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Review

New trends for a classical enzyme: Papain, a biotechnological success story in the food industry

Jesús Fernández-Lucas [a, b, *], Daniel Castañeda [b], Daniel Hormigo [a, **]

[a] Applied Biotechnology Group, Department of Pharmacy and Biotechnology, School of Biomedical Sciences, Universidad Europea de Madrid, Urbanización El Bosque, Calle Tajo s/n, 28670 Villaviciosa de Odón, Madrid, Spain
[b] Grupo de Investigación en Desarrollo Agroindustrial Sostenible, Department of Agroindustrial Engineering, School of Environmental Sciences, Universidad de la Costa, Cra. 55 #58-66, Barranquilla, Colombia

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ABSTRACT

Background: In recent years, proteases have arisen as standard biocatalysts in many industrial processes in different fields, such as pharmaceutical, medicine, detergent manufacturing and food science. Among them, papain is undoubtedly one of the most frequently studied and widely used proteases in the food industry around the world. However, the latest advances in recombinant papain expression systems, genetically engineered biocatalysts, new purification and isolation strategies, and enzymatic immobilization will enhance the development of new applications of papain, as well as improve and optimize classical applications.

Scope and approach: This review addresses not only the latest advances in classic applications, such as meat tenderization and protein hydrolysates, but also the most innovative applications in different industries such as food, animal feed, bioactive peptides production, water treatment, baking and brewing, among many others.

In addition, papain is a perfect example of a successful industrial enzyme that covers all the steps of the biocatalytic cycle that are necessary for the industrial implementation of any biocatalyst. This cycle includes the production and extraction of the enzyme concerned (from natural or recombinant sources), functional and structural characterization, genetic improvement, immobilization and, finally, industrial application. This review describes the complete biocatalytic cycle of papain.

Key findings and conclusions: Papain is clearly a case of industrial and commercial success over the last 40 years. The key to this success has been continual biotechnological and process engineering innovation, which has opened up a new range of possibilities for this exciting biocatalyst. However, further efforts are needed in protein engineering and characterization of new mutants to reach the full potential of this enzyme.

1. Introduction

Within the global industrial enzymes market, the food industry enzymes sector is the major segment and is expected to grow from nearly $1.5 billion in 2016 to $1.9 billion in 2021 (BCC Research Biotechnology Report, 2011. Enzymes in industrial applications: Global markets).

Proteases (also called peptidases or proteinases) are enzymes that hydrolyse the peptidic linkages in protein into shorter fragments (peptides) and eventually into their components, amino acids. Proteases are important enzymes in living organisms, being involved in many different biological processes.

Protease enzymes are used in a large variety of applications, mainly in the detergent and pharmaceutical industries, followed by the food industry. Since proteases represent more than 60% of the enzyme market share, with an expected CAGR (compound annual growth rate) of 5.3% from 2014 to 2019, they are the most important type of commercialized enzymes in the world. Leading producers worldwide include Novo Industries, DSM, DuPont Industries, BASF, Genencor International, and Roche (Feijoo-Siota & Villa, 2011;

* Corresponding author. Applied Biotechnology Group, Department of Pharmacy and Biotechnology, School of Biomedical Sciences, Universidad Europea de Madrid, Urbanización El Bosque, Calle Tajo s/n, 28670 Villaviciosa de Odón, Madrid, Spain.
** Corresponding author.
E-mail addresses: jesus.fernandez2@universidadeuropea.es (J. Fernández-Lucas), daniel.hormigo@universidadeuropea.es (D. Hormigo).

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Kumar, Singh, Sangwan, & Gill, 2014).

According to the nature of the active site, proteases can be divided into seven mechanistic classes: serine proteases, S (EC 3.4.21); cysteine proteases, C (EC 3.4.22); aspartic and glutamic proteases, D or E (EC 3.4.23); metalloproteases, M (EC 3.4.24); threonine proteases, T (EC 3.4.25); and asparagine peptide lyases, N (EC 4.3) (Rawlings, Barrett, & Bateman, 2011). According to the MEROPS database, cysteine proteases are divided into ten clans, and most plant cysteine proteases belong to the C1 family, also known as the papain family (papain-like proteases) Rawlings, Barrett, & Bateman, 2010). Papain-like proteases are found in most known organisms, such as viruses, protozoa, plants, invertebrates, and vertebrates. Most of the C1 family members are endopeptidases, but some of them display a wide variety of activities, including aminopeptidases, dipeptidyl peptidases and enzymes with both exo- and endo-peptidase activities (Feijoo-Siota & Villa, 2011; Novinec & Lenarčič, 2013; Rawlings et al., 2010).

Papain (EC 3.4.22.2), also called Papaya proteinase I (PPI), is a 23.4 kDa, 212 residue cysteine endopeptidase belonging to subfamily C1A of papain-like proteases (Rawlings et al., 2010). The commercial importance of papain is mainly due to its strong proteolytic activity against a broad range of protein substrates, and because it is active across a broad range of operational conditions. These interesting characteristics have allowed papain to lead the proteases market, outselling other plant derived proteases, such as bromelain (*Ananas comosus*) and ficin (*Ficus carica*), as well as fungal source proteases (Market Research Future, 2017. Global Meat Tenderizing Agents Market Research Report- Forecast to 2023). Major producers of papain include India, Sri Lanka, Democratic Republic of Congo, Zaire, Tanzania, Uganda, Mexico, Brazil and Argentina. As regards the global papain market, the main importers are concentrated in Europe and USA, with a market size of about 150–200 and 300–400 tons per year, respectively. Furthermore, the Japanese market is relatively small (less than 50 tons per year). Some of the main limiting factors of the global and sustainable supply of papain are the high climatic dependence of papaya crops, as well as the economical and political issues of some producing countries (IDEA, 2000. Commercialisation Bulletin 13 Papain Report). This fact makes the search for alternative sources of papain a priority for producing companies. Fortunately, the latest advances in recombinant papain expression may provide a solution to this problem.

This review presents papain as an industrial success paradigm in Biocatalysis, where all the steps necessary for the industrial implementation of any enzyme have been effectively addressed (Fig. 1). The review provides a detailed description of each step, including the different strategies of isolation and purification from natural and recombinant sources, operational conditions, genetic modifications to improve its functional characteristics, enzyme immobilization, and industrial applications. It also describes the most recent trends in meat tenderization, dairy industry, production of protein hydrolysates and bioactive peptides, food allergens removal, brewing and baking industry, animal feed and water treatment, among other fields. Finally, we look at some industrial uses of papain not strictly focused on the food industry, but which demonstrate the enormous versatility of this exciting enzyme, such as certain biomedical approaches, antimicrobial food packaging, caries removal agent, or tooth-whitening additive in toothpastes.

