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Molecules of Interest

Plant cysteine proteinases: Evaluation of the pharmacological activity

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ABSTRACT

Cysteine proteinases are involved in virtually every aspect of plant physiology and development. They play a role in development, senescence, programmed cell death, storage and mobilization of germinal proteins, and in response to various types of environmental stress. In this review, we focus on a group of plant defensive enzymes occurring in germinal tissue of Caricaceae. These enzymes elicit a protective response in the unripe fruit after physical stress. We propose that these enzymes follow a strategy similar to mammalian serine proteinases involved in blood clotting and wound healing. We show evidence for the pharmacological role of plant cysteine proteinases in mammalian wound healing, immunomodulation, digestive conditions, and neoplastic alterations.

1. Introduction

Plant proteinases are responsible for protein metabolism, a fundamental network of reactions required during the life cycle. Proteinases mainly involve two groups of enzymes: exoproteinases, which act on the ends of protein substrates and designated as amino- or carboxypeptidases, and endoproteinases acting on the interior of protein substrates. Classification of endoproteinases rests on the type of residue at the active site. The hydroxyl group of serine or threonine proteinases and the cysteine group of cysteine proteinases are the nucleophile during catalysis of one group of endoproteinases, while activated water is the nucleophile for aspartic-, glutamic- and metalloproteinases. Rawlings and Barrett (1993) used structural and evolutionary criteria to group proteinases in families and clans. MEROPS database annotates each proteinase according to this classification, and is available at http://merops.sanger.ac.uk/ (Rawlings et al., 2008). As of February 14, 2008 the database lists 91,142 sequences and provides 410 PDB (Protein Data Bank) (http://www.pdb.org/pdb/home/home.do) entries distributed among 196 families and 51 clans.

Cysteine proteinases account for approximately 16% of the total sequences deposited, in agreement with the distribution cited in 2004 for genes of cysteine proteases in Arabidopsis (Schaller, 2004). To date, they are represented by 70 families belonging to 12 different clans, while in 2004 were described by 40 families and 6 clans (Grudkowska and Zagdańska, 2004). Fifty-eight of these families belong to 9 clans, namely; CA, CD, CE, CF, CH, CL, CM, CN and C-, sharing a catalytic mechanism involving the cysteine nucleophile. The other 12 families are grouped into 3 clans (PA, PB and PC) of mixed mechanism, as they contain exemplars of serine and threonine peptidases. Most plant cysteine proteinases belong to the papain (C1) and legumain (C13) families.

The structure of enzymes from C1 family shows the typical papain-like fold described by Drenth et al. (1968), composed of two domains, an α-helix-rich (L) domain and a β-barrel-like (R) domain, separated by a groove containing the active site formed by residues Cys25 and His159 (papain numbering), each one on each domain (Fig. 1).

The physiological functions attributed to plant cysteine proteinases include the build up and breakdown of storage proteins during seed germination, organ senescence and programmed cell death (Huffaker, 1990). Most important perhaps is their involvement in the proteasome proteolytic pathway affecting several metabolic processes, such as hormone signaling, cell cycle, embryogenesis, morphogenesis, flower development, oxidative stress (Watanabe and Lam, 2005). The relevance of cysteine proteinases cannot underscore the key role played by the their natural inhibitors, many of them proteins that by interacting with these enzymes modulate their activity, but this subject will not be discussed in this review (for review see Grudkowska and Zagdańska, 2004). This study briefly focus on the main sources of plant cysteine proteinases trying to establish a link between their cell localization and their putative physiologic function, followed by a discussion of evidence attributing a pharmacological role to some of these enzymes.

