Bromelain: Applications and Purification Strategies

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ABSTRACT

Bromelain is a plant protease present in different parts of the pineapple [*Ananas Comosus* (Linn.) Merr.] plant. It is an enzyme of high commercial interest which can be attributed to the wide therapeutic and industrial applications it possesses. As such efficient purification and recovery of the enzyme is utmost necessary on a large scale basis. The review highlights the numerous applications of the protease enzyme and further discusses the various purification techniques beginning from simpler ultrafiltration to the more complex and specific column chromatography that are being commonly employed nowadays. Effort has been made to compare the purification strategies extensively and suggest the best among the techniques which is cost-effective, has high output and can be used on a large scale production basis.

Keywords: Bromelain, Pineapple, Ananas Comosus, Purification, Enzyme, Column chromatography, Ultrafiltration

INTRODUCTION

Pineapple belongs to the family Bromeliaceae and is abundantly grown in several tropical and sub-tropical countries. It has widely been used as a medicinal plant by the natives of the countries.[1] The top ten countries producing pineapple as per the 2013 statistics (Fig.1) are Costa Rica, Brazil, Philippines, Thailand, Indonesia, India, Nigeria, China, Mexico and Columbia.[2] The medicinal efficacy of the plant can be attributed to an enzyme called Bromelain.[3] Bromelain is a crude extract, basically a protease enzyme obtained from the fruit or stem of the pineapple [*Ananas Comosus* (Linn.) Merr.] plant.[4] The concentration of bromelain is the highest in the stem of the pineapple as compared to the fruit and thus, extraction of the same is quite cheaper due to the lack of economic value of the stem which is generally a waste by-product.[5] The enzyme bromelain that is extracted from stem is called stem bromelain (EC 3.4.22.32) and that from the fruit is known is fruit bromelain (EC 3.4.22.33).[6] Other undesirable parts of pineapple such as core, peel, leaves and crown also contains the enzyme, but in very smaller quantity.[7] Bromelain, the enzyme is composed of a mixture of both thiol proteases and non-proteases. The proteases contribute to form a major part of the bromelain while the non-proteases present are carbohydrates, glucosidases, cellulases, phosphatases, peroxidases and glycoproteins.[8] The isoelectric point for stem bromelain is at 9.5 and that of fruit bromelain is at 4.6.[9] Bromelain has been

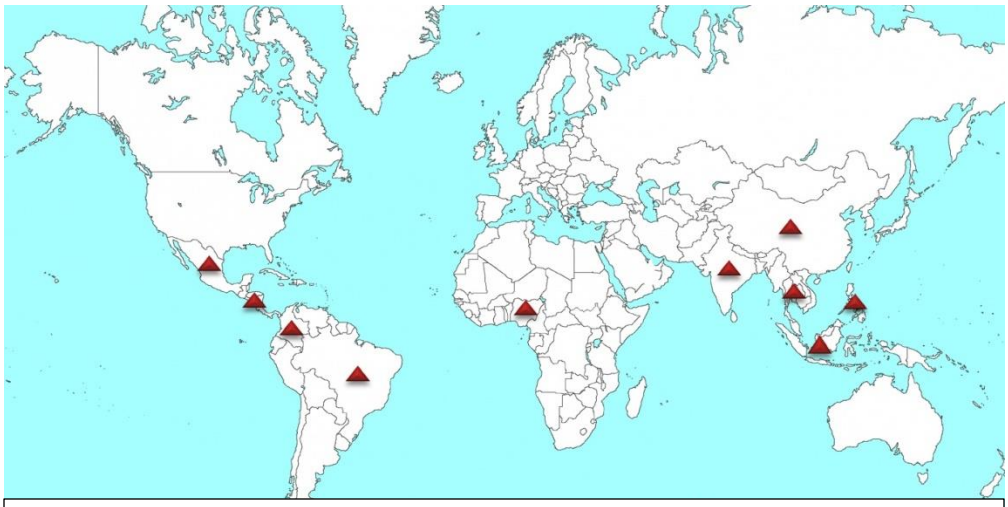

Fig.1. Top ten countries producing Pineapple

accepted universally as a phytotherapeutical drug[10] and has wide clinical applications like tumor suppression, anti-inflammatory property, improving action of antibiotics [11] and as an anti- cancer agent. [8]

The review aims at highlighting the various therapeutic and commercial applications of the enzyme bromelain. Further, effort has also been made to discuss the various extraction process employed and th level of purification achieved therein to draw a meaningful conclusion.