2. Production and purification strategies

2.1. Plant derived papain

Papain can be obtained from the latex of the papaya plant (*Carica papaya*), which is a natural source of other endopeptidases, such as chymopapain (EC 3.4.22.6), caricain (EC 3.4.22.30) and glycyl endopeptidase (EC 3.4.22.25). In fact, papain is a minor constituent (5–8%) among the papaya endopeptidases (Feijoo-Siota & Villa, 2011).

Purification of papain from papaya latex has traditionally been carried out using precipitation methods, reaching high yields of up to 53 g of crude enzyme per kg of latex. Although these methods have routinely been used in industry, they can only achieve up to 39% purity of papain (Nitsawang, Hatti-Kaul, & Kanasawud, 2006). An alternative and more efficient purification strategy involves the use of various chromatographic techniques (ion exchange or affinity). In these types of techniques the initial pre-processing of the latex is essential before samples can be applied on a chromatography column. This processing consists in a sun- or spray-drying process after latex extraction from plant (Feijoo-Siota & Villa, 2011).

Current techniques usually include aqueous two-phase systems (ATPS). These systems may be composed of two polymers in aqueous solution, or one polymer and salt in aqueous solution. ATPS techniques have shown great potential for downstream processing of papain and other proteases, since they allow clarification, concentration and purification of the target product in a one-pot process (Nitsawang et al., 2006). In this respect, it is worth mentioning that polymer–salt–water systems have aroused greater interest in the industry, since they are cheaper and show less viscosity than polymer–polymer–water systems. In this regard, polyethylene glycol–phosphate based systems are the most employed (Rocha & Nerli, 2013). Recent studies have demonstrated that the use of alginate as macro-ligand in PEG based systems improves papain purification. In a recent study, Rocha et al. (2016) described a PEG-citrate buffer system able to purify papain from latex with a 20% of recovery. They observed that by adding alginate (0.1% w/w) and $CaCl_2$ they could recover up to 72% of papain and recycle PEG for purification. This strategy is very promising, since it is low cost, easy to scale up, accurate and environmentally friendly (Rocha et al., 2016).

In 2014, He et al. reported an efficient method of large scale papain purification from unclarified papaya juice feedstock in batch adsorption system. In this study, the authors employed a reversed phase expanded bed adsorption chromatography (RP-EBAC) using a FastlineTM-10-EBAC column packed with AmberliteTM-XAD-7HP. This technique allowed the authors to purify papain in a single operation with a purification factor of 7.04 and a purity of 75% (He, bin Tuan Chik, & Chong, 2014).

After the purification process, crude papain usually has to be treated with reducing agents in order to protect the free cysteine thiol groups from oxidation, preserving its protease activity. When needed, free thiol groups of papain can be regenerated by the addition of low molecular mass thiols, such as cysteine or dithiothreitol.

2.2. Recombinant papain

The high world demand of papain for industrial uses makes necessary the search for alternative sources from traditional extraction from the latex of *C. papaya*. Despite the high recovery of papain from *Carica papaya* plant material (up to 53 g of crude enzyme per kg of wet latex), this natural source presents several drawbacks. First, papain only represents about 5–8% of total cysteine proteases in the latex, which entails an expensive and ponderous isolation strategy (Nitsawang et al., 2006). Furthermore, isolated papain is highly susceptible to oxidation, so it must immediately be conserved at low temperatures and direct air contact must be avoided. Another major disadvantage is the dependence of papaya crops on external factors, such as climate, soil, pests, etc., which avoids a continuous supply of papain.

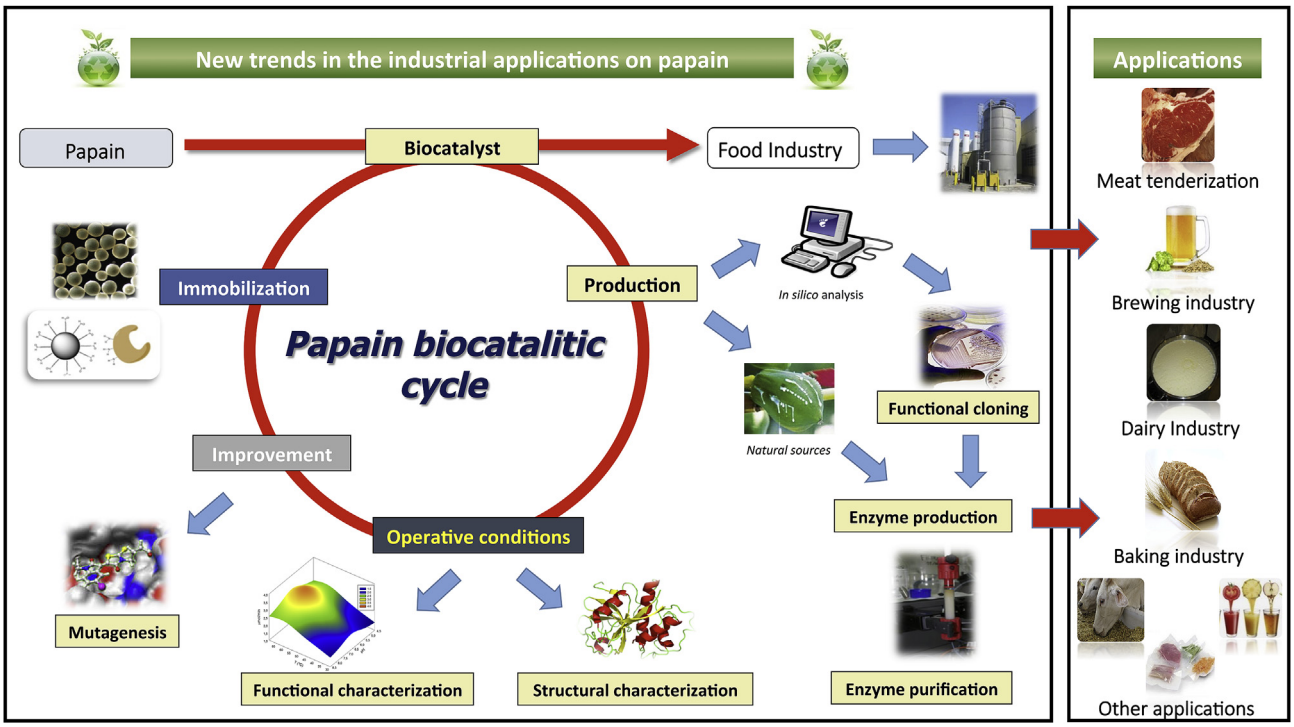

Fig. 1. Complete biocatalytic cycle of papain.