2. The distribution of plant cysteine proteinases

Plant cysteine proteinases can be present in virtually every organ, such as PsCYP15A a protease from pea roots (Vincent and

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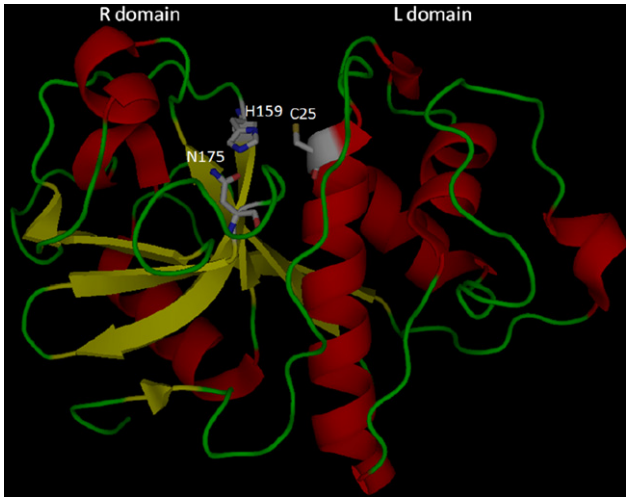

Fig. 1. Three-dimensional model of papain. The structure of papain [PDB code 1ppn (Pickersgill et al., 1992)] is shown in cartoon style. R-L domains and the catalytic-site residues cysteine, histidine and asparagine are indicated.

Brewin, 2000), GP2 and GP3 from ginger rhizomes (Kim et al., 2007), from sweet potato (*Ipomoea batatas*) roots (Huang et al., 2005); FLCP-1 and FLCP-3 from *Phaseolus leaves* (Popovic et al., 1998), bromelain (Rowan et al., 1990) and ananain (Lee et al., 1997) from stems and the most ubiquitous group found in fruits, i.e. balansain I, macrodontain I in Bromeliaceae (Pardo et al., 2000; Lopez et al., 2000), araujiain in Asclepiadaceae (Priolo et al., 2000). This account does not cover every finding available; it merely represent examples described to date, providing support for our assertion. For a full listing, we suggest visiting the MEROPS site mentioned in Section 1.

The ubiquitin (Ub)/26S proteasome proteolytic pathway engages cysteine proteinases in a variety of processes aimed at selectively removing specific intracellular proteins. Through this process the proteasome controls much of cell physiology, growth, photomorphogenesis, trichome development, floral homeosis, environmental adaptation to stress, circadian rhythms, disease resistance, hormonal control and senescence (for reviews, see Pickart, 2001; Smalle and Vierstra, 2004; Schaller, 2004). The senescence control includes the caspase-like enzymes involved in programmed cell death, although gene identification for these enzymes remains unclear (Bonneau et al., 2008). Two groups of proteases have been proposed as candidates to encode caspase-like activities in plants: the vacuolar processing enzymes (VPEs) and the metacaspases (Woltering et al., 2002). They are related by sequence and tertiary structure to animal caspases (Aravind and Koonin, 2002) and have been associated with programmed cell death (PCD) processes in plants (Hoeberichts et al., 2003; Rojo et al., 2003).

Legumain are Asn-specific cysteine proteinases with extra-cytoplasmic localization in vacuoles or cell walls. They act temporarily as precursor-processing or protein-degrading enzymes, depending on the conformational state of their substrates undergoing development- or environment-dependent changes. They are classified into three major groups, those from seeds that are expressed during seed maturation, from vegetative tissues, expressed in vegetative organs and from early embryogenesis expressed in fruit tissue and cotyledons, however, it is not unusual that a particular enzyme manifests itself also in a different group (Hara-Nishimura et al., 1998; Müntz and Shutov, 2002; Gruis et al., 2004). Temporal controlled expression is also an attribute of caspase-like proteinases reflecting the "standing by" condition of this group of enzymes (Bosch and Franklin-Tong, 2007). While animal caspases specifically cleave their targets next to aspartic sites, as yet the site specificity of plant caspase-like enzymes it is not clear.

Calpain, a calcium-dependent cysteine protease, plays an essential role in basic cellular processes including cell proliferation, apoptosis, and differentiation by regulating both, physical interaction and biochemical communication between cells and the extracellular environment (Carragher, 2007). In contrast to other relatively promiscuous degradative proteases, calpains cleave only a restricted set of protein substrates and use complex substrate-recognition mechanisms, involving primary and secondary structural features from target proteins (Croall and Ersfeld, 2007).

