Agriways 7(1): 50-57. June 2019

ISSN: 2321-8614 (Print)
ISSN: 2454-2318 (Online)

Review Article

PLANT PROTEASES: SOURCES, PRODUCTION METHODS AND VARIOUS COMMERICIAL APPLICATIONS

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Received: 06 April 2019/Accepted: 22 June 2019

Abstract Proteolytic enzymes catalyze hydrolysis of peptide bonds in proteins and one of the most widely used industrial enzymes. Proteases are found in a wide diversity of sources such as plants, animals and microorganisms but plant sources are preferred for the production of proteases due to various economic advantages. This review displays an overview on proteases chiefly from sources of plants but microbial and animal proteases are also discussed briefly. Plant proteases have potential for applications in different industries including dairy, bakery, beverages and pharmaceutical. They act as an important industrial enzyme occupying about 60% of total enzyme market. Plant proteases assume an imperative part in various ventures, above all in the food preparing and medical purposes.
Key words: Proteases, proteolytic enzymes, zymogen, commercial importance, pharmaceutical

1. Introdution

Protease is also known as peptidase. Proteases are a class of enzymes that results in hydrolysis of peptide bonds. Polypeptides chains are formed with the help of peptide bonds by linking amino acids to each other (Kinda *et al.,* 1995). Proteolysis process in case of plants is not restricted to produce amino acids, carbon, and nitrogen for the creation of new molecules (Pogany *et al.,* 2015). These enzymes also serve as essential mediators of various biological activities as they cleave specific peptide bonds in key proteins and enzymes, involving unalterable reactions, and thus are involved in the regulation of growth and development (Kurepa *et al.,* 2009). These enzymes occur in wide range of plants, animals and microorganisms. They play a great role in physiological processes, for example zymogen activation by proteolysis, tumor growth and transport of secretory protein across membranes, cell growth and tissue arrangement (Souza *et al.,* 2015). In worldwide market about 60% are proteolytic enzymes (Monti *et al.,* 2000). These enzymes show different properties such as substrate specificity, catalytic mechanisms, active site, pH and optimal temperature. Due to wide range of pH and temperature, enzymes from plant sources have great industrial applications including food and pharmaceutical industries as well as eco-friendly and non-toxic. Plant-based proteases with high amount of proteases have been conventionally used for a long time for the treatment of antitumorals (Ostuki *et al.,* 2010), cancer, digestion disorders and immune-modulation problems (Mello *et al.,* 2008).

2. Sources of protease enzyme

Protease enzyme plays various physiological roles in all living organisms and therefore, they are present in a wide range of sources such as plants, animals and microorganisms (Kurepa *et al.,* 2009).

2.1 Animal protease: These enzymes are derived from animals such as pancreatic trypsin, chymotrypsin, pepsin and rennin. Trypsin enzyme is derived from intestine of animals therefore it acts as intestinal digestive enzyme. It shows various applications like biocontrol of insect and pests and few medical appications. Pancreatic chymotrypsin has analytical and diagnostic applications. Pepsin enzyme is extracted from intestine of vertebrates and acidic in nature so it is widely used in laundry industy for conversion of rennet into active rennin. Rennin enzymes are used in dairy industry industry for prepration of good flavoured curd (Verma, 2014).

2.2 Microbial protease: Most of the microorganisms (bacteria, fungi, actinomycetes) are used in the production of protease at large scale. The production of new recombinant enzymes increases with the use of microbes because they show fast growth rate (Bruinenberg *et al.,* 1994). Microbes secrete mainly two types of enzymes such as extracellular and intracellular proteolytic enzymes.