THERAPEUTIC APPLICATIONS OF BROMELAIN

Human body can absorb significant quantity of bromelain. Around 12g/day of bromelain can be consumed by humans without any side effects.[12] The high degree of absorption may be responsible for the efficiency of bromelain in treatment of several disorders (Fig.2) as observed in various clinical studies.

Bromelain being an inhibitor of blood platelet aggregation helps in minimizing the chances of arterial thrombosis and embolism[13], hence effective in treatment of cardiovascular diseases like coronary heart disease, cerebrovascular disease, rheumatic heart disease, congenital heart disease, peripheral artery disease and hypertension.[14] Bromelain also counter acts against certain intestinal pathogens like *E.coli* and *Vibrio cholera* that are mainly responsible in causing diarrhea. There are mainly two different mechanisms suggested based on different studies conducted. One of the mechanisms suggests that bromelain exhibit the counteraction effect by interacting with the secretory signaling pathways of the intestine, which includes calcium dependent signaling cascades and cyclic monophosphatase.[15] While the other mechanism suggests that bromelain proteolytically modifies attachment sites of certain specific glycoprotein receptors present in the mucosa of the intestine and thus prevents attachment of the bacteria to those receptors.[16] 35% bromelain in a lipid base formulated as a cream is helpful in removal of damaged tissues from wounds and burns. It also accelerates the healing process, which is attributed to the presence of escharase which has a non-proteolytic and non-hydrolytic action activity against the normal protein.[17] For the removal of damaged tissues, bromelain pose to be a safe, non-invasive and better alternative as compared to surgery which is more painful and associated with many risks.[18] Several studies have also reported bromelain to have immuno-modulatory effect. Application of bromelain in cells *In vitro,* activates natural killer cells. Bromelain also possess anti-microbial activity. It shows potential anti-helminthic [19] and anti-candida effects.[20] Bromelain also increases antibiotic potentiation. Bromelain combined with antibiotics is more effective than antibiotic alone. This combined therapy has been helpful in curing bronchitis, pyelonephritis, pneumonia, cutaneous staphylococcus infection.[21] Bromelain has also successfully been used as a digestive enzyme in various intestinal disorders.[22] Post colonic surgery administration of bromelain in rats, acts against surgery induced decreased intestinal motility, increases the weight of stool, water content and improves defecation.[23] Research studies also have found that bromelain is able to decrease the occurrence and severity of colitis in mice.[24] Bromelain has also been reported to be successfully used in the treatment of mild ulcers. Study was