Papain was the first cysteine proteinase to be characterized as a latex component in *Carica papaya*, and became a protein model in structural and kinetic studies for cysteine proteinases. The relatively easy of producing milligram amounts of this enzyme stems from its high concentration amounting to about 1% of the total protein in papaya fruit latex before ripening, thus providing another example for the temporal expression of these enzymes. Papain is found in latex along with five isoforms of chymopapains, proteinase omega, and proteinase IV. It is likely that other short lived proteinases also occur in latex, but their identity has not yet been confirmed in latex preparations, obtained by tapping the plant fruit by non-rigorous collection protocols. Early work demonstrated the presence of up to six proteinase activities during latex coagulation in *C. papaya* (Moutim et al., 1999). Papain-like enzymes participate in protein degradation and N-mobilization during seed germination, leaf senescence (Kato and Minamikawa, 1996; Beers et al., 2004; Otegui et al. 2005), PCD (Belenghi et al., 2004) and as deterrents against plant pathogens, explaining their high levels before fruit maturation (Konno et al., 2004). The pesticide effect depends on the proteolytic activity, as E-64 inhibited papain losses protective effect. For a revision of the protective activity found in latex components, see Moussaoui et al. (2001).

Carica candamarcensis (also known as *Vasconcellea cundinamarcensis*), a member of the Caricaceae family common to many countries in Latin America, contains highly active proteolytic enzymes in its latex (Bravo et al., 1994). A latter study demonstrated that a protein fraction from *C. candamarcensis* has therapeutic potential because it stimulates proliferation of fibroblastic cells (Silva et al., 2003).

3. Pharmacological actions of plant proteinases

3.1. Proteinases as antihelminthics and other digestive disorders

Antihelmintic activity is a recognized feature attributed to vegetable cysteine proteinases. Extracts or ingredients of six different plant species were tested against exsheathed infective larvae of *Haemonchus contortus* using a modified methyl-thiazolyl-tetrazolium (MTT) reduction assay and pyrantel tartrate as reference anthelmintic. Bromelain, the enzyme complex of the stem of *Ananas comosus* (Bromeliaceae), the ethanolic extracts of seeds of *Azadirachta indica* (Meliaceae), *Caesalpinia crista* (Caesalpiniaceae), *Vernonia anthelmintica* (Asteraceae), the ethanolic extracts of the whole plant of *Fumaria parviflora* (Papaveraceae) and of the fruit of *Embelia ribes* (Myrsinaceae) showed an anthelmintic efficacy of up to 93%, relative to pyrantel tartrate (Thomson et al., 2001; Hördegen et al., 2003, 2006). In a similar study, the anthelmintic efficacy of cysteine proteinases from papaya, pineapple, fig, kiwi fruit and Egyptian milkweed *in vitro* using the rodent gastrointestinal nematode *Heligmosomoides polygyrus* assay was analyzed. Following a 2 h incubation period, all the cysteine proteinases, with the exception of the kiwi fruit extract, caused marked damage to the cuticle of *H. polygyrus* adult male and female worms, reflected by loss of surface cuticle layer. Efficacy was comparable in both sexes

of worms, was dependent on the presence of cysteine at the active site and was completely inhibited by the cysteine proteinase inhibitor, E-64. LD_{50} values indicated that the purified proteinases were more efficacious than the proteinases in the crude latex, with purified ficin, papain, chymopapain, Egyptian milkweed latex extract and pineapple fruit extract containing fruit bromelain, displaying the most efficacious effect (Stepek et al., 2005).

The anti-inflammatory properties of plant cysteine proteinases were also demonstrated. In a clinical study, the histological severity of inflammatory bowel disease (IBD) was determined in IL-10−/− mice treated orally with bromelain *in vivo*. Daily treatment with oral bromelain decreased the incidence and severity of spontaneous colitis in C57BL/6 IL-10−/− mice. Bromelain also significantly decreased the clinical and histological severity of IBD when administered to piroxicam-exposed IL-10−/− mice with established colitis. Proteolytically active bromelain was required for anti-inflammatory effects *in vivo* without adverse effects (Hale et al., 2005).