The expression of recombinant papain in different microorganism-based systems makes it possible to overcome these drawbacks while significantly increasing the papain production capacity. Papain is synthesized as a pre-pro-peptide with 345 amino acid residues. This precursor contains a signal peptide (residues 1 to 18), a spacer peptide (19–33) and the mature enzyme (134–345). The most abundant amino acids in papain precursor protein are glycine (35), tyrosine (28) and valine (24), while histidine (3) and methionine (5) are present in the lowest amounts.

Papain precursor can be cloned and expressed in *Escherichia coli*, but recombinant enzyme forms inactive inclusion bodies. However, they can be renatured to yields about 3 mg of active papain/L (Taylor et al., 1992). In this regard, Choudhury, Roy, Chakrabarti, Biswas, and Dattagupta (2009) described an optimized purification and refolding protocol, reaching 400 mg/L of a His-tagged fusion pro-papain from *E. coli*. However, several problems can arise during the refolding process, such as autoproteolysis, oxidation of the active site and formation of incorrect disulfide bonds.

Eukariotic expression systems have also been studied for production of recombinant papain. Vernet et al. (1990) obtained 0.3 mg/L of soluble papain by using a baculovirus/insect expression system. Higher concentrations of 1.7 mg/L have been reported by Ramjee, Petithory, McElver, Weber, and Kirsch (1996) using the yeast *Saccharomyces cerevisiae* as expression system. More recently, Werner, Hirth, Rupp, and Zibek (2015) described the expression of a codon-optimized His-tagged papain in *Pichia pastoris*. After purification 463 mg/L enzyme can be obtained, matching the highest production reported to date using *E. coli* as host (400 mg/L) (Choudhury et al., 2009). Moreover, this codon-optimized papain can be purified in a single step from the culture medium, and it showed a 1.4 times higher enzymatic activity towards the chromogenic peptide Z-phenylalanine-arginine-paranitroanilide compared with a commercial papain. In this study, the authors demonstrate that the expression of papain is better in *P. pastoris* than in *E. coli* system (Werner et al., 2015).

It is worth noting that the traditional process of papain production from papaya crops (up to 53 g of crude enzyme per kg of wet latex) takes about 11–13 months from the transplant of *Carica papaya* until the fruit is ready to produce the latex (Kamphuis, Kalk, Swarte, & Drenth, 1984). Comparing these data with those obtained by Werner et al. (2015) in *Pichia pastoris*, it can be estimated that a small culture in a 1000-L batch fermenter could yield 463 g of papain in as little as 2–3 days. Taking into account this enormous difference in yields as well as the advantages that recombinant production offers regarding purification processes, irrespective of political, economic and climatic factors, in our opinion the production of recombinant papain will completely displace traditional production in the medium term.

3. Operational conditions

3.1. Functional characterization

Regarding its enzymatic activity, papain has a broad-spectrum endopeptidase activity over a pH range of 5–8 and optimal temperature of 65 °C (Polaina & MacCabe, 2007).

The specificity of papain has been extensively examined using different substrates, such as milk, haemoglobin, collagen discs and labelled collagen products, synthetic peptides, gelatine (You, Regenstein, & Liu, 2010), casein (Morais, Silva, Oliveira, & Silvestre, 2004), chitosane (Pan, Zeng, Foua, Alain, & Li, 2016), rice bran protein concentrates (Ahmadifard, Murueta, Abedian-Kenari, Motamedzadegan, & Jamali, 2016), soybean proteins (Shutov et al., 2013), potato protein isolate (Waglay & Karboune, 2016), immunoglobulins including sheep IgG, rabbit IgG, chicken IgY and fish IgM, bovine serum albumin (BSA), lipid transfer protein (LTP), and whey proteins (α-lactalbumin and β-lactoglobulin) (Chalabi, Khademi, Yarani, & Mostafaie, 2014), among many others (Table 1).

According to the model proposed by Schechter and Berger (1967), papain interacts with at least seven residues of the

Table 1
Optimum pH and temperature for papain with different substrates.

Substrate	pH	T (°C)	References
Meat	7–8	60–65	(Bekhit et al., 2014)
Gelatin	7.5	56.8	(You et al., 2010)
Casein	7.5	37	(Morais et al., 2004)
Chitosan	4.55	44.42	(Pan et al., 2016)
Rice bran proteins concentrates	8	28	(Ahmadifard et al., 2016)
Soybean (β-conglycin)	5.6	30	(Shutov et al., 2013)
Potato protein isolate	8	50	(Waglay & Karboune, 2016)
Sheep and rabbit IgG	7	37	(Chalabi et al., 2014)
Fish IgM	5.5–8.5	37	(Chalabi et al., 2014)
LTP	7.2	37	(Chalabi et al., 2014)

substrate, four amino acid residues in N-terminal direction from the cleaved bond (named P1-P4), and three in C-terminal direction (named P1′-P3′). Authors also proposed seven corresponding sites on the enzyme (sites S1-S4 and S1′-S3′) which are responsible for the recognition and cleavage of polypeptide (Novinec & Lenarčič, 2013; Schechter & Berger, 1967). However, three decades later, the structural analysis of reported papain structures support this definition to some extent, and only a few sites were confirmed in papain-like proteases (sites S2, S1 and S1′) (Turk, Gunčar, Podobnik, & Turk, 1998).

In general, papain-like proteases have broad specificity and the major determinant is the residue in the P2 position of the substrate. In this sense, Papain specificity is controlled by the S2 site, a hydrophobic core that accommodates the P2 side chain of the substrate. Due to this, papain shows high specificity for amino acids with hydrophobic or aromatic side chains, such as Val, Phe and Tyr, at this position. However, papain does not recognize Val in P1′ (Choe et al., 2006; Groves, Coulombe, Jenkins, & Cygler, 1998; Amri & Mamboya, 2012; Novinec & Lenarčič, 2013; Turk et al., 1998).

3.2. Structural characterization

Despite the limited sequence homology, cysteine proteases display a conserved core structure, composed of two clearly differentiated interacting domains, an α-helix and a β-barrel-like that have been termed the L- and R-domains according to their position in the standard orientation (Fig. 2) (Novinec & Lenarčič, 2013). Papain structure displays a globular protein with three disulfide bonds and two catalytic L- and R-domains. The active site is located at the interface of L- and R-domains in the form of a V-shaped cleft and is formed by a cysteine (Cys), a histidine (His), an asparagine (Asn) and a glutamine residue (Gln), which are conserved in all papain-like proteases (Fig. 2). In the mature protein, Cys_{25} and His_{159} form an ion pair substrate-binding pocket stabilized by Asn_{175}, which orients the His_{159} imidazolium ring. The sulfhydryl group of Cys_{25} performs a nucleophilic attack on the carbonyl carbon of a substrate's peptide bond, leading to an unstable tetrahedral intermediate. This intermediate spontaneously collapses to regenerate the carbonyl group, leading to an acyl enzyme complex, which is hydrolysed into the free enzyme and the N-terminal portion of the substrate (Fig. 3) (Cstorer & Ménard, 1994). Another important residue is Gln_{19}, which precedes the Cys_{25} and is believed to help in forming an oxyanion hole by stabilizing the tetrahedral intermediate. Asn_{175} forms a hydrogen bond to His_{159} (showing a similar role to the aspartic acid in the catalytic triad of serine proteases), but is not necessary for catalysis (Vernet et al., 1995). Finally, it is interesting to note that Trp_{177} is involved in the generation of the nucleophilic character of Cys_{25}/His_{159} ion pair (Fig. 2).