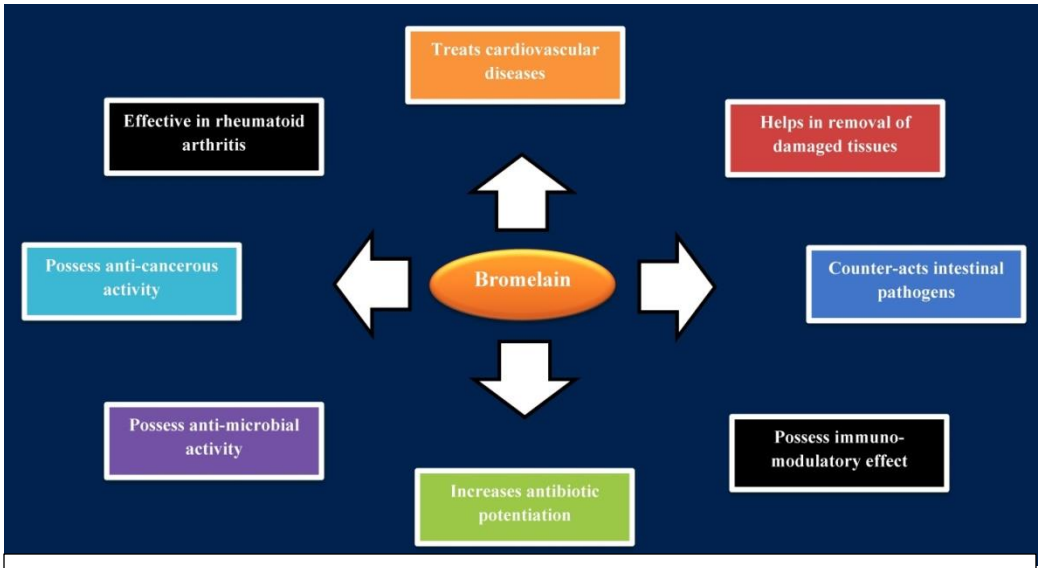

Fig.2. Bromelain in therapeutic applications

undertaken on two patients suffering from colitis who did not respond to any conventional medicines. Bromelain was administered to them in addition to the drugs, thus resulting in rapid recovery.[25] Bromelain along with other enzymes and vitamins has also been found to slow down renal diseases in rat models.[26]

Patients suffering from knee osteoarthritis were administered with bromelain for a month, which resulted in significant reduction of pain and stiffness among the patients.[27] There was some contrasting result obtained in another study wherein bromelain when administered to a group of individuals suffering from knee arthritis for 12 weeks did not bring about any improvement.[28] A study conducted in the 1960's has suggested that bromelain may be beneficial in rheumatoid arthritis.[29] Bromelain's anti-inflammatory property against rheumatoid arthritis is more effective or better than indomethacin, piroxicam, etodolac, naproxen, ketoprofen and diclofenac.[30] In combination to other enzymes like papain and quercetin, bromelain has also been used to improve symptoms of patients suffering from category III chronic prostatitis.[31] Bromelain has also been successful in reducing reactivity and sensitivity to airway irritants in case of acute asthma.[32] Bromelain is believed to may have certain anti-cancer activity as indicated by several human and animal studies.[33,34] Influence of bromelain on tumor lines like P-388 leukemia, Lewis lung carcinoma, sarcoma (S-37), ADC-755 mammary adenocarcinoma and Ehrlich ascetic tumor shows in-vivo anti-tumoral or anti-leukemic activity.[35] The anti-cancer effect of bromelain may be attributed to its impact on various molecules involved in cell signaling as well as on inflammatory, immune and homeostatic pathways.[8]

Bromelain has a high value as an enzyme in the field of health care and therapeutics. It stands as a natural, non-toxic and better alternative to various chemicals and artificially synthesized medicines. Alongside the therapeutic applications, the enzyme also possesses quite a good number of industrial applications.

INDUSTRIAL APPLICATIONS OF BROMELAIN

The wide industrial applications of bromelain (Fig.3) can be attributed to the proteolytic activity posed by the enzyme. The industrial applications moreover increase the commercial value of the enzyme. This part of the review deals with some of the important industrial applications of bromelain. Bromelain has a long history of being used in meat tenderization and is commercially available in the market under brand names like Knorr and McCormick. Bromelain is quite efficient in hydrolyzing myofibril proteins present in the meat like nebulin, actomysin and titin.[36] Extensive proteolysis of beef, squid and chicken has been observed upon treatment of up to 20% (w/w) bromelain. Further, bromelain could act as a good substitute as a hydrolysisng agent for oyster meat in place of hydrochloric acid.[37] The enzyme is also used in baking industry to enhance solubility, improve relaxation of dough and prevent shrinkage, thus allowing the dough to rise evenly during baking.[38] Bromelain has also been used to produce flour for wheat allergic patients by enzymatic fragmentation of constituent allergens present and is known as the hypoallergenic flour.[39] Bromelain also inhibits oxidation of phenols to quinones in fruits, thus acting as an anti-browning agent.[40] In animal feed, bromelain is used for estimation of degradation of protein in cereals, hays, protein concentrates, forages and silages in ruminants.[41] Application of bromelain in silk industry during cocoon cooking effectively reduces the softening time.[42]