Finally, cysteine proteases are recognized as useful options in cases of digestive obstructive conditions (phytobezoar) by vegetable fibers. Papain and cellulase appeared to be effective in the management of phytobezoars in a number of patients studied without manifesting adverse effects (Walker-Renard, 1993; Dwivedi et al., 2001; Baker et al., 2007). Also, countless formulations containing plant cysteine proteases are commercially available in the Web to treat a variety of digestive disorders.

3.2. Proteinases and the immunological system

Serine and cysteine proteinases from both animals and plants have been shown to modulate the immune response in several ways (Trevani et al., 1996). By reversible reacting to circulating α2-macroglobulin and α1-protease inhibitor, plant proteinases interfere with binding of TGF-β and IL1-β. A long list of cell-surface molecules is modulated by plant cysteine proteinases, CD4, CD6, CD7, CD8, CD14, CD16, CD21, CD29, CD38, CD44, CD41, CD42a, CD45RA, CD48, CD49, CD51, CD54, CD57, CD58 CD62L, CD128a, and CD128b interfering with the process of cell communication and probably enhancing CD2-mediated T cell stimulation (Hale and Haynes, 1992; Munzig et al., 1994; Sakalová et al., 2001; Hale et al., 2002). This seems to be a selective process, as other cell-surface molecules remain unchanged or slightly change (Eckert et al., 1999). Particularly, bromelain enhances T-cell dependent immunity *in vivo* and blocks T-cell responses *in vitro* (Mynott et al., 1999; Engwerda et al., 2001), increase IFN-γ-dependent TNFα, IL-1b, and IL-6 production by human PBMC and enhance antigen-independent binding of T cells to monocytes *in vitro* (Hale and Haynes, 1992; Desser et al., 1993). These changes are also observed with papain from *C. papaya* (Desser and Rehberger, 1990). The removal of surface markers from leukocyte and colon epithelial cells is evident soon after (10 min) bromelain oral administration, arguing for a direct role of the protease (Hale, 2004). In granulocytes, bromelain acts by producing oxygen reactive species, but in many of these studies bromelain was given together with mammalian proteolytic enzymes, thus casting doubts on the identity of the enzyme responsible for these effects (Zavadova et al., 1995).

Many of these effects were studied in connection with the inflammatory response. In fact, Seligman showed in 1962 the anti-inflammatory action of bromelain, and since then a number of clinical studies sustain the use of bromelain extracts as anti-inflammatory therapeutics. This activity is mediated by an increase of serum fibrinolytic activity (Ako et al., 1981), decrease in plasma fibrinogen and bradykinin levels resulting in diminished vascular permeability leading to reduction of oedema and pain (Brien et al., 2004); also, by decreasing levels of prostaglandin (Gaspani et al., 2002); inhibiting lymphocyte T cell signal transduction (Mynott et al., 1999); and through modulation of certain immune cell surface adhesion molecules (Barth et al., 2005). Conversely, papain induces human eosinophils to degranulate and to produce superoxide anion. The E-64 inhibitor abolished the activation by papain suggesting that the protease activity is required to trigger eosinophil response. It is likely that this action in eosinophiles is mediated by protein G-linked receptors (Miike and Kita, 2003). As it stands, it appears that bromelain and papain depending on the target cell display opposite effects.

3.3. Proteinases and wound healing

In response to injury, mammalian tissues exposed to trauma respond concurrently and quickly halting blood loss, keeping the area aseptic and by closure of compromised tissue by coagulation and healing. Injury to a blood vessel triggers activation of blood platelets and plasma coagulation system, leading to formation of a blood clot containing platelets and fibrin. During these events a cascade involving the sequential activation of plasma serine proteases takes place, culminating with the generation of thrombin, which converts plasma fibrinogen into a fibrin clot that prevents further bleeding (Macfarlane, 1964).

Intriguingly, a similar phenomenon has been observed in latex-containing plant from Caricaceae. Following fruit injury, bleeding transiently proceeds until a clot forms around the wounded area (Fig. 2). During latex coagulation a number of peptides are being processed in a non-random manner. Furthermore, peptide processing occurs concomitantly with the sequential activation of proteolytic enzymes, as in mammals (Moutim et al., 1999). At this time, during blood coagulation several factors become activated initiating a healing process required for regeneration of necrotic tissue (Singer and Clark, 1999). Interestingly, thrombin, the serine protease involved in blood clot formation, also acts as a mitogen for many cells, by proteolytic cleavage and activation of cognate PAR receptors (Déry et al., 1998).