Extracellular proteases show high commercial value and multiple applications in detergent, food, dairy, pharmaceutical, leather, diagnostic, waste management and silver recovery. In world-wide market protease are used as 60% of industrial enzyme. They find commercial application for toothpastes as antiplaque and antitartar, cosmetics and for the recovery of silver from used X-ray films (Ishikawa *et al.,* 1993). Intracellular enzymes are mostly applicable in various metabolic and cellular processes such as sporulation, cell differentiation, enzyme maturation, hormones and help in activation of Bt-based biopesticides (Hasnain, 1992).Various bacterial and fungal species are the major source of acidic, neutral and alkaline activity. Proteases from fungal source have lower reaction rate and heat stability than wide range of bacterial proteases such as *Bacillus clausii, B. cereus, B. licheniformis, B. sphaericus, B. subtilis, B. sterothermophilus, B. mojavensis, B. megaterium, B. brevis, B. anthracis, B. thuringiensis B. circulans B. coagulans, B. marmarensis, B. firmus, B. stratosphericus, B. polymyxa, B. Lentus, B. alcalophilus, B. amyloliquifaciens, B. subtilis, B.intermedius, B. thermoruber, Bacillus pumilus B. cohnii, B. fastidiosus, B. pseudofirmus, B. pantotheneticus, B. aquimaris, B. proteolyticus, B. laterosporus, B. coagulans, B. amovivorus, B. flexus, B. horikoshii, Pseudomonas aeruginosa, P. fluorescens, P. putida, Aromonas hydrophila, Serratia liquefaciens, Flavobacterium balustinum, Exiguobacterium* sp. which show broad substrate specificity (Jisha *et al.,* 2013; Aruna *et al.,* 2014; Souza *et al.,* 2015). The enzyme which shows hydrolytic properties belong to *Aspergillus, Humicola, Mucor, Penicillum, Rhizopus, Thermomyces etc* (Wu *et al.,* 2006). Bacterial alkaline proteolytic enzymes show pH range 8-12 and optimal temperature of 50 -70^0 C. Due to these properties they are used in detergent, food, laundry, silk and leather industry as well as fungal proteases show high stability and range with broader substrate specificity at extreme conditions. In industry fungal protease are produce in solid state fermentation process (Maurer, 2004).

2.3 Plant protease: Proteases from various plant sources are extensively used in drug and food industry. Most explored enzymes are bromelain, ficin and papain which are extracted from different fruits like *Ananas comosus, Ficus caric and Carica papaya.* These fruits contains large amount of protease which are highly used in brewing, meat softening, digestion, milk- clotting, cancer treatment, tissue damage and viral treatment. In unripe papaya fruit contains high quality of papain proteases than riped papaya (Gonzalez *et al.,* 2011).

Medicinal plants act as source of protease and give high yield of protease. *Azadirachta indica* also known as neem and belongs to family *Meliaceae*. This plant has various medicinal uses such as antipyretic, blood purifier of detoxifiers as well as used for manufacturing of various health and cosmetic products including bath powders, soaps, shampoos, cream and lotions because neem shows antiviral, antineoplastic, antibiotic, anti-inflammatory, antiseptic, insecticidal, anti-hyperglycemic activity etc. (Gupta A and Chaphalkar, 2015a ; Gupta A and Chaphalkar, 2015b).

Terminalia arjuna commonly known as Arjun tree and belongs to family *Combretaceae.* The bark of this tree is very useful because it contains high amount of Coenzyme Q 10. This enzyme is helpful to reduce blood pressure and heart tonic is prepared from its bark when processed with milk. Traditionally, Arjuna tea is very useful to relieve pain in kidneys as well as to break kidney stones. In medical field Arjuna is widely used to cure various health problems like kidney, liver and gall bladder (Gupta *et al.,* 2014; Gupta and Chaphalkar, 2015).

Calotropis gigantea commonly known as Akk and belongs to family *Apocynaceae.* Entire plant is very beneficial and showed medicinal properties. The dry form of plant used as a good tonic and its flowers are bitter in taste but act as best digestive, stomachic, antihelmintic and tonic. Usually this plant is used to cure common health problems like cold, asthma, cough, fever, nausea, vomiting etc. (Gupta and Chaphalkar, 2016 a; Gupta and Chaphalkar, 2016b).

Mimusops elengi commonly known as Spanish cherry and belongs to family *Sapotaceae.* This plant is commonly found in parks. Different parts of plants i.e bark, flower, fruits and seeds are widely used as Ayurvedic medicine. Mostly it is used for cure dental problems such as loose teeth and bleeding gums (Gupta *et al.,* 2016).

Jasminum grandiflorum commonly known as jasmine plant and belongs to family *Oleaceae.* The flowers of this plant are used for extraction of protease. The stamens showed higher protease activity at pH 4.0 and it is widely used for wound healing, skin diseases, ulcers, pruitus (Ramundo and Gray 2008; Sandeep and Paarakh 2009). The root of the plant is helpful to cure paralysis, mental disability, chronic constipation, flatulence, sterility, ring worm, leprosy and skin diseases (Sulaiman *et al.,* 2012). The leaves of the plant are used in fixing loose teeth, leprosy, skin diseases, dysmenorrhoea, ulcers and wounds (Umamaheswari *et al.,* 2007).