In order to highlight the substrate binding-mode, a three-dimensional scheme of all described binding interactions is shown using the crystal structure of papain complexed with CLIK148, a representative cathepsin L-specific inhibitor (Fig. 4) (Tsuge et al., 1999). On one side, the hydrogen bond formed between Asn_{175} and His_{159} stabilizes the substrate-binding pocket and also orients the His_{159} imidazolium ring. On the other side, the

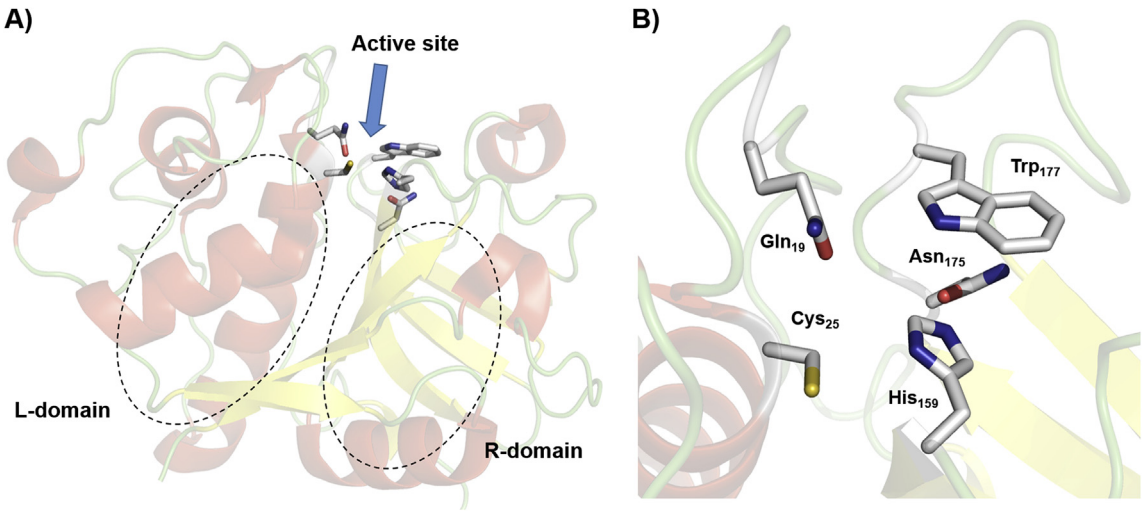

Fig. 2. A) Three-dimensional structure of papain (PDB accession code 1PPN). The arrow indicates the situation of active site. **B)** Catalytic residues in the active site of papain. Cys_{25} and His_{159} are forming the catalytic ion pair.

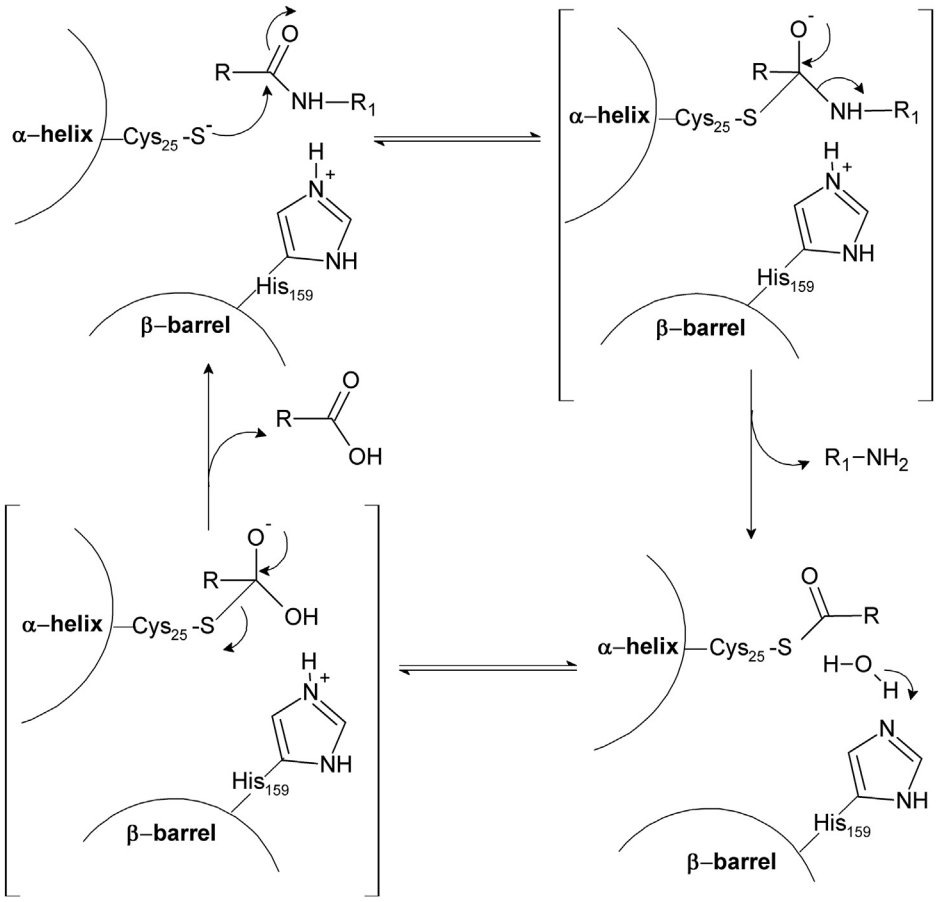

Fig. 3. Catalytic mechanism of papain.