The similarities between latex coagulation in Caricaceae and the mammalian coagulation process, led us to propose that some analogous factors may be present in both systems. If putative analogies do occur, it is possible that some plant metabolites intervening during plant healing, may also act during the healing process ensuing clot formation of mammals. To gain further insight into this hypothesis, our group examined for substances in latex from *C. candamarcensis* for their ability to stimulate mammalian cell proliferation. This prospect was rewarded when it was demonstrated that a protein fraction from *C. candamarcensis* acted as a mitogen

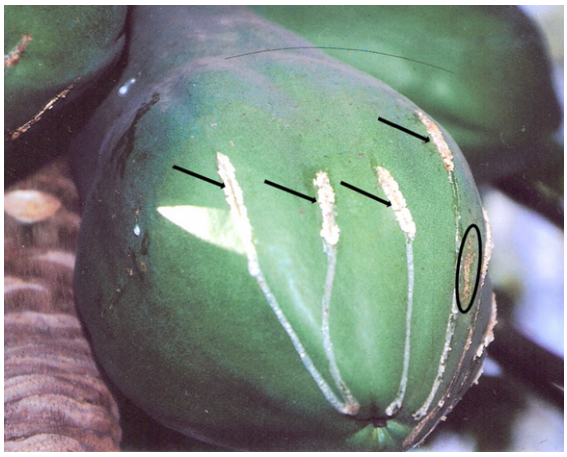

Fig. 2. Latex bleeding and clot formation in Carica papaya unripe fruit. Several longitudinal incisions on the fruit are shown (arrows). Below each incision latex coagulation is under way. The ellipse shows the site of a 15-day incision already healed.

on fibroblast and epithelial cells (Silva et al., 2003) and in a subsequent report it was showed that two proteinases (CMS2MS2 and CMS2MS3) were responsible for this effect (Gomes et al., 2005). In contrast, the proliferative effect of papain attained 15% above control, suggesting that this property is specific for some proteolytic enzymes. The putative novel function for a proteinase from Caricaceae may explain the traditional use of *C. candamarcensis* fruit as a digestive and skin therapeutic (Soplin et al., 1996).

Other evidence confirmed the healing potential of these proteases. A fraction obtained from *C. candamarcensis* latex designated as P1G10 displayed a gastric ulcer protective effect when given to animals prior to indomethacin (Mello et al., 2008). By using ranitidine, the H_2 receptor antagonist and omeprazole, the inhibitor of gastric H+/K pump, as positive controls, we showed that P1G10 displays a protective effect similar to these controls. Also, by using an acetic acid model of gastric lesion, we confirmed the healing effect of P1G10. A prior study showed that papain from *C. papaya* latex was effective in protecting histamine induced ulcer in rats, by blocking the acid secretion (Chen et al., 1981). Based on the similarities between papain and the cysteine proteinases from *C. candamarcensis* (Pereira et al., 2001), it is likely that they share the mode of action.

In addition to the inhibition of acid secretion, the healing property of P1G10 might be related to its proliferative effect in gastric cells since these are epidermal cells, and we demonstrated that these cells are stimulated to proliferate by the protease CMS2MS2. Further evidence confirmed that the proliferative effect of mitogenic proteinases correlates with the activation of MAP-kinases, the pathway that triggers mitosis (Gomes et al., 2005). However, while the healing effect of P1G10 in acetic acid assays following a week-long period of treatment can be explained by its proliferative action, it is uncertain whether in short-term studies involving indomethacin, a similar mechanism could be invoked. In this case, a repair mechanism designated as gastric mucosal restitution involving EGF may explain the short-term protective effect (Yanaka et al., 2002). In support of this notion, the presence of EGF determinants in some cysteine proteinases from *C. candamarcensis* latex and the structural similarity between cysteine proteinases and a hormone growth factor from plerocercoids were described (Phares and Kubic, 1996; Silva et al., 2003). Also, the increase in neovascularization observed following local treatment of P1G10 using a rodent implant model and the enhanced healing in physically induced dorsal dermal abrasions of hairless mice supports this mechanism (Christiano et al., unpublished results).