Canavalia ensiformis commonly known as jack bean is a leguminous plant. It is cultivated in whole world but commonly found in Central America. In agriculture, it is used as a green cover for the nutritional enrichment of soils, because it is resistant to insects and microbes and control the harmful effects of nematodes and fixes nitrogen efficiently (Defferrari *et al.,* 2011 and Kokalis *et al.,* 2013). The jack bean seeds contain high amount of proteases (Hoorn, 2008 and Janska *et al.,* 2010). The

legume plants are helpful to regulate both protein and general metabolisms to express high levels of proteases and their specific inhibitors (Lopez, 2009 and Gome *et al.,* 2011). The protease from plant sources has the advantages of high activity, ready availability, and low cost of production (Gonzalez *et al.,* 2011).

3. Properties of protease: The properties of these enzymes are based on the catalytic mechanism, specificity and its catalytic site. Proteases are divided into two groups on the based on the site of action on polypeptide chains i.e. exopeptidases and endopeptidase. Exopeptidases are involved in hydrolysis beacuse they act on terminal end of polypeptide chains. Endopeptidases initiate degradradation by breaking inner region of the polypeptide chains away from its terminal end. On the basis of catalytic site they are sub-classified as serine, aspartic, cysteine and metalloproteases (Bah *et al.,* 2006).

3.1 Serine protease: Serine protease contains a serine residue in its active site and involved in various biochemical and physiological processes. These proteases are mostly found in trees, crops to legumes and herbs but most abundant in fruits (Rawling and Barret, 2004). Serine proteases are extracted from various parts of plants such as latex, seeds, flowers, leaves and roots. *Euphorbia neriifolia* is an Indian spurge tree. Neriifolin, a chymotrypsin – a serine protease is extracted from the latex of this tree and these enzymes act as milk clotting enzymes (Yadav *et al.,* 2011 and Yadav *et al.,* 2012). Religiosin B and C –serine protease isolated from the latex of *ficus religiosa* is peepal tree (Kumari *et al.,* 2012; Sharma *et al.,* 2012). A new serine protease i.e prunifoline is extracted from the latex of *Euphorbia prunufolia* –medicinal plant. It is isolated by acetone precipitation method and purified by using DEAE-C (Diethylaminoethyl-cellulose) column chromatography. This enzyme is widely used in food and biotechnological industries as a strong candidate (Khan *et al.,* 2011).

3.2 Cysteine protease: In the catalytic mechanism of cysteine proteases (also known as thiol proteases), these involve a cysteine group in their active site. These enzymes are having property of being active over different ranges of temperature and pH and have great potential in food, biotechnology and pharmaceutical industries. In different plant tissues cysteine proteases are present in excessive amount and offer an attractive alternate (Gonzalez *et al.,* 2011). Various *ficus* species possess specific properties; ficin is isolated from the latex of *ficus racemosa* showed an activity to digest casein (Devaraj *et al.,* 2008). Ripe fresh fruit of kiwifruits contain Actinidin. Ginger rhizomes contain ginger proteases having the molecular mass of 36kDa (Hashim *et al.,* 2011). Papain is an important cysteine proteases, its main role is to control key processes at various levels of plant defense. Papain- like cysteine protease (PLCPs) is major enzymes in plant apoplast and papain is a member of protease family (Richau *et al.,* 2012).

3.3 Aspartic protease: These are acidic proteases and optimally active at pH 3 to 4 and cleave the peptide bonds between non-polar amino acid residues (Domingos *et al.,* 1992). Aspartic proteases such as cardosins and cyprosins are commonly found in mature cardoon flowers but not found in leaves or seeds (Cordeiro *et al.,* 1998). Cardosin A is extracted in large quantity from pistils of *Cynara cardunculus* and its dry flowers contain three types of cryposins which have milk-clotting activity (Heimgartner *et al.,* 1990).

3.4 Metallo proteases: In the catalytic activity of metallo proteases, these require divalent metal ions, such as zinc, cobalt or manganese. Due to sequestering effect of chealting agents on the metal ions, the metallo proteases are sensitive which are involved in catalytic mechanisms. These enzymes have wide range of applications in drug development by having substrate specificity (Kumar *et al.,* 2008).

4. Methods of proteases: Proteases can be obtained naturally from various plant parts such as root, seed, flower and its latex or through in-vitro propagation techniques.

4.1 In vitro propagation technique: Proteases which are used in diary industry have been produced by plant tissue culture. Proteases which are extracted from the culture of *Mirabilis jalapa* which are produced by in vitro contains higher yield as compared to natural plant (Tamer and Mavituna, 1997). Aspartic protease were produced at pH 8.1 from the homogenized cell suspension culture of *Centaurea calcitrapa* and the proteases were extracted by centrifugation of homogenate and then lyophilized the supernatant for the production of enzyme (Reis *et al.,* 2000). In optimized conditions; the aspartate protease was produced from the callus culture of *Silybum marianum* (Cimino *et al.,* 2006). To characterize aspartate proteolytic enzymes were produced from the callus culture of *Cynara cardunculus* (Oliveira *et al.,* 2010).

