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Research review paper

Production of plant proteases *in vivo* and *in vitro* — A review

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ABSTRACT

In the latest two decades, the interest received by plant proteases has increased significantly. Plant enzymes such as proteases are widely used in medicine and the food industry. Some proteases, like papain, bromelain and ficin are used in various processes such as brewing, meat softening, milk-clotting, cancer treatment, digestion and viral disorders. These enzymes can be obtained from their natural source or through *in vitro* cultures, in order to ensure a continuous source of plant enzymes. The focus of this review will be the production of plant proteases both *in vivo* and *in vitro*, with particular emphasis on the different types of commercially important plant proteases that have been isolated and characterized from naturally grown plants. *In vitro* approaches for the production of these proteases is also explored, focusing on the techniques that do not involve genetic transformation of the plants and the attempts that have been made in order to enhance the yield of the desired proteases.

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1. Introduction

In recent decades, interest in plant natural products has grown rapidly. The number of industrially employed enzymes of plant origin is still small but growing fast. In this respect, proteases are the most commercially important enzymes due to their multiple applications in the food, pharmaceutical and detergent industries, as well as in the preparation of leather and wool, among others (Doran, 2002). Proteolytic enzymes produced commercially are used in processes such as brewing, tenderization of meat and dairy processing. The

Abbreviations: AP, aspartic protease; ATP, adenosine triphosphate; BAP, 6-benzylaminopurine; ClpP, proteolytic subunit of Clp,caseinolytic protease; CP, cysteine protease; FtsH, temperature sensitive H protease; IBA, indole-3-butyric acid; LAP, leucine aminopeptidase; MP, metalloprotease; MS, Murashige and Skoog; NAA, naphthaleneacetic acid; SP, serine protease.

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most widely utilized plant proteases are papain, bromelain and ficin, extracted from *Carica papaya*, *Ananas comosus* and *Ficus carica*, respectively. The present review summarizes the achievements in production of commercially relevant plant proteases both *in vivo* and *in vitro*. The focus will be on *in vivo* production of proteases from the four major endoprotease classes and on the tissue culture techniques that do not involve genetic modification used to improve *in vitro* production of these compounds.

1.1. Proteases

Proteases are enzymes able to hydrolyze peptide bonds. They can act near the ends of polypeptide chains (exopeptidases) or within them (endopeptidases) (Palma et al., 2002). The term "protease" will be used in this paper to refer to "proteases", "peptidases" and "proteolytic enzymes" for clarity. Exoproteases have been differentiated according to their substrate specificity as aminopeptidases, which are able to cleave peptides at the N-terminus, and carboxypeptidases, which degrade peptides at the C-terminus. Endoproteases are classified according to their catalytic mechanism, which implies specificity in the enzyme active site. The MEROPS Database (Rawlings et al., 2010) considers seven families of proteases: Aspartic, Cysteine, Glutamic, Metallo, Asparagine, Serine and Threonine. In plants, five classes of endoproteases have been described: Serine, Cysteine, Aspartic, Metallo and Threonine (Rawlings et al., 2010).

Proteases constitute the most important group of industrial enzymes currently in use, with an important role in the food and detergent industries, and more recently in leather processing and as therapeutic agents (Walsh, 2002). Measuring hydrolytic activity on synthetic substrates is a simple way to know the cleavage specificity of these enzymes, which provides important information for biotechnological applications, as with the production of bioactive peptides from food proteins (Silva and Malcata, 2005a). Some peptides are hidden and inactive in the original proteins, but when liberated they can have diverse biomedical applications, as antihypertensive or antioxidant agents, among others (Perpetuo et al., 2003).

Typically, proteases contain an autoinhibitory prodomain that must be removed to activate the enzyme (Bryan, 2002). The activity of many proteases also depends on pH, indicative of the compartment where they are localized and on the presence of endogenous protease inhibitors or activators (van der Hoorn et al., 2004). Among the functions assigned to proteolysis are: the removal of abnormal/misfolded, modified, and mistargeted proteins; the supply of amino acids needed to make new proteins; contribution to the maturation of zymogens and peptide hormones by limited cleavages; the control of metabolism and homeostasis by reducing the abundance of key enzymes and regulatory proteins; and the cleavage of targeting signals from proteins prior to their final integration into organelles (Palma et al., 2002).