A different application of plant cysteine proteinases results from their ability to remove necrotic tissue (Ayello and Cuddigan, 2004). Accordingly, several prescriptions containing papain are marketed in USA as debriders (Ford et al., 2002; Melano et al., 2004). However, to exert debrider action the concentration of cysteine proteinases must be 30–100 times higher than the pharmacological dose required for wound healing in our experimental model, although, it is not denied that debriding doses may contribute to some extent with the healing process. However, in experiments to measure the dose dependant proliferative effect of proteinases from *C. candamarcensis*, the proliferative effect was abolished above 50 nM or higher protein concentration.

For efficient slough debriding, proteinases must be unspecific to facilitate digestion of diverse substrates located at the site of the wound. Moreover, in some pharmaceutical formulations, urea is included because it does not inhibit proteolytic activity while enhancing substrate denaturation. In this regard, the cysteine proteinases from Caricaceae including those from *C. candamarcensis* show collagenolytic and fibrinolytic activities favoring their use in this type of application (Hamaguchi et al., 1984; Teixeira et al., unpublished results) while, this is not a specific attribute for Caricaceae, as other plant cysteine proteinases share collagenolytic activity (Kim et al., 2007).

3.4. Plant proteinases as antitumorals

More than 40 years after its introduction, 5-fluorouracil (5-FU) remains the most used chemotherapeutic agent for the treatment of many types of cancer (Malet-Martino and Martino, 2002). Severe side effects like leukopenia, neutropenia, thrombocytopenia, alopecia and diarrhea result from 5-FU treatment (Shibuya et al., 2004), supporting the search for less aggressive drugs. Plants have played an important role as source of effective anticancer agents, and it is significant that 60% of currently used anticancer agents are derived from natural sources, including plants, marine organisms, and microorganisms (Fikrat, 2001; Rajkapoor et al., 2007).

Initially, pharmaceutical preparations containing mixtures of various proteolytic enzymes including papain and bromelain have been used as adjuvant in the treatment of malignant diseases, despite the lack of knowledge of their mode of action. It was demonstrated that oral administration of crude bromelain brought remarkable remissions of malignant tumors with relatively little side effects to cancer patients (Gerard, 1972). More recently the inhibitory effect of crude extracts and bromelain fractions on tumor cells growth and invasion were confirmed (Grabowska et al., 1997). Initial experiments indicate that the effects after oral administration of polyenzyme preparations are related to the induction of cytokine production by human peripheral blood mononuclear cells (Desser et al., 1993). The antimetastatic effect of bromelain on certain tumours, seems to be linked to changes of the expression at receptors, known to be important in glioma cell invasion, namely $\alpha 3\beta 1$ integrin and the hyaluronan receptor CD44 (Batkin et al., 1988; Grabowska et al., 1997). By this action, bromelain reversibly affects cell surface proteins, protein translation, and intracellular signaling pathways, confirming the hypothesis that cleavage of integrins and CD44 prevent their function as receptors, inhibiting the invasive capacity of glioma cells (Tysnes et al., 2001). The reduction of cell proteins can be explained by selective proteolysis of surface proteins, since transcriptional profiling of these genes revealed no obvious alterations. A description of the surface protein known to be affected was presented in Section 3.2, and suggests that bromelain-mediated removal of these proteins is to some extent selective. However, the expression of the EGF receptor was also decreased after bromelain treatment suggesting the involvement of additional antitumoral mechanisms (Tysnes et al., 2001). Immunological studies already mentioned in Section 3.2 propose that bromelain stimulates activation of TNFα, IL-1b and T cells and decrease levels of TGF-β.