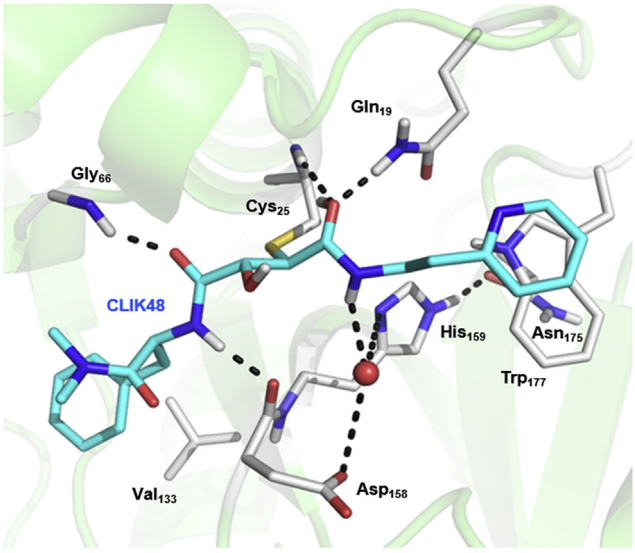

Fig. 4. Crystal structure of CLIK148 (cyan C atoms with heteroatom coloring) bound in the active site of papain. The hydrogen bonding interactions between ligand and protein are draw as black dashed lines. Water molecule is draw as red sphere. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sulfhydryl group of catalytic Cys_{25} is covalently bonded to the C2 atom of CLIK148. Apart from that, CLIK148 is stabilized in the active site by a network of hydrogen bonds. A hydrogen bond is formed between the nitrogen atom of both Cys_{25} and Gln_{19} and the oxygen atom O1 of CLIK148. Besides, the Gly_{66} peptide nitrogen is hydrogen bonded to the O3 atom of CLIK148. Another hydrogen bond is formed between an oxygen atom of Asp_{158} and the nitrogen atom N5 of CLIK148. As shown in Figs. 2 and 4, a water molecule is located in the active site, near the residue His_{159}. In this regard, three hydrogen bonds are formed from N7 atom of CLIK148, carbonyl oxygen of Asp_{158} and N1 atom of His_{159} to the water molecule. Finally, several hydrophobic interactions among CLIK148 with Trp_{177} (responsible for the stacking of pyridine ring of the substrate) and Val_{133} are shown.

4. Biocatalyst improvement

4.1. Genetic modifications

As mentioned above, several studies have addressed papain mutations to increase expression levels. Other studies, however, have told us more about the catalytic mechanism of this enzyme, such as that carried out by Gul et al. (2008), who demonstrated that Asp_{158} does not affect to nucleophilic character in the Cys_{25}-His_{159} dyad as previously thought, whereas Trp_{177} showed a key role. Choudhury, Biswas, Roy, and Dattagupta (2010) developed single ($Lys_{174}\rightarrow Arg$), double ($Lys_{174}\rightarrow Arg$, $Val_{32}\rightarrow Ser$) and triple mutants of papain ($Lys_{174}\rightarrow Arg$, $Val_{32}\rightarrow Ser$, $Gly_{36}\rightarrow Ser$), increasing the thermostability of the biocatalyst. The triple mutant was the most thermostable, increasing its half-life by 45 min at 60 °C and at 65 °C compared with wild type papain. In addition, the triple mutant had

a faster inactivation rate beyond T_{max}, which is very interesting for industrial implementation.

Recent studies have demonstrated that the specificity of papain can be modified by replacing the residue Ile_{86} in its pro-peptide region. These mutations ($Ile_{86} \rightarrow$ Phe, $Ile_{86} \rightarrow$ Leu and $Ile_{86} \rightarrow$ Ala) can block the specificity determining S2-subsite of the catalytic cleft of the protease in its zymogen form and significantly improve the macroscopic kinetic parameters (K_m and k_{cat}) of the enzyme (Dutta, Choudhury, Roy, Dattagupta, & Biswas, 2016). Several of these studies and others are summarized in Table 2.

5. Papain immobilization

Enzyme immobilization provides an excellent base for increasing enzyme stability and half-life, also simplifying downstream processing. Thus, many different methods exist for enzymatic immobilization (physical adsorption, entrapment, copolymerization or covalent attachment, among others), and several natural and synthetic supports have been assessed for their efficiency in the process. Since papain has been utilized in a large number of industrial applications, numerous efficient strategies on a variety of carriers have been described in the current literature, such as starch gel (Sangeetha & Abraham, 2006), nitrilon fibre functionalized with amine groups (Li, Xing, & Ding, 2007), cotton fabric (Xue, Nie, Zhu, Li, & Zhang, 2010) or sepharose (Homaei, Sajedi, Sariri, Seyfzadeh, & Stevanato, 2010), between many others.

More recent papain immobilization strategies have been included: generation of porous cross-linked enzyme aggregates (*p*-CLEAs) (Wang et al., 2011); the use of iron oxide magnetic nanoparticles (g-Fe_2O_3/Fe_3O_4) activated with thiophene and triazole as enzyme linkers (Xin, Si, & Xing, 2010); encapsulation in biosilica matrix through a biomimetic silicification process induced by papain (Zhou, Wang, Jiang, & Gao, 2013); magnetic gold nanocomposites modified with 3-(mercaptopropyl) trimethoxy silane (MPTS) (Sahoo, Sahu, Bhattacharya, Dhara, & Pramanik, 2013) (Fig. 5); and crosslinking with a glutaraldehyde to p*oly (vinyl alcohol*) PVA nanofibers prepared by electrospinning (Moreno-Cortez et al., 2015).

6. Industrial applications of papain

Functional properties of papain have developed an increasing interest in a wide range of industrial uses, mainly in meat tenderization, foods, feeds, brewing and the textile industry. Papain has also been described as an active additive in tooth-bleaching dentifrices and skin products. This part of the review summarises the main applications of this enzyme (Table 3).

6.1. Meat tenderization

The use of exogenous proteases to improve meat tenderness has become an increasing focus of interest recently. It is a priority for the meat industry to be able to cover the increasing demand for guaranteed tender meat and give added value to lower-grade meat cuts.

Many approaches have been based on improving post-mortem tenderness, such as mechanical tenderization, water content enhancement, and different enzymatic treatments (Pietrasik & Shand, 2011). Traditionally, meat is softened by autoproteolysis (mainly mediated by cathepsins and capains), keeping it at 4 °C for 7–10 days.

Under optimal operating conditions (pH around 7–8 and temperature between 60 and 65 °C) papain is able to hydrolyse almost any protein present in muscle tissue, as well as tendons and ligaments, which makes it a potent meat softener (Bekhit, Hopkins, Geesink, Bekhit, & Franks, 2014).

