilayers separated by an aqueous medium, with a spherical structure with amphiphilic molecules and can encapsulate hydrophilic substances in the aqueous core and lipophilic substances in the interior of lipid bilayers [86]. The fluidity of lipid bilayers allows structural flexibility, eases the interaction with cell membranes, and has the ability to incorporate water and fat-soluble compounds. Furthermore, lipid bilayers are biodegradable, biocompatible and non-immunogenic [87]. Conventional liposomes are composed of phospholipids with negative or positive charges that prevent vesicle aggregation, and of cholesterol that increases their stability in suspensions. *In vivo* long-lasting liposomes are obtained by different methods, including coating the liposome surface with natural hydrophilic components such as monosialoganglioside (GM1) and phosphatidylinositol, or synthetic hydrophilic polymers, specifically PEG. The hydrophilic surface layer of these polymers increases the circulation time and prevents association with opsonins (antigen-bound molecules that facilitate phagocytosis) in plasma[88].

PEGs inhibit molecular recognition and uptake by cells of the mononuclear phagocytic system [92]. Modification of the liposome surface with PEG can circumvent these problems because of the increased stability [87]. The most commonly used lipids in liposome formulations are phosphatidylcholines, phosphatidylserine, phosphatidylglycerol and sphingomyelins, which form a stable bilayer in aqueous solution. Phosphatidylcholines are the compounds most frequently used in liposome formulation studies because of their great stability against variations in pH or salt concentration in the medium due to both positive and negative charges [89].

3.7 Methods for Incorporating Proteins and Peptides

The preparation of polymeric systems depends on the efficiency of the methodologies used for enzyme incorporation, which allow the modulation of structures, compositions and physiological properties of these proteins [90,91]. The choice of a preparation methodology will depend on the polymer and the solubility of the biopharmaceuticals to be encapsulated. The methods most frequently employed for the incorporation of peptides and proteins are multiple emulsion, phase separation and spray drying [92].

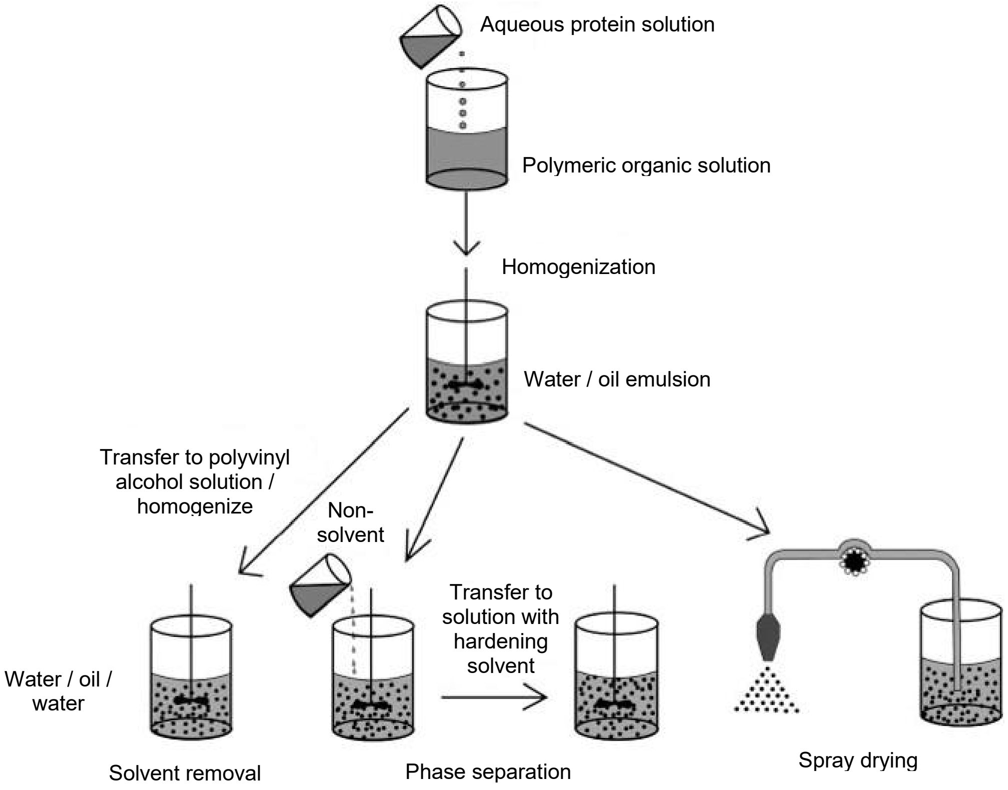

Figure 3 – Nanotechnology scheme for incorporating proteins and/or peptides into polymeric drug delivery systems (Adapted from Mundargi et. al., 2008) [92].

The first step consists of obtaining a water-in-oil emulsion by dispersing an aqueous solution with the protein or peptide to be encapsulated in an organic solvent, already containing the dissolved polymer. With this method, the solution is emulsified with a large amount of aqueous medium to form a water-in-oil-in-water multiple emulsion, where the protein is in the internal aqueous phase and the polymer in the organic (or oily) phase. Polymeric systems are further formed by solvent removal (Figure 3). In the phase separation method, a non-solvent is added under stirring to the water-in-oil emulsion, where the protein is in the internal aqueous phase and the polymer in the organic (or oily) phase, inducing agglomeration of protein molecules and transforming the stable colloidal system into immiscible solutions of different concentrations [61].