Proteases have a first place in the world market of enzymes, estimated at ~US$3 billion (Leary et al., 2009), since they play an important role in biotechnology, given that proteolysis changes the chemical, physical, biological, and immunological properties of proteins. Hydrolysis of food proteins, for example, is carried out for various reasons: improvement of the nutritional characteristics, retarding deterioration, modification of different functional properties (solubility, foaming, coagulation, and emulsifying capacities), prevention of undesired interactions, change of flavors and odors, and removal of toxic or inhibitory factors, among others (Pardo et al., 2000). One of the most important applications of proteases in the food industry is the use of rennet in cheese making. Milk-clotting enzymes have been found in almost all kinds of plant tissues and it appears to be a general rule that all proteolytic enzymes possess the ability to clot milk under proper conditions (Tamer and Mavituna, 1997).

1.2. Plant proteases

Plant proteases are involved in many aspects of plant physiology and development (reviewed in van der Hoorn, 2008). They play a pivotal role in processes such as protein turnover, degradation of misfolded proteins, senescence and the ubiquitin/proteasome pathway (Beers et al., 2000). Proteases are also responsible for the post-translational modification of proteins by limited proteolysis at highly specific sites (Schaller, 2004). They are involved in a great diversity of cellular processes, including photoinhibition in the chloroplast, defense mechanisms, programmed cell death, and photomorphogenesis in the developing seedling (Estelle, 2001). Proteases are thus involved in all aspects of the plant life cycle ranging from movilization of storage proteins during seed germination to the initiation of cell death and senescence programs (Schaller, 2004).

Proteases have been identified and studied from the latex of several plant families such as *Asteraceae*, *Caricaceae, Moraceae, Asclepiadaceae, Apocynaceae* and *Euphorbiaceae* (Domsalla and Melzig, 2008). Most plant-derived proteases have been classified as cysteine proteases and more rarely as aspartic proteases (Rawlings et al., 2010). Proteolytic enzymes derived from plants are very attractive since they can be active over a wide range of temperature and pH (Uhlig, 1998). A number of industrial processes involve the breakdown of proteins by proteases, some of which are extracted from plants.

Enzyme preparations from plant extracts have been used in industrial processes for a long time, even before much was known about the nature and properties of the enzymes. The great majority of commercial enzymes have been obtained mainly from microbial sources but plant enzymes are becoming increasingly important, with applications in industrial processes, biotechnology and pharmacology. Proteases like papain, bromelain and ficin are employed in different industrial processes and medicine (Uhlig, 1998). Some of these papain-like proteases are currently used in the food industry for cheese, brewing and beverage industries for the preparation of highly soluble and flavored protein hydrolysates (papain-like proteases), as a food complement (Kleef et al., 1996; La Valle et al., 2000; Losada, 1999) to soften meats and dehydrated eggs (Bailey and Light, 1989; Lawrie, 1985; Miller, 1982) and for the production of emulsifiers, among other uses (Pardo et al., 2000). Uses in other industries include culture medium formulation (Headon and Walsh, 1994), isolation of genetic material (Genelhu et al., 1998) and the use of keratinases in the leather industry for dehairing and bating of hides to substitute toxic chemicals (Foroughi et al., 2006). Also, they are used in the production of essential amino acids such as lysine and for the prevention of clogging of wastewater systems (reviewed in Rao et al., 1998).

Proteases also have an important application in the pharmaceutical industry. Plant extracts with a high content of proteolytic enzymes have been used in traditional medicine for a long time. They have been used for the treatment of cancer (Batkin et al., 1988; Targoni et al., 1999), as antitumorals (Guimaraes-Ferreira et al., 2007; Otsuki et al., 2010), for digestion disorders (Kelly, 1996; Mello et al., 2008), and swelling and immune-modulation problems (Leipner et al., 2001; Lotti, 1993; Melis, 1990; Otsuki et al., 2010). A good example is bromelain, derived from pineapple, which has been shown to be capable of preventing edema, platelet aggregation and metastasis due to it's capacity of modifying cell surface structures by peptide cleavage. Salas et al. (2008) reviewed the pharmacological activity of plant cysteine proteases, emphasizing their role in mammalian wound healing, immunomodulation, digestive conditions, and neoplastic alterations.

Many of the plant proteases used in the above mentioned industries come from plants that are cultivated in tropical or subtropical, geographically remote, areas which are subject to political instability, drought, disease and changing land use patterns, among other environmental factors. In addition, the long cultivation periods between planting and harvesting make selection of high-yielding strains