There are numerous studies in which the meat softening effect of papain has been evaluated when enzyme is injected into the animal before being slaughtered, allowing a homogenous distribution of papain in the meat of the animal. Today, this technique presents ethical concerns and is not used, since the injection of active papain can cause suffering and stress in animals. An alternative is the injection of inactive papain. For this purpose, papain is usually treated with hydrogen peroxide to oxidize the catalytic cysteine and then injected into the animal. Once the animal is sacrificed, the anoxic conditions induce the reduction of catalytic cysteine and thus the reactivation of papain (Bekhit et al., 2014). However, this ante-mortem method presents some drawbacks, mainly due to the difficulty in predicting the level of tenderization, since this depends on different physiological factors of the animal. Some problems that may appear are related to differences in texture as compared with high quality meat slices, over-tenderization, undesired tastes or smells, or degradation of organs that may be of commercial interest.

Post-mortem application is generally acceptable for lower-grade meat cuts. Papain is supplied commercially in powder and liquid forms (e.g. PANOL®, LIQUIPANOL® T100), as well as combined with other proteases such as bromelain (e.g., ENZECO® DUAL PROTEASE). Several commercial preparations may include other ingredients (salt, phosphates or flavour enhancers such as sodium glutamate),

Table 2
Mutant variants of papain.

Mutation	Host	Result	References
$Lys_{174} \rightarrow$ Arg $Lys_{174} \rightarrow Arg/Val_{32} \rightarrow$ Ser $Lys_{174} \rightarrow Arg/Val_{32} \rightarrow Ser,Gly_{36} \rightarrow$ Ser	*E. coli*	Increase thermal stability	(Choudhury et al., 2010)
$Ile_{86} \rightarrow$ Phe $Ile_{86} \rightarrow$ Leu $Ile_{86} \rightarrow$ Ala	*E. coli*	Modification of substrate specificity	(Dutta et al., 2016)
$Gln_{19} \rightarrow$ Glu $Gln_{19} \rightarrow$ His $Gln_{19} \rightarrow Asn/Ser_{21} \rightarrow$ Ala $Gln_{19} \rightarrow$ Ala $Gln_{19} \rightarrow$ Ser	Baculovirus-insect cell expression system and *S. cerevisiae*	Elucidation of the role of Gln19 in the active site	(Ménard et al., 1991) (Ménard et al., 1995)
$Val_{133} \rightarrow Ala/Ser_{205} \rightarrow$ Glu $Val_{133} \rightarrow Ala/Val_{157} \rightarrow Gln/Ser_{205} \rightarrow$ Glu	Baculovirus-insect cell expression system *a*	Modification of substrate specificity of S2	(Khouri et al., 1991)
$Gly_{19} \rightarrow$ Glu	Baculovirus-insect cell expression system	Nitrile hydratase activity into papain	(Dufour, Storer, & Ménard, 1995)

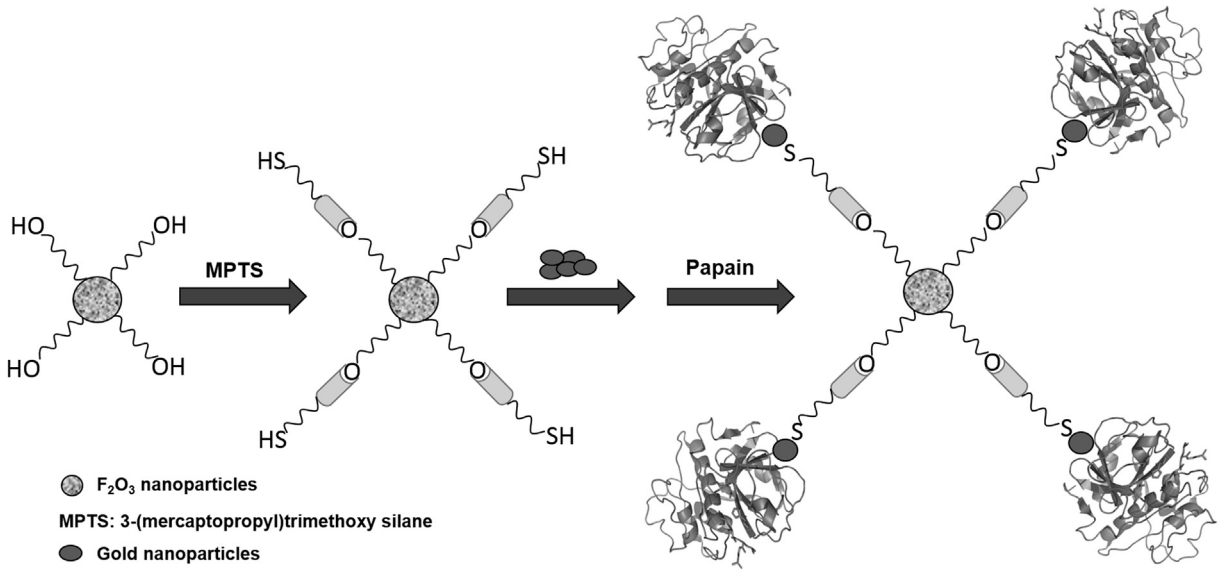

Fig. 5. Schematic representation of the immobilization of papain on magnetic gold nanoparticles.

Table 3
Industrial applications of papain.

Application	Mode of action	References
Meat tenderization	Hydrolysis of connective tissue and myofibrillar proteins.	(Pietrasik & Shand, 2011) (Bekhit et al., 2014)
Production of protein hydrolysates	Hydrolysis of proteins to form peptides of varying sizes with multiple applications: nutritional supplement, pharmaceutical ingredient, flavor enhancer, bioactive properties, etc.	(Polaina & MacCabe, 2007) (Agyei & Danquah, 2011) (Liu et al., 2016) (Meinlschmidt et al., 2016)
Dairy industry	Production of semisoft cheese or cream cheese, improve the meltability and stretchability of cheese	(Mahajan & Chaudhari, 2014) (Hejazin & El-Qudah, 2009) (Abe et al., 2015)
Baking industry	Increase of protein solubility, and reduction of allergenic protein content of cereals.	(Polaina & MacCabe, 2007) (Kong et al., 2007) (Li et al., 2016)
Animal feed	Estimation of protein degradation in ruminant feed. Production of bioactive peptides	(Polaina & MacCabe, 2007) (Choi et al., 2016) (Mo et al., 2016)
Brewing and wine industry	Degradation of several insoluble protein aggregates formed during and after beer fermentation, wine stabilization agent.	(Polaina & MacCabe, 2007) (Esti et al., 2013)
Bioethanol industry	Papain acts as a deflocculating agent avoiding yeast flocculation during fermentation.	(Silva et al., 2015)
Water treatment	Binding of heavy metals, such as mercury, due to presence of four sulfhydryl groups on papain active site.	(Metin & Alver, 2016)
Tooth whitening	Removal of stains, plaque, and food debris from the outer tooth surface.	(Münchow et al., 2016) (Chakravarthy & Acharya, 2012)
Biomedicine	Treatment of burn wounds, esophageal obstruction, caries removal, activity against gastrointestinal nematodes, tissue repairing of venous ulcers, antibacterial activity, etc.	(Anuar, Zahari, Taib, & Rahman, 2008) (Bussadori et al., 2014) (Ribeiro et al., 2015) (Morse et al., 2016)

and they are available to be used directly by processors and households.