Spray drying (SD) is performed from a water/oil emulsion with polymeric particles loaded with therapeutic proteases, which must be homogeneous to allow greater precision and dose-by-dose reproducibility (Figure 3). For this purpose, SD is used to obtain polymeric particles loaded with therapeutic enzymes in the form of dry powder. It is a drying method for obtaining "post-dry" from a liquid phase which is widely used in the food, pharmaceutical, polymer and chemical industries. In the case of encapsulation of therapeutic proteases into spheres or capsules, the dry powder can be obtained from a solution, suspension or emulsion. Proteases are best preserved in the "post-dry" form, which increases stability during storage by eliminating water] thus, SD is also used as a method of preservation. This is a reproducible and fast technique, which can be scaled up and produce stable particles without the need for lyophilization. SD is a continuous process divided into four stages: atomization, mixing of droplets with drying gas, evaporation, and product separation. Its limitation is the solvent evaporation that does not allow the production of particles on a large scale [93].

A physicochemical method of double emulsion is the most suitable for the nanoencapsulation of hydrophylic proteins. It is conceptually simple, and consists of the preparation of a primary water/oil emulsion by sonication of an aqueous solution containing the protein and an organic polymer solution. This emulsion constitutes the internal phase of the second emulsion, also prepared by sonication, whose external phase is an aqueous solution with a surfactant. The preparation of nanoparticle formulations by this methodology requires the presence of an emulsifying agent to stabilize the dispersed phase into a water/oil/water multiple emulsion. The emulsifying agent, in this case, is required to prevent aggregation and coalescence of particles [94].

The development of new protein formulation techniques, mainly for plant proteases, in the form of micro/nanoparticles has increased the stability, efficiency and specificity of these biopharmaceuticals for medicinal use, and has decreased their toxicity.

The newest strategy for protein formulation from nanoparticles has been investigated using natural materials from plant extracts, bacteria, fungi, yeasts, algae, and biomolecules (enzymes and polysaccharides), which provide differentiated characteristics such as protection, reduced toxicity and stability of the formulation of nanoparticles, in addition to a high yield and low production cost [95,96]. Sustainability initiatives that use green chemistry to improve and/or protect our global environment are becoming focal issues in many fields of research [94], and the use of various biological entities has received considerable attention in the field of nanobiotechnology [98]. In order to reduce the risks of toxicity to living organisms and the environment, green synthesis uses lower concentrations of metal ions and water-soluble, biocompatible, non-toxic compounds [99,100].

The principles of green chemistry are fundamental for the implementation of sustainable processes for environment preservation, and they are: economy of atoms; synthesis of less toxic products, as well as solvents and residues used in the process; search for energy efficiency; use of renewable sources of raw material; avoiding the formation of derivatives; catalysis; real-time analytics for pollution prevention; intrinsically safe chemicals for accident prevention [101]. The use of extracts from different parts of plants such as leaves, stems, roots, seeds, and fruits, and plant biomass, play an important role in these processes [102,101].

The green chemistry method can provide a wide variety of types, sizes and shapes of nanoparticles, and as the growth phase length increases, the nanoparticles aggregate to form nanospheres, nanotubes, nanoprisms, nanohexahedra and a variety of other irregularly shaped nanoparticles. In the formation phase, these nanoparticles acquire the most favorable conformation from an energetic point of view, and this process is strongly influenced by the stabilizing capacity of plant extracts. The metal ion reduction process for the formation of nanoparticles is affected by the nature of the extract that contains active biomolecules in different combinations and concentrations, by the reaction mixture pH, temperature, reaction time, concentration and by the electrochemical potential of a metal ion [100]. The first step is mixing an aqueous solution of a metallic salt with a water-based extract. Next, the reduction of this metal solution converts metal ions from their mono, bi or trivalent oxidation states to zero valence states and the nucleation is initiated. In the nucleation phase, there is a reduction of metallic ions as well as the nucleation of reduced metallic atoms due to the electrostatic interactions between the positive charges of metallic ions and the negative charges of the carboxylic groups of the plant protease. Then, during the growth phase the small adjacent nanoparticles spontaneously fuse into larger particles, a process accompanied by increased thermodynamic stability of the nanoparticles, i.e., the reduction of ions is initiated by the components of biological materials, favoring the generation of the neutral metal (M0) that will agglomerate and trigger a phenomenon called nucleation. The biomolecules of the extract act as covering agents to coat and stabilize the nanoparticles. The last step is the formation of metallic nanoparticles that will determine the final shape (Figure 4) [103].