4.2 Cardunculus plant as natural source: The proteases have been mostly extracted from their natural plant parts such as flowers, seeds, leaves and roots. The crude extract of enzyme which acts as coagulant also produced from the dried or crushed flowers of cardoon and these flowers were soaked in water at room temperature for a changeable time period (Roseiro *et al.,* 2003). Another method of enzyme extraction is: firstly, dried *cardoon* flowers were grinding with crude kitchen salt and strainer is formed when laying the paste on a cotton cloth and enzymes are solubilized by percolation with warm milk (Souca and Malcata, 2002). The stigmae and style part of dried *Cynara cardunculus*

contains cardosin A and cardosin B proteases (Silva *et al.,* 2003).

4.3 Dubiumin is a serine protease. It is mainly extracted from the *Solanum dubium* seeds at different pH and salt concentrations. It shows actitivity at pH value 9.3 and optimum pH is 11.0 and retaining thermostability at 60^0 C after 1 hour and acts optimally at 70^0 C for 30 minutes. Moreover, it shows high stability with use of different denaturants and organic solvents (Ahmed *et al.,* 2009). Hieronymain protease is extracted from the unripe fruits of *Bromelia hieronymi* and this enzyme have ability to hyrolyze milk whey proteins and bovine casein so it is widely used in diary industry for the production of cheese and milk protein hydrolysates (Bruno *et al.,* 2010). Onopordosin is a partially purified protease. It is extracted from the *Onopordum acanthium* flowers and these enzymes act as best plant coagulant agent at pH 2.5 to 4.4, used in cheese making as well as they have tendency to act alone or in combination with a starter in the process of proteolysis (Brutti *et al.,* 2012). The coagulation activity was found in the extract of artichoke and the enzyme to be used in the cheese making with increasing concentrations of calcium, milk-clotting activity increased hyperbolically. The concentration was saturated at 50mM. These proteases were extracted from *Cynara scolymus* and purified. Cynarases are glycoproteins with milk clotting activity. In case of cynarases A and C purification decrease the specific coagulant activity, whereas cynarase B increasess its specific clotting activity. There is possible use of this enzyme in diary industry for cheese making due to their result indication (Chazzara *et al.,* 2007).

4.4 Plant extract from various sources:

4.4.1 Coriander plant: Crude extract of protease is prepared from various fresh fruits and vegetables such as star fruit, sweet potato, brinjal, mango, cucumber, lime and curry leaf. For the extraction of protease 500 g sample is used by cut the leaves and submerged in phosphate buffer at 50mM and pH 7 in the ratio of 1:2 (w/v). After that centrifuge the samples at 10,000 X for 15 minutes at 4^0 C (Baskarn *et al.,* 2014).

4.4.2 Euphorbia prunifolia plant: The protease is mainly found in the latex of *Euphorbia* plant. For prepration of extract sample was collected early in the morning for allowing the milky latex (El-Bendary *et al.,* 2007). In four folds of muslin cloth the homogenized latex was purified and filtered. Filterate then centrifuged at 15,000 rpm at 4^0 C for 45 minutes. Supernatant of latex enzymes is known as 'crude enzyme' or 'centrifugal fraction' and used for protease enzyme assay (Badgujar and Mahajan, 2010).

4.4.3 Plant leaves: The protease was isolated and their specific activity with optimum pH was studied from the dried leaves of *Nicotiana tobaccum*, green leaves of *Murraya koenigii, Moringa oleifera* and *Coriandrum sativum.* The proteases were also studied and compared. The samples were prepared by homogenization of leaves in pestle and mortar with phosphate buffer (pH 7.0). The supernatant was collected through centrifugation at 10,000 rpm for 10 minutes and subjected for further studies (Sharmila *et al.,* 2012).

Jasmine flower: Different parts *of Jasminum grandiflorum* plant such as corolla, stamens and gynoecium along with calayx and lobes were collected and stored at -20^0 C after weighing it. The two organs such as calayx and the gynoecia usually do not contribute to property of floral extract but they both together are useful for the determination of total protein content and protease activity at different pH range with buffers such as citrate phosphate buffer 50mM was used for the pH range 4, 5 and 6 and sodium phosphate buffer 50mM was used for the pH range 7, 8. Extractions of samples were carried out at different pH range with 0.01% (w/v) ascorbic acid. Extracts were filtered, followed by centrifugation at 2000g for 15 minutes. Supernatant was collected and used for protein and enzyme assay as a source (Vidyalakshmi and Selvi, 2013).