On the other hand, the role of proteolytic activity in the antitumoral effect is unclear since Batkin et al. (1988) showed that heat-inactivated bromelain did not lose the antimetastatic capacity on mice infected with Lewis lung tumor C57B1/6. This controversial observation might be settled if purified bromelain preparations were utilized in pharmacological studies. A recent study using purified bromelain preparations shows *in vivo* antitumoral/antileukemic activity in a panel of tumor lines composed by: P-388 leukemia, sarcoma (S-37), Ehrlich ascitic tumor (EAT), Lewis lung carcinoma (LLC) and ADC-755 mammary adenocarcinoma. The major antitumoral activity was observed in mice bearing EAT ascites, the latter effect being superior to 5-FU, the positive control for these experiments. From these data, it was concluded that the antitumoral effect depends on the bromelain molecule, but, it is uncertain if the proteolytic function must be intact to exert the antitumoral effect (Báez et al., 2007). This question has been raised by other authors (Batkin et al. 1988; Tysnes et al., 2001) and more recently our group found that the mitogenic effect of CMS2MS2 on fibroblastic cells does not depend on the proteolytic activity, as the

E-64 inhibited enzyme preserves proliferative activity (Gomes et al., unpublished data).

Fastuosain the cysteine proteinase from *Bromelia fastuosa* (25 kDa) that shares 79.4% identity with bromelain is another example of a plant proteinase with antitumoral effect (Cabral et al., 2006). The protective effect is attained by intraperitoneal administration starting concomitantly with the intravenous challenge with melanoma B16F10-Nex2 cells. Tumor lung colonization occurred after 21 day in controls, unless treatment with fastuosain or bromelain was adopted. In addition, no metastatic nodules were detected in the myocardium, brain, kidney, liver, and spleen of infected animals treated with either protease. The suggested mechanism invokes the proteolytic depletion of CD44, based on the loss of tumor cell adherence by CD44 (Guimarães-Ferreira et al., 2007). Alternatively, treatment with protease stimulates production of host antiproteinases that may react with endogenous cysteine proteinases, like cathepsin B, which are associated with tumor progression and metastasis (Frohlich et al., 2001). In support of this notion, the resulting antifastuosain antibody reacted to membrane cathepsin B reducing by half the growth of B16F10-Nex2 cells. Furthermore, this cytotoxic effect is increased by addition of complement (Guimarães-Ferreira et al., 2007), supporting the notion that these enzymes act through multiple mechanisms.

Similar findings support the antitumoral action of papain from *C. papaya*. The growth rate, invasiveness and metastasis of both the B16 melanoma and Lewis lung carcinoma were inhibited in mice immunized with papain, increasing the mean survival of treated mice relative to the tumor-bearing untreated controls. The protection is mediated by serum antibodies cross-reacting with cathepsin-B- and cathepsin-H-like endopeptidases isolated from B16 melanoma cells (Bellelli et al., 1990), as seen for fastuosain. Further studies showed that long-term rectal administration of an enzyme mixture containing papain, trypsin and chymotrypsin displayed antitumoral effect in C57Bl6 inbred mice inoculated with Bl6 melanoma cells (Wald et al., 1998). In addition, the growth inhibition of primary tumors and metastasis was correlated with decreased expression of CD44 and CD54 molecules in animals exposed to proteolytic enzymes, suggesting that serine and/or cysteine proteinases suppress B16 melanoma, and restrict metastatic dissemination in C57B16 mice (Wald et al., 2001). Our group has shown that in melanoma murine models P1G10 from *C. candamarcensis* reduces the tumor size and the frequency and number of lung metastases, the latter by about 75%, with a significant increase in survival (Figueiredo et al., unpublished data).

Taken together these findings clearly show the beneficial effects of plant cysteine proteases as principles endowed with antitumoral and antimetastatic effects.

3.5. Other applications

This review focuses on some pharmacological effects attributed to cysteine proteinases, ignoring other therapeutical options. For instance, the efficacy and safety of cysteine proteinases was established in enzymatic debridements of burns (Rosenberg et al., 2004) and we observed a 25–30% increase in the epithelization rate following periodical applications of P1G10 proteases in rodent subjected to dorsal burns induced by dry-heat (Latini et al., unpublished data). In these experiments the pharmacologically effective dose was about 0.1%, which is equivalent to the dose used for dermal healing described in Section 3.3. A different approach has been used when using cysteine proteinases as debrider as an alternative to mechanical cleansing for rapid removal of dead tissue. In these protocols the pharmacological concentration of proteases ranges between 2% and 5% (Rosenberg et al., 2004). A number of reports attribute cartilage remodeling and anthirheumatic properties to cysteine proteinases, the latter effect probably related to the immunomodulatory actions attributed to these enzymes.