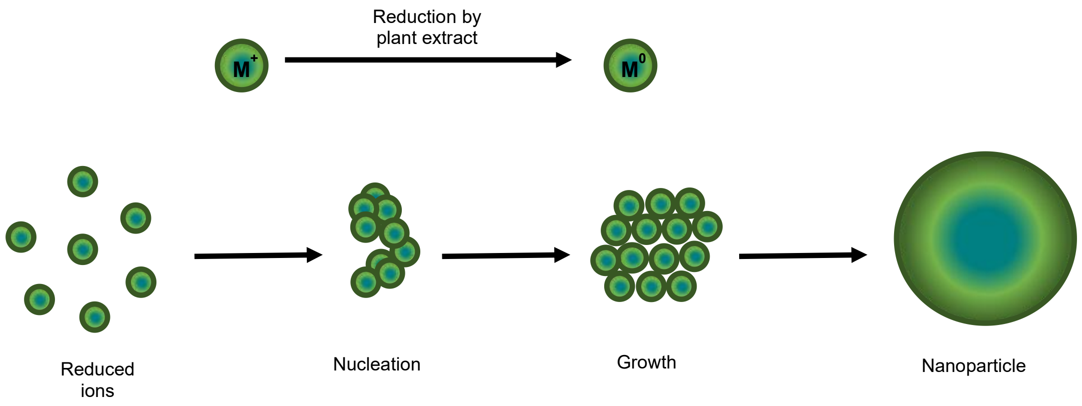

Figure 4 Steps of formation of metallic nanoparticles (Adapted from Toledo, 2021) [103].

Other studies have shown the efficiency of these types of formulations, such as the synthesis of silver nanoparticles and the use of a propolis extract and dragon blood sap with antimicrobial action [97], suggesting their application in hospital infections, or the aqueous extract of *Brosimum gaudichaudii* leaves in the application of an electrochemical nanobiosensor [104].

Thus, green chemistry has many advantages over the traditional method in the production of nanoparticles such as the use of an aqueous plant extract acting as a stabilizing and reducing agent during their formation. Its aqueous-based synthesis process is an ecological, direct and simple method that does not require specialized equipment [103].

4. Conclusion

Therapeutic plant proteases have gained an important place in the treatment of various diseases and conditions. They are specific, have great catalytic power, high stability and low cost of acquisition. Formulations developed with polymeric nanoparticle strategies are the most suitable for protecting proteases and directing them to the therapeutic target. An in-depth literature analysis allowed us to conclude that polymeric nanoparticles, hydrogels, liposomes and dendrimers are excellent protease transporters, protecting them against general degradation.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

Declaration of competing interest

The authors declare that there was no conflict of interest.

References

[1]. Henriques SR, Ferreira JWS, Muniz MP, Albuquerque UP. Adaptive memory and evolution of the human naturalistic mind: Insights from the use of medicinal plants. PLoS One. 2019; 14 (3). DOI: 10.1371/journal.pone.0214300.

[2]. Rehman HM, Cooper JW, Lam HM, Yang SH. Legume biofortification is an underexploited strategy for combatting hidden hunger. Plant Cell Environ. 2019; 42 (1): 52-70. DOI: 10.1111/pce.13368.

[3]. Turunen P, Rowan AE, Blank K. Single-enzyme kinetics with fluorogenic substrates: lessons learnt and future directions. FEBS Lett. 2014; 588 (19): 3553-63. DOI: 10.1016/j.febslet.2014.06.021

[4]. Chauhan V M , Zhang H, Dalby PA, Aylott J W. Advancements in the co-formulation of biologic therapeutics. J Control Release. 2020; 327: 397-405. DOI: 10.1016/j.jconrel.2020.08.013.

[5]. Botelho SM. Novas estratégias de administração de proteínas e peptídeos. Coimbra. Dissertação do Programa de Pós-Graduação da Faculdade de Farmácia da Universidade de Coimbra. 2014.

[6]. Prado FK. Desenvolvimento de método analítico para quantificação de antineoplásico em sistemas de liberação controlada de fármacos. Dissertação do Programa de Pós-Graduação da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo. 2019.

[7]. Vizovišek M, Vidmar R, Drag M, Fonović M, Salvesen GS, Turk B. Protease specificity: Towards *in vivo* imaging applications and biomarker Discovery. Trends in Bioche Sci. 2018; 43 (10): 829-44. DOI: 10.1016/j.tibs.2018.07.003.

[8]. Bond JS. Proteases: History, discovery, and roles in health and disease. J Biol Chem. 2019; 294 (5): 1643-51. DOI: 10.1074/jbc.TM118.004156.

[9]. Rawlings ND, Barrett AJ, Thomas PD, Huang X, Bateman A, Finn RD. The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. Nucleic Acids Res. 2018; 46(D1): D624–32. DOI: 10.1093/nar/gkx1134.

[10]. Li Q, Yi L, Marek P, Iverson BL. Commercial proteases: Present and future. FEBS Lett. 2013, 587 (8): 1155–63. DOI: 10.1016/j.febslet.2012.12.019.

[11]. Silva-López RE. Immunocytochemistry of proteases in the study of *Leishmania* physiology and host-parasite interaction in applications of immunocytochemistry In: Dehghani H, editor. Applications of immunocytochemistry, InTech, Rijeka. 2012; 267–96. DOI: 10.5772/32954.