6.2. Dairy industry

Due to their ability to hydrolyse the specific peptide bonds to generate casein and macropeptides, proteases have been extensively used as biocatalysts in milk clotting processes in the dairy industry. One of the most important uses of proteases is cheese manufacturing. Traditionally, dried calf stomachs or dried enzyme extracts (animal rennet) have been employed for better controlled cheese making. Due to limited availability of calf stomachs, it was necessary to find new proteases with similar properties, in order to be used as biocatalysts in the industrial production of cheese. The potential of plant proteases in cheese making has been reported in this regard (Jacob, Jaros, & Rohm, 2011).

Arlene, Prima Kristijarti, and Ardelia (2015) studied the effect of papain in the production of Cheddar cheese with different types of milk (cow, goat and soy), concluding that papain displayed highly proteolytic activity on milk but with a very irregular clotting performance. These results suggest that papain is not a good biocatalyst for the production of hard cheese. However, papain shows significant advantages over other proteases, such as better accessibility, lower price, greater availability in large quantities, and more resistance to extreme pH and temperatures. In this respect, if clotting performance could be controlled, papain could be used as a

rennet enzyme replacement for the production of hard cheese.

On the contrary, Mahajan and Chaudhari (2014) report the use of papain in the production of semisoft cheese or cream cheese. Hejazin and El-Qudah (2009) improved the meltability and stretchability of Nabulsi cheese by papain addition. Finally, an interesting article describes how Abe, Wu, Kim, Fujii, and Abe (2015) developed a new method for soymilk cream production using a simple three-step process, including papain digestion, heat treatment and low-speed centrifugation.

6.3. Production of protein hydrolysates

Protein hydrolysates are widely used in industry with many different applications: nutritional supplement, pharmaceutical ingredient, flavor enhancer, nitrogen source for growth media for microbial, plant, and animal cell culture, in cosmetics and in beverages. Currently, the industrial production of protein hydrolysates includes acid, alkali, and enzyme hydrolysis, among which the latter-mentioned is acquiring the most importance in the food and pharmaceutical industries. The enzymatic process is milder and facilitates control of the degree of hydrolysis, which makes the process efficient and reproducible. The operational parameters for enzymatic hydrolysis have been extensively studied, the most important being temperature, hydrolysis time, pH, and degree of hydrolysis (DH) (Agyei & Danquah, 2011).

As a result of hydrolysis, the parent protein forms peptides of varying sizes depending on the enzyme employed and the operational parameters. The peptides with a positive effect on health are termed bioactive peptides, and they can regulate several physiological functions. Many bioactive qualities of protein hydrolysates with papain have been widely studied, such as antitumor, antioxidant, antidiabetic, inhibition of angiotensin−I converting enzyme, modulation of immune system and mineral binding ability (Nesse, Nagalakshmi, Marimuthu, & Singh, 2011).

Due to their high antioxidant properties, fish protein hydolysates (FPH) have aroused special interest (Elavarasan & Shamasundar, 2016; Gajanan, Elavarasan, & Shamasundar, 2016). An important aspect of industrial production of FPH is related to the concentration methods that can be employed, such as oven, freeze or spray drying. Among them, freeze drying is the preferred operation in industry, since it causes less damage to peptides. However, high capital and running costs have led to a search for alternate drying methods. Recently, Elavarasan and Shamasundar (2016) compared the effect of oven- and freeze-drying on the antioxidant properties of FPH with papain from *Cirrhinus mrigala,* suggesting that oven drying may be advantageous.

Moreover, enzymatic conversion of fish frame waste to protein hydrolysate could be a solution for minimizing the pollution issues related to seafood processing operations, and a way to add value to processing by-products.

Another recent application of papain for the production of antioxidant hydrolysates is the Chinese walnut (*Juglans regia* L.) hydrolysates (Liu et al., 2016). The authors hydrolysed walnut proteins with different proteases including papain. This study revealed that hydrolysates obtained with papain showed a yield and purity of 16% and 81%, respectively, higher than others. Moreover, approximately 99% of peptides had a molecular weight lower than 1500 Da. Furthermore, the bioassay indicated that peptides obtained with papain exhibited one of the highest antioxidant properties.

In addition, papain can be used to reduce the level of allergenicity of soy protein isolates (SPI), while sensory, technical and functional properties can be improved. It is worth mentioning that papain proved to be the most suitable protease for improving functionality and sensory characteristics, effectively reducing the molecular weight of SPI (Meinlschmidt, Sussmann, Schweiggert-Weisz, & Eisner, 2016).

Another interesting application of papain in the production of protein hydrolysates is reported by Damodaran (2007). In this work, the author studied the inhibition of ice crystal growth in ice creams by employing gelatine hydrolysates, previously digested with papain. These results are very important, because understanding the molecular interactions responsible for ice crystal growth inhibition by peptides from gelatine hydrolysate would greatly facilitate the development of new peptide cryoprotective agents with enhanced antifreeze activity.

6.4. Baking industry

Wheat is one of the most harvested cereals throughout the world. Nevertheless, wheat contains proteins able to cause allergic reactions in 0.4−1.3% of children and 0.2−0.9% of adults.

Wheat grain contains different allergenic proteins such as gliadins (a, b, c and x), that conserve the allergenic epitope Gln-Gln-Gln-Pro-Pro. Papain has been used to produce hypoallergenic flour suitable for allergic consumers, since it is able to recognize this -Gln-Pro rich motif. Li, Yu, Goktepe, and Ahmedna (2016) described a complete removal of gliadins of wheat flour by a sequential enzymatic treatment with alcalase and papain. This treatment was found to be more effective in reducing allergenic protein content and increasing solubility compared with other proteases (Li et al., 2016).

Another issue related to gluten in the baking industry is that it turns insoluble and expands to form lattice-like structures when it is hydrated. Therefore, gluten should be hydrolysed to obtain more mouldable dough. The use of proteases such as papain improves the quality of the dough, preventing it from contracting, and enhancing its solubility, softness and rising during baking process (Kong, Zhou, & Qian, 2007; Polaina & MacCabe, 2007).

6.5. Brewing and wine industry

Papain is a very commonly used enzyme in the brewing industry, especially in the production of light and clear beers, due to their high chill-proofing potential (Polaina & MacCabe, 2007). The treatment of beer with papain (among other proteases) allows the degradation of several insoluble protein aggregates formed during and after beer fermentation, without any side effects on its organoleptic properties. These high protein levels are responsible for several undesired effects in beer, such as hazes, high viscosity and excess foaming.