Table 1
Therapeutic properties assigned to plant cysteine proteases

Protease	Clan/family	Organism	Therapeutic properties
Papain	CA/C1	*Carica papaya*	Antihelmintic (Stepek et al., 2005) gastric phytobezoar (Dwivedi et al., 2004) gastric antiulcerogenic (Chen et al., 1981) debridement (Ayello and Cuddigan, 2004) antitumoral (Bellelli et al., 1990)
Bromelain	CA/C1	*Ananas comosus*	Antihelmintic (Hördegen et al., 2006) anti-inflammatory (Seligman, 1962) burn debridement (Rosenberg et al.,2004) antitumoral (Báez et al., 2007)
Ficin	CA/C1	*Ficus carica*	Antihelmintic (Stepek et al., 2005)
Fastousain	NC	*Bromelia fastuosa*	Antitumoral (Cabral et al., 2006)
Chymopapain	CA/C1	*Carica papaya*	Antihelmintic (Stepek et al., 2005)
P1G10	NC	*Carica candamarcensis*	Wound healing (Mello et al., 2005) gastric antiulcerogenic (Mello et al., 2008)

NC: Not classified.

A summary of the various therapeutic applications described in this review is presented in Table 1. Bromelain and papain, the two most cited proteinases are cultivars indigenous from America widely distributed throughout the world since the Spaniard colonization. Moreover, while some of these applications are backed by one or few citations, others such as the antitumoral actions are ubiquitously cited, most likely due to the relevance of this field. For space considerations we did not cite most of these studies but in no way this diminishes the importance of these findings.

4. Concluding remarks

The findings presented in this review establish a clear association between plant cysteine proteinases and therapeutic treatment of digestive disorders, dermal and gastric ulcers of different origins, immunological modulation, and tumoral/metastatic disorders. The therapeutic effect observed in these various conditions, does not mean a myriad of cellular targets affected by these enzymes, as probably the immunological and antitumoral actions respond to a similar mechanism. The finding of a proliferative property associated with selected proteinases from *C. candamarcensis* discloses a novel property, although about 30 years ago, a report described that *in vitro* DNA synthesis was stimulated by bomelain but not by papain (Zetter et al., 1976). In accordance, *in vitro* assays with papain failed to reveal a significant increase of DNA synthesis (15%) measured by thymidine incorporation into DNA, while CMS2MS2 showed a robust 60–80% increase compared to a control without protease, thus reinforcing the notion that cysteine proteinases exhibit differential effects depending on the target and the enzyme source.

Yet, the possible association between cysteine proteinases and proliferative effects is not new, since a previous report showed that a cysteine proteinase of plerocercoids shares a growth factor property within the same molecule (Phares and Kubic, 1996) and extension sequences at the C-terminus of some members of the papain family contain domains of homology to animal proteins of the epithelin and granulin families. Epithelins and granulins are 6 kDa proteins that modulate growth of animal cells, while the function of plant granulins-like proteins remains unknown (Yamada et al., 2001).

In recent years, the signaling role by mammalian proteinases has become evident, however, this function has been mostly attributed to serine proteinases. Of the many isoforms of the mamma-

lian cysteine proteinase cathepsin, it is proposed that isoforms B and L might be involved in ulcerogenic and carcinogenic processes. On the other hand, the cysteine proteinases from *C. candamarcensis* described in this review, act as healers of chemically induced gastric ulcers and as antitumorals. Both, cathepsins and cysteine proteinases from Caricaceae belong to the same family of proteases (papain superfamily), which facts raise the question, why two group of enzymes displaying strong homology, act putatively in opposed manner. We propose that minor structural differences between these enzymes may explain the apparent contradiction. Future research should address the structural differences between mammalian and plant cysteine proteinases.

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