[12]. Mandujano-González V, Villa-Tanaca L, Anducho-Reyes MA, Mercado-Flores Y. Secreted fungal aspartic proteases: A review. Rev Iberoam Micol. 2016; 33 (2): 76-82. DOI: 10.1016/j.riam.2015.10.003.

[13]. Baker PJ, Numata K. Polymerization of peptide polymers for biomaterials applications. Polymer Science. IntechOpen, 2013. DOI: 10.5772/46141.

[14]. McCauley JA, Rudd MT. Hepatitis C virus NS3/4a protease inhibitors. Curr Opin Pharmacol. 2016; 30: 84-92. DOI: 10.1016/j.coph.2016.07.015.

[15]. Van der Hoorn RAL, Jones JDG. The plant proteolytic machinery and its role in defence. Curr Opin Plant Biol. 2004; 7 (4): 400-7. DOI: 10.1016/j.pbi.2004.04.003.

[16]. Van der Hoorn RAL. Plant proteases: From phenotypes to molecular mechanisms. Annu Rev Plant Biol. 2008; 59: 191–223. DOI: 10.1146/annurev.arplant.59.032607.092835

[17]. Kurepa J, Wang S, Li Y, Smalle J. Proteasome regulation, plant growth and stress tolerance. Plant Signal Behav. 2009; 4 (10): 924–27. DOI: 10.4161/psb.4.10.9469.

[18]. Craik CS, Page MJ, Madison EL. Proteases as therapeutics. Biochem. 2011; 435 (1): 1–16. DOI: 10.1042/BJ20100965.

[19]. Schwaminger SP, Fraga-García P, Eigenfeld M, Becker TM, Berensmeier S. Magnetic separation in bioprocessing beyond the analytical scale: from biotechnology to the food industry. Front Bioeng Biotechnol. 2019; 7: 233. DOI: 10.3389/fbioe.2019.00233

[20]. Gavrilescu M, Chisti Y. Biotechnology-a sustainable alternative for chemical industry. Biotechnol Adv. 2005; 23 (7-8): 471-99. DOI: 10.1016/j.biotechadv.2005.03.004.

[21]. de Souza PM, Bittencourt MLA, Caprara CC, de Freitas M, de Almeida RPC, Silveira D, et al. A biotechnology perspective of fungal proteases. Braz J Microbiol. 2015; 46 (2): 337-46. DOI: 10.1590/S1517-838246220140359.

[22]. Oliveira LG, Mantovani SM. Transformações biológicas: contribuições e perspectivas. Quim. Nova. 2009; 32 (3): 742-56. DOI: 10.1590/S0100-40422009000300018.

[23]. Chapman J, Ismail AE, Dinu CZ. Industrial applications of enzymes: recent advances, techniques, and outlooks. Catalysts. 2018; 8: 238. DOI:10.3390/CATAL8060238.

[24]. Vellard M. The enzyme as drug: application of enzymes as pharmaceuticals. Curr Opin Biotechnol. 2003; 14 (4): 444-50. DOI: 10.1016/s0958-1669(03)00092-2.

[25]. Raheem AA, Johnson M, Ralph D, Garaffa G. Collagenase *Clostridium histolyticum*: a novel medical treatment for Peyronie's disease. Minerva Urol Nefrol. 2018; 70 (4): 380-85. DOI: 10.23736/S0393-2249.18.03118-1.

[26]. Kido H, Takahashi E, Kimoto T. Role of host trypsin-type serine proteases and influenza virus-cytokine-trypsin cycle in influenza viral pathogenesis. Pathogenesis-based therapeutic options. Biochimie. 2019; 166: 203-13. DOI: 10.1016/j.biochi.2019.09.006.

[27]. Leite AP, de Oliveira BGRB, Soares MF, Barrocas DLR. Use and effectiveness of papain in the wound healing process: a systematic review. Rev Gaucha Enferm. 2012; 33 (3): 198-207. DOI: 10.1590/s1983-14472012000300026.

[28]. Sato K, Egami F. Studies on ribonucleases in takadiastase. J Biochem. 1957; 44 (11): 753-67.

[29]. Pacheco JS, Silva-López RE. Study of the proteolytic activity of the tropical legume *Crotalaria spectabilis*. Z Naturforsch C J Biosci. 2012; 67 (9-10): 495– 509. DOI: 10.1515/znc-2012-9-1008.

[30]. Silva-López RE, Gonçalves RN. Therapeutic proteases from plants: biopharmaceuticals with multiple applications. J Appl Biotechnol Bioeng. 2019; 6 (2): 101–9. DOI:10.15406/jabb.2019.06.00180.

[31]. Menou A, Duitman JW, Crestani B. The impaired proteases and anti-proteases balance in idiopathic pulmonary fibrosis. Matrix Biol. 2018; 68-69: 382-403. DOI: 10.1016/j.matbio.2018.03.001.

[32]. Silva-López, RE. Debridement applications of bromelain: a complex of cysteine proteases from pineapple. Adv Biotechnol Microbiol. 2017; 3 (5): 124-6. ISSN: 2474-7637.