In addition, due to the broad substrate specificity displayed by papain, this protease could find useful application in winemaking. In this respect, several reported studies have tested the effect of papain as a wine stabilization agent. In a recent study, Esti, Benucci, Lombardelli, Liburdi, and Garzillo (2013) evaluated papain activity under wine-like conditions, showing that this protease could be applied efficiently as a biocatalyst in the wine industry.

6.6. Animal feed

Approximately 90% of food energy and nutrients for ruminants is provided by low-cost forages. Therefore, quantification of soluble nitrogenous compounds in the rumen is essential to evaluate the quality of feed. Adding proteases like papain to animal feed can increase protein assimilation and bio-availability, which translates into significant economic savings (Polaina & MacCabe, 2007).

Along these lines, the Hong Kong Agriculture, Fisheries and Conservation Department has employed papain to hydrolyse proteins of soy meal for fish feed. In this study, they supplement

traditional pellets with soybean hydrolysates and use them for feed three different species of marine fishes (*Rhabdosargus sarba, Epinephelus bleekeri* and *Trachinotus blochii*). After 340 days of treatment, they observed more relative weight gain and better growth yield in fishes fed with papain treated pellets (Mo, Lau, Kwok, & Wong, 2016). Moreover, Choi, Lam, Mo, and Wong (2016) used mixtures of papain and bromelain to hydrolyse food wastes employed to make fish feed (final composition of proteases at 1–2%). They observed that the use of feeds supplemented with these mixtures of proteases improved the growth, lipid accumulation and immunity of grass carp (*Ctenopharyngodon idella*).

6.7. Other industrial applications

Due to papain's strong proteolytic activity, it has been employed as a versatile tool in the food industry, but it has many other application fields, such as biomedicine, dental or textile industry, among others. Here we want to report some industrial uses of papain not strictly focused on the food industry, but which reveal the enormous versatility and commercial potential of this enzyme.

Applications of papain in medicine over the last five years include the treatment of proteinaceous esophageal food impaction using papain and other proteolytic enzymes as an initial treatment for all patients with esophageal obstruction (Morse, Wang, Donahue, Garrity, & Allan, 2016); the treatment of mild to moderate acne with a fixed combination of 0.1% hydroxypinacolone retinoate (synthetic ester of 9-cis-retinoic acid), 1% retinol in glycospheres and 2% papain in glycospheres in aqueous gel (Veraldi et al., 2015); and tissue repairing of venous ulcers employing low concentrated papain gels (Ribeiro et al., 2015).

Nowadays, the use of papain is increasingly common in the dental industry, where the proteolytic and antibacterial properties of papain seem to be an efficient and safe alternative for caries removal before restorative procedures are undertaken.

Papain has also been employed as a chemo-mechanical caries removal agent (CMCR), first commercialized in 2003 as Papacarie, which is a mixture of papain, chloramine, toluidine blue and salts in a thickening emulsion (Bussadori et al., 2014). Other similar chemo-mechanical caries removing agents based on papain are Apacaries (Juntavee et al., 2014), papEdent (Subramaniam & Gilhotra, 2011) and CarieCare (Venkataraghavan et al., 2013).

One the other hand, papain has extensively been described as an active ingredient in dentifrices. There are numerous types of toothpaste containing various enzymes in order to remove stains from teeth (Chakravarthy & Acharya, 2012). More recently, Münchow, Hamann, Carvajal, Pinal, and Bottino (2016) also described the stain removal effect of papain- and bromelain-based dentifrices applied to enamel.

Another interesting example is the use of papain in antimicrobial food packaging. Cynthya, Prabhawathi, and Mukesh (2014) immobilized papain on polyurethane films to avoid microbial contamination on cheese. Experimental results showed that this derivative could be used as effective agent control and reduce the growth of *Staphylococcus aureus* biofilm formation. In the same way, Manohar, Prabhawathi, Sivakumar, and Doble (2015) tested the antimicrobial effect of immobilized papain derivatives against *Acinetobacter* spp. and *S. aureus.*

Other interesting uses of papain include water treatment for removal of heavy metals (Metin & Alver, 2016), or preventing yeast flocculation in the bioethanol industry (Silva, Rosa, Carvalho, & Oliva-Neto, 2015).

7. Conclusions

Papain is undoubtedly one of the most frequently studied and widely used proteases in the industry worldwide. Despite the fact that papain can cause an allergic response in humans (Díez-Gómez, Quirce, Aragoneses, & Cuevas, 1998; Mansfield, Ting, Haverly, & Yoo, 1985), papain is generally recognized as safe (GRAS) as a direct human food ingredient according to Code of Federal Regulations of US Food and Drug Administration (U. S. Food and Drug Administration, 2016). Moreover, according to the FAO/WHO food standards, papain is a food additive that may be used in the foods under the conditions of good manufacturing practices (GMP) as outlined in the Preamble of the Codex GSFA (FAO/WHO Codex Alimentarius Commission, 2016).

Although one cysteine protease inhibitor has been predicted to be in the papaya genome (Paull et al., 2008) and such inhibitor is also suspected to be stored in the papaya latex (Azarkan, El Moussaoui, Van Wuytswinkel, Dehon, & Looze, 2003), specific papain inhibitors, which might compromise enzyme activity in papaya latex extracts with relevance to the food industry, have so far not been found.

Papain has been used in the food industry for decades and today continues to dominate the world market for industrial proteases. This is mainly due to its better operational characteristics, such as temperature stability and half-life, in comparison with its main competitors. However, growing market demand is outpacing supply of papain, which has boosted prices in recent years and is holding back the growth of the papain market, in turn favouring the market for other proteases such as bromelain or fungal proteases.

The latest advances in recombinant papain expression systems, especially yeast-based systems, will help overcome these difficulties and provide the market with a cheaper and more sustainable supply of papain. In addition, the new strategies of purification and isolation, genetic modification and enzymatic immobilization will improve the development of new applications of papain, especially in the food industry, making some processes profitable, which is currently not the case with traditional production methods.

Papain remains a very important product in the meat, brewing and dairy industry, where the challenge is to improve the operational properties of papain and to reduce its supply. Regarding the latest trends, most of the efforts are focused on the production of bioactive peptides and functional foods that may have antioxidant, antitumor, or hypoallergenic properties.

In this respect, further efforts are needed in the functional characterization of recombinant of papain, as well as in the generation of new mutant variants of papain with improved properties. In this sense, directed evolution, molecular docking, molecular dynamics and genetic engineering could be very important tools in order to improve activity and stability of the enzyme.

Compliance with ethical standards

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals conducted by any of the authors.

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