5. Applications:

5.1 Protease use in industry: In food industry protease are widely used as basic enzyme. For example preservation of various fruit juices for long term storage and the catalyzed sequence of these enzymes in helpful in the production of milk products such as candies (Srilakshmi *et al.,* 2014). These are highly efficient for the modification of food proteins by improving flavour, nutritional value, solubility and reducing allergenic compounds as well as these are showing functional properties like coagulation, emulsification, gel strength, fat binding and foaming etc (Tavano, 2013).

In beverage industry: Protease are act as substrate and used in the production of hard and soft drinks by carrying chain of biochemical reactions from low temperature range to harsh temperature conditions (Srilakshmi *et al.,* 2014).

In diary industry: These are highly applicable in diary industry for manufacturing of cheese because they have ability to hydrolyze peptide bonds in food proteins for the production of casein and macropeptides as well as helpful in tenderization of meat.Some alkaline proteases are used in the production of soy products such as soy souce (Rao *et al.,* 1998; Kumar and Takagi, 1999).

In baking industry: Protease is widely used in bread making because their ability to ensure dough uniformity, reduce dough consistency and maintain gluten strength. These things are helpful in improving the bread texture and flavour (Sandoval *et al.,* 2016).

5.2 Therapeutic applications of plant proteases: Plant proteases play an important role in the treatment of

different disorders in humans. The pineapple plants are the best source of Bromelain, is a type of protease. It is widely used in treatment of inflammatory diseases because it acts as natural anti-inflammatory agent. Bromelain is able to regulate the expression of m-RNAs encoding pro-inflammatory cytokines by individual immune cells. Specific molecules on the cell surface are cleaved by proteases that are responsible for the migration and activation of leukocytes. The inflammatory response interrupts by inhibiting the signal transduction between immune cell and colon epithelial cell as a result it is helpful to reduce inflammation that are responsible for joint pain (Bhattacharya, 2008; Onken *et al.,* 2008). Bromelain also have anti-cancer properties. Proteolysis process carried by bromelain is a main pathway useful in the treatment of breast, lung and malignant cancer by promoting cell death and tumor development (Pillai *et al.,* 2013 and Pillai *et al.,* 2014). Protease enzymes which are extracted for medicinal plants have antiviral activity (Ashfaq and Idrees 2014; Gupta *et al.,* 2016). Because these enzymes plays an interpretative role in the life cycle of various viruses without damage in the host cell (Mahajan and Badgujar, 2010). The most applicable plant protease enzyme such as papain, bromelain, keratinases etc. showed several physiological processes. For example: digestion of food proteins, protein turnover, cell division, blood clotting cascade, signal transduction, processing of polypeptide hormones, apoptosis etc.(Roy *et al.,* 2005; Mahajan and Badgujar, 2010). Serine protease named "Prunifoline" enzyme which is extracted from latex of *Euphorbia prunifolia* plant is used for the treatment of toothaches, nervine diseases, dropsy and palsy, deafness to kill the maggot in wounds (Mahajan and Badgujar, 2010).

The roots of *Jasmine grandiflorum* is very effective against various ailments such as paralysis, mental debility, chronic constipation, sterlity, ring worm, leporsy and skin diseases (Sulaiman *et al.,* 2012). The leaves and flowers of the plant are very useful in fixing loose teeth, leporsy, ulcers, wounds, dysmenorrhoea and vitiated conditions of pitta (Sandeep and Paarakh, 2009).

6. Proteases help in plant immunity: Papain protease is isolated from the wound –exuding latex of papaya plant. These enzymes are activated during wounding (Azarkan *et al.,* 2006) as well as they provide protection against insects because of toxic chemical present in papaya leaves (Kohno *et al.,* 2004). In the case of maize leaves, accumulation of Maize insect resistance1 (Mir 1) increases the resistance because itself acts as ethylene dependent, long –distance transport signal useful against root feeding herbivores and corn leaf aphids (Louis *et al.,* 2015).

Conclusion

Proteases have shown their potential role in different industries and various plant sources (leaves, stamens, flowers, latex and roots) exist for the efficient production of these enzymes to meet continuously increasing demand because of their precise range of action and property of being active over a wide range of temperature and pH attracted the attention of biotechnologists worldwide. Plants are better source due to natural and easy cultivation and no harmful effect on humans. These hydrolytic enzymes are efficiently involved in food industry for enhancing nutritional value, digestibility, flavour and reducing allergenic compounds as well these enzymes are being utilized in cure of respiratory tract tissues, cardiovascular disease, inflammation and cancer. Due to these special features, proteases are an emerging class of enzymes that possess diverse medical applications.

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