[33]. Feijoo-Siota L, Villa T G. Native and Biotechnologically Engineered Plant Proteases with Industrial Applications. Food Bioprocess Technol. 2011; 4: 1066–88. DOI:10.1007/s11947-010-0431-4.

[34]. Silva MZR, Oliveira JPB, Ramos MV, Farias DF, de Sá CA, Ribeiro JAC, et al. Biotechnological potential of a cysteine protease (CpCP3) from *Calotropis procera* latex for cheesemaking. Food Chem. 2020; 307: 125574. DOI: 10.1016/j.foodchem.2019.125574.

[34]. Siritapetawee J, Khunkaewla P, Thumanu K. Roles of a protease from *Euphorbia resinifera* latex in human anticoagulant and antithrombotic activities. Chem Biol Interact. 2020; 329: 109223. DOI: 10.1016/j.cbi.2020.109223.

[36]. Storer AC, Ménard R. Papain. Handbook of Proteolytic Enzymes. 2013; 2: 1858-61. DOI: 10.1016/B978-0-12-382219-2.00418-X.

[37]. Novinec M, Lenarčič B. Papain-like peptidases: structure, function, and evolution. Biomol Concepts. 2013; 4 (3): 287–308. DOI: 10.1515/bmc-2012-0054.

[38]. Rawlings ND, Barrett AJ, Finn R. Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res. 2016; 44 (D1): D343–50. DOI: 10.1093/nar/gkv1118.

[39]. Amri E, Mamboya F. Papain, a plant enzyme of biological importance: a review. Am J Biochem Biotech. 2012; 8 (2): 99–104. DOI: 10.3844/ajbbsp.2012.99.104.

[40]. Dos Anjos MM, Da Silva AA, De Pascoli IC, Mikcha JMG, Machinski M, Peralta RM et al. Antibacterial activity of papain and bromelain on *Alicyclobacillus* spp. Int J Food Microbiol. 2016; 216: 121–26. DOI: 10.1016/j.ijfoodmicro.2015.10.007.

[41]. Moraes D, Levenhagen MA, Costa-Cruz JM, Costa-Netto AP, Rodrigues RM. In vitro efficacy of latex and purified papain from *Carica papaya* against *Strongyloides venezuelensis* eggs and larvae. Rev Inst Med Trop. 2017; 59: e7. DOI: 10.1590/S1678-9946201759007.

[42]. Mota VS, Turrini RNT, Poveda VB. Antimicrobial activity of *Eucalyptus globulus* oil, xylitol and papain: a pilot study. Rev Esc Enferm USP. 2015; 49 (2): 215–19. DOI: 10.1590/S0080-623420150000200005.

[43]. Müller A, Barat S, Chen X, Bui KC, Bozko P, Malek NP et al. Comparative study of antitumor effects of bromelain and papain in human cholangiocarcinoma cell lines. Int J Oncol. 2016; 48 (5): 2025-34. DOI: 10.3892/ijo.2016.3411.

[44]. Shindo T, Van de Hoorn RAL. Papain-like cysteine proteases: key players at molecular battlefields employed by both plants and their invaders. Mol Plant Pathol. 2008; 9 (1): 119–25. DOI:

[45]. Chen YY, Lu YH, Ma CH, Tao WW, Zhu JJ, Zhang X. A novel elastic liposome for skin delivery of papain and its application on hypertrophic scar. Biomed Pharmacother. 2017; 87: 82–91. DOI: 10.1016/j.biopha.2016.12.076.

[46]. De Lencastre Novaes LC, Jozala AF, Lopes AM, Santos-Ebinuma VC, Mazzola PG, Pessoa Jr A. Stability, purification, and applications of bromelain: a review. Biotechnol Prog. 2016; 32 (1): 5–13. DOI: 10.1002/btpr.2190.

[47]. Taussig SJ, Batkin S. Bromelain, the enzyme complex of pineapple (*Ananas comosus*) and its clinical application. J Ethnopharmacol. 1988; 22 (2): 191–203. DOI: 10.1016/0378-8741(88)90127-4.

[48]. Bhui K, Prasad S, George J, Shukla Y. Bromelain inhibits COX-2 expression by blocking the activation of MAPK regulated NF-kappa B against skin tumor-initiation triggering mitochondrial death pathway. Cancer Lett. 2009; 282 (2): 167–76. DOI: 10.1016/j.canlet.2009.03.003.

[49]. Secor ER, Szczepanek SM, Castater CA, Adami AJ, Matson AP, Rafti ET et al. Bromelain inhibits alergic sensitization and murine asthma via modulation of dendritic

cells. Evid Based Complement Alternat Med. 2013; 2013: 702196. DOI: 10.1155/2013/702196.

[50]. Jayachandran S, Khobre P. Efficacy of bromelain along with trypsin, rutoside trihydrate enzymes and diclofenac sodium combination therapy for the treatment of TMJ Osteoarthritis - A randomised clinical trial. J Clin Diagn Res. 2017; 11 (6): ZC09-ZC11. DOI: 10.7860/JCDR/2017/25771.9964.

[51]. Mynott TL, Guandalini S, Raimondi F, Fasano A. Bromelain prevents secretion caused by *Vibrio cholerae* and *Escherichia coli* enterotoxins in rabbit ileum *in vitro*. Gastroenterology. 1997; 113 (1): 175-84. DOI: 10.1016/s0016-5085(97)70093-3.

[52]. Baeyens-Volant D, Matagne A, El Mahyaoui R, Wattiez R, Azarkan M. A novel form of ficin from *Ficus carica* latex: purification and characterization. Phytochem. 2015; 117: 154–67. DOI: 10.1016/j.phytochem.2015.05.019.

[53]. Shahidi S, Jamili S, Ghavam Mostafavi P, Rezaie S, Khorramizadeh M. Assessment of the inhibitory effects of ficin-hydrolyzed gelatin derived from squid (*Uroteuthis duvauceli*) on breast cancer cell lines and animal model. Iran J Allergy Asthma Immunol. 2018; 17 (5): 436-52. DOI: 10.18502/ijaai.v17i5.302.

[54]. Raskovic B, Lazic J, Polovic N. Characterisation of general proteolytic, milk clotting and antifungal activity of *Ficus carica* latex during fruit ripening. J Sci Food Agric. 2016; 96 (2): 576–82. DOI: 10.1002/jsfa.7126.

[55]. Storpirtis S, Gonçalves JE, Chiann C, Gai MN. Ciências farmacêuticas: Biofarmacotécnica. Rio de Janeiro: Ed. Guanabara Koogan, 2009.

[56]. Nasciutti PR. Desenvolvimento de novos fármacos. Pós-Graduação em Ciência Animal da Escola de Veterinária e Zootecnia da Universidade Federal de Goiás, 2012.

[57]. Loyd V, Allen Jr, Nicholas GP, Howard CA. Formas Farmacêuticas e Sistemas de Liberação de Fármacos. 9ª ed, Ed. Artmed, 2013.

[58]. Melo CS, Cunha Jr AS, Fialho SL. Formas farmacêuticas poliméricas para a administração de peptídeos e proteínas terapêuticos. Rev Ciênc Farm Básica Apl. 2012; 33 (4): 469-77. ISSN 1808-4532.

[59]. McClements DJ. Encapsulation, protection, and delivery of bioactive proteins and peptides using nanoparticle and microparticle systems: A review. Adv Colloid Interface Sci. 2018; 253: 1-22. DOI: 10.1016/j.cis.2018.02.002.

[60]. Almeida AJ, Souto E. Solid lipid nanoparticles as drug delivery systems for peptides and proteins. Adv Drug Del Rev. 2007; 59 (6): 478-90. DOI: 10.1016/j.addr.2007.04.007.

[61]. Feitosa RC, Geraldes DC, de-Araújo BVL, Costa JSR, Oliveira-Nascimento L. Pharmacokinetic aspects of nanoparticle-in-matrix drug delivery systems for oral/buccal delivery. Front Pharmacol. 2019; 10: 1057. DOI: 10.3389/fphar.2019.01057.

[62]. Bizerra A, Silva V. Sistemas de liberação controlada: Mecanismos e aplicações. Revista Saúde e Meio Ambiente. 2016; 3 (2): 1-12. ISSN: 2447-8822.

[63]. Moeller EH, Jorgensen L. Alternative routes of administration for systemic delivery of protein pharmaceuticals. Drug Discov Today: Technol. 2008, 5 (2–3): e89-94. DOI: 10.1016/j.ddtec.2008.11.005.

[64]. Silva AC, Lopes CM, Lobo JMS, Amaral MH. Delivery systems for biopharmaceuticals. Part I: Nanoparticles and Microparticles. Curr Pharm Biotechnol. 2015; 16 (11): 940-54. DOI: 10.2174/1389201016666150731112532.

[65]. Lin YW, Zhou QT, Hu Y, Onufrak NJ, Sun S, Wang J et al. Pulmonary pharmacokinetics of colistin following administration of dry powder aerosols in rats. Antimicrob Agents Chemother. 2017; 61 (11): e00973-17. DOI: 10.1128/AAC.00973-17.

[66]. Steiner V, Öhlinger K, Corzo C, Salar-Behzadi S, Fröhlich E. Cytotoxicity screening of emulsifiers for pulmonary application of lipid nanoparticles. Eur J Pharm Sci. 2019; 136: 104968. DOI: 10.1016/j.ejps.2019.104968.

[67]. Mitragotri S, Burke PA, Langer R. Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies. Nat Rev Drug Discov. 2014; 13 (9): 655-72. DOI: 10.1038/nrd4363.

[68]. Griffin BT, Guo J, Presas E, Donovan MD, Alonso MJ, O'Driscoll CM. Pharmacokinetic, pharmacodynamic and biodistribution following oral administration of nanocarriers containing peptide and protein drugs. Adv Drug Deliv Rev. 2016; 106 (Pt B): 367-80. DOI: 10.1016/j.addr.2016.06.006.

[69]. Muheem A, Shakeel F, Jahangir MA, Anwar M, Mallick N, Jain GK et al. A review on the strategies for oral delivery of proteins and peptides and their clinical perspectives. Saudi Pharm J. 2016; 24 (4): 413-28. DOI: 10.1016/j.jsps.2014.06.004.

[70]. Blanco E, Shen H, Ferrari M. Principles of nanoparticle design for overcoming biological barriers to drug delivery. Nat Biotechnol. 2015; 33 (9): 941–51. DOI: 10.1038/nbt.3330.

[71]. Bai JP, Chang LL, Guo JH. Effects of polyacrylic polymers on the lumenal proteolysis of peptide drugs in the colon. J Pharm Sci.1995; 84 (11): 1291-94. DOI: 10.1002/jps.2600841107.

[72]. Silva C, Ribeiro A, Ferreira D, Veiga F. Administração oral de peptídios e proteínas: Estratégias gerais para aumento da biodisponibilidade oral. Rev.Bras. Cienc. Farm, 2002; 38 (2): 125-140. DOI: 10.1590/S1516-93322002000200002.

[73]. Réus M, Carmignan F, Lemos Senna E, Machado de Campos A. Nanopartículas poliméricas na administração tópica ocular de fármacos. Lat. Am. J. Pharm. 2009; 28 (1): 125-32. ISSN 0326-2383.

[74]. Zhang L, Wang S, Zhang M, Sun J. Nanocarriers for oral drug delivery. J Drug Target. 2013; 21 (6): 515-27. DOI: 10.3109/1061186X.2013.789033.

[75]. Wang L, Liu Y, Zhang W, Chen X, Yang T, Ma G. Microspheres and microcapsules for protein delivery: Strategies of drug activity retention. Curr. Pharm. Des., 2013; 19 (35): 6340-52. DOI: 10.2174/1381612811319350010.

[76]. Reis CP, Neufeld RJ, Ribeiro AJ, Veiga F. Nanoencapsulation II. Biomedical applications and current status of peptide and protein nanoparticulate delivery systems. Nanomedicine; 2006; 2 (2): 53-65. DOI: 10.1016/j.nano.2006.04.009.

[77]. Lu XY, Wu DC, Li ZJ, Chen GQ. Polymer nanoparticles. Prog Mol Biol Transl Sci. 2011; 104: 299-323. DOI: 10.1016/B978-0-12-416020-0.00007-3.

[78]. Qodratnama R, Serino LP, Cox HC, Qutachi O, White LJ. Formulations for modulation of protein release from large-size PLGA microparticles for tissue engineering. Mater Sci Eng C Mater Biol Appl. 2015; 47: 230-6. DOI: 10.1016/j.msec.2014.11.003.

[79]. Liu Y, Lu W, Wang J, Zhang X, Zhang H, Wang X, et al. Controlled delivery of recombinant hirudin based on thermo-sensitive Pluronic® F127 hydrogel for subcutaneous administration: In vitro and in vivo characterization. J Control Release. 2007; 117 (3): 387-95. DOI: 10.1016/j.jconrel.2006.11.024.

[80]. Sokker HH, Abdel Ghaffar AM, Gad YH, Aly AS. Synthesis and characterization of hydrogels based on grafted chitosan for the controlled drug release. Carbohyd Polym. 2009; 75 (2): 222-29. DOI: 10.1016/j.carbpol.2008.06.015.

[81]. Hoare TR, Kohane DS. Hydrogels in drug delivery: progress and challenges. Polymer. 2008; 49 (8): 1993-2007. DOI: 10.1016/j.polymer.2008.01.027.

[82]. Kashyap N, Viswanad B, Sharma G, Bhardwaj V, Ramarao P, Kumar MNV. Design and evaluation of biodegradable, biosensitive *in situ* gelling system for pulsatile delivery of insulin. Biomaterials. 2007; 28 (11): 2051-60. DOI: 10.1016/j.biomaterials.2007.01.007.

[83]. Nanjwade BK, Bechra HM, Derkar GK, Manvi FV, Nanjwade VK. Dendrimers: emerging polymers for drug-delivery systems. Eur J Pharm Sci. 2009; 38 (3): 185-96. DOI: 10.1016/j.ejps.2009.07.008.

[84]. Pimenta LFC. Influência de dendrímeros e da iontoforese na penetração da protoporfirina IX em tumores cutâneos. Dissertação da Faculdade de Ciências Farmacêuticas de Ribeirão Preto da Universidade de São Paulo, 2013. DOI: 10.11606/D.60.2013.tde-18122013-093754.

[85]. Onoue S, Yamada S, Chan HK. Nanodrugs: pharmacokinetics and safety. Int J Nanomedicine. 2014; 9: 1025-37. DOI: 10.2147/IJN.S38378.

[86]. D'Abreu DP. Sistemas de nanopartículas híbridas para veiculação de DNA. Dissertação da Faculdade de Farmácia, da Universidade de Lisboa, 2017. http://hdl.handle.net/10451/36115.

[87]. Coutinho JA. Nanopartículas na entrega eficaz e segura de fármacos ao cérebro por via nasal Experiência Profissionalizante na vertente de Farmácia Comunitária, Hospitalar e Investigação. Dissertação em Ciências da Saúde – Universidade da Beira Interior, 2015. http://hdl.handle.net/10400.6/5324.

[88]. Madigan MT, Martinko JM, Dunlap PV, Clark DP. Microbiologia de Brock. 14. ed. Porto Alegre: Artmed. 2016; 1160.

[89]. Batista CM, Carvalho CMB, Magalhães NSS. Lipossomas e aplicações terapêuticas: Estado da arte. Braz J Pharma Sci. 2007; 43 (2): 167-79. DOI: 10.1590/S1516-93322007000200003.

[90]. Dés Rieux A, Fievez V, Garinot M, Schneider Y, Préat V. Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach. J Control Release. 2006; 116 (1): 1-27. DOI: 10.1016/j.jconrel.2006.08.013.

[91]. Grabnar PA, Kristl J. The manufacturing techniques of drug-loaded polymeric nanoparticles from preformed polymers. J Microencapsul. 2011; 28 (4): 323-35. DOI: 10.3109/02652048.2011.569763.

[92]. Mundargi RC, Babu VR, Rangaswamy V, Patel P, Aminabhavi TM. Nano/micro technologies for delivering macromolecular therapeutics using poly(d,l-lactide-*co*-glycolide) and its derivatives. J Control Release. 2008; 125 (3): 193-209. DOI: 10.1016/j.jconrel.2007.09.013.

[93]. Ré MI. Formulating Drug Delivery Systems by Spray Drying. Drying Technol. 2006; 24 (4): 433–46. DOI:10.1080/07373930600611877.

[94]. Mishra DK, Ashish KJ, Prateek KJ. Review on Various Techniques of Microencapsulation. Int J Pharma Chem Sci. 2013; 2 (2): 962-77. ISSN: 2277–5005.

[95]. Lee KX, Shameli K, Mohamad SE, Yew YP, Mohamed-Isa ED, Yap HY et al. Bio-mediated synthesis and characterisation of silver nanocarrier, and its potent anticancer action. Nanomaterials (Basel). 2019; 9(10): 1423-41. DOI: 10.3390/nano9101423.

[96]. Mathur P, Jha S, Ramteke S, Jain NK. Pharmaceutical aspects of silver nanoparticles. Artif Cells Nanomed Biotechnol. 2018; 46 (sup1): 115–26. DOI: 10.1080/21691401.2017.1414825.

[97]. Freitas, I. Síntese Verde para obtenção de Nanopartículas de Prata a partir de Extratos Naturais. Monografia da Universidade Federal do Rio de Janeiro (Curso de Engenharia de Materiais), 2019; 60 p.

[98]. Mane Gavade SJ, Nikam GH, Dhabbe RS, Sabale SR, Tamhankar BV, Mulik GN. Green synthesis of silver nanoparticles by using carambola fruit extract and their antibacterial activity. Advances in Natural Sciences: Nanosci Nanotechnol. 2015, 6 (4): 1-6. DOI: 10.1088/2043-6262/6/4/045015.

[99]. Oliveira GZS, Lopes CAP, Sousa MH, Silva LP. Synthesis of silver nanoparticles using aqueous extracts of *Pterodon emarginatus* leaves collected in the

summer and winter seasons. International Nano Letters. 2019; 9: 109-17. DOI: 10.1007/s40089-019-0265-7.

[100]. Sena AEC, Ramos AL, Vianna-Faria FSED. Avaliação da síntese de nanopartículas de prata sob diferentes concentrações do extrato de *Copaiba multijuga* (Heine). Scientia Naturalis. 2019; 1 (1): 10-16. ISSN: 2596-1640.

[101]. Lopes JR. Síntese de nanopartículas de prata *(*NPsAg*)* em soluções aquosas de fibroína de seda e gelatina. 2017, 115p. Dissertação da Universidade Estadual de Campinas, 2017.

[102]. Kuppusamy P, Yusoff MM, Maniam GP, Govindan N. Biosynthesis of metallic nanoparticles using plant derivatives and their new avenues in pharmacological applications – An updated report. Saudi Pharm Journal. 2016; 24 (4): 473-84. DOI: 10.1016/j.jsps.2014.11.013.

[103]. Toledo, ACO. Desenvolvimento, caracterização e avaliação das atividades biológicas de nanopartículas de prata e de ouro, obtidas por síntese verde, a partir do extrato aquoso das sementes de *Pterodon emarginatus* Vogel (Fabaceae) associadas à gentamicina e ao ácido hialurônico. Tese de doutorado em Ciências Farmacêuticas da Universidade Estadual de Ponta Grossa. 2021; 169p.

[104]. Albernaz VL. Síntese verde de nanopartículas de prata com extrato aquoso de folhas de *Brosimum gaudichaudii*, caracterização fisicoquímica, morfológica e suas aplicações no desenvolvimento de um nanobiossensor eletroquímico. Dissertação do Instituto de Ciências Biológicas da Universidade de Brasília, 2014.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.