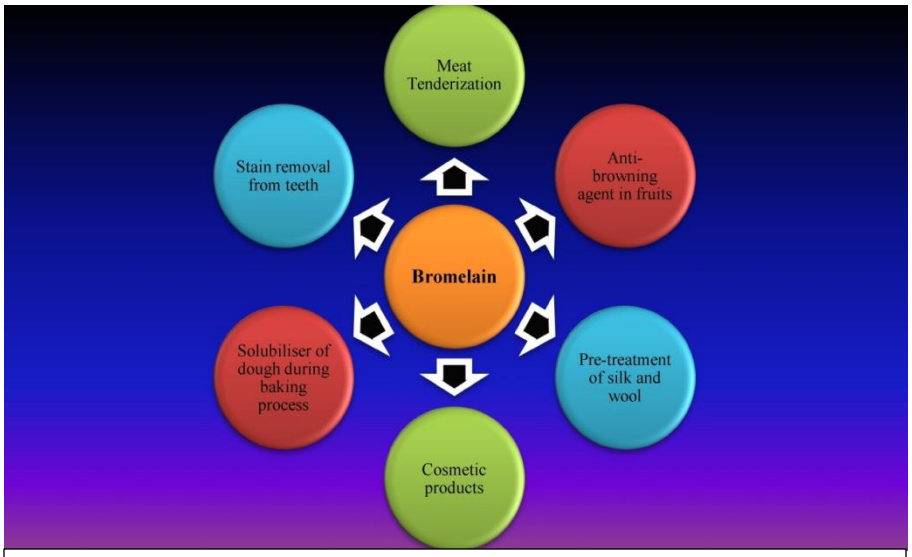

Fig.3. Industrial applications of bromelain

Studies reveal that pre-treatment of silk and wools with bromelain removes scales and impurities and improves the quality.[43] Bromelain is also one of the active ingredients in many cosmetic products which help in alleviating several skin related problems like acne, wrinkles and dry skin.[44] Bromelain also reduces swelling of skin after treatment or cosmetic surgeries.[45] Toothpaste containing bromelain and papain has been found to remove extrinsic stains from teeth more efficiently and has a better lightening effect as compared to regular Colgate. The only limitation in this case being that the prepared formulation containing the enzymes had a short shelf life due to the proteolytic nature of the enzymes present in it.[46]

The wide applications of bromelain, makes it one of the most industrially important enzyme and thus efficient strategies are to be followed for purification of the enzyme.

STRATEGIES FOR EFFICIENT PURIFICATION OF BROMELAIN

A variety of strategies are followed for proper purification of the enzyme. The review also intends to discuss and compare amongst these strategies and find out the best possible one. Techniques that are being widely used in the purification of bromelain are ultrafiltration, salt precipitation, aqueous two phase system, reverse micelle system and chromatography.

Ultrafiltration

This process is widely used in large scale processes and makes use of membranes that can separate proteins with molecular weight ranging from 3 kDa to 100 kDa. The protein remains trapped behind the membrane and is continuously recycled until the desired level of purity is achieved.[47] Howsoever, ultrafiltration is a long separation process and increased protein loading may cause the membrane to clog.

Salt precipitation

Salt precipitation generally makes use of high concentration of precipitating agents like ammonium sulfate to reduce the solubility of proteins in a solution. This reduced solubility, precipitates the proteins and the mechanism is known as salting out. The whole process is carried out at low temperature to prevent the denaturation of the proteins. Howsoever, the protein recovery needs other additional steps like centrifugation and dialysis to remove contaminants present along with it.[48] There has been use of several organic solvents like ethanol, methanol and other alcohols and ketones for precipitation of the proteins. These organic solvents replace a part of the water, thus precipitating the proteins. However, the efficiency of ethanol precipitation process is not as good as compared to that of ammonium sulfate. The purification achieved by ammonium sulfate (4.44 fold) is more than twice that achieved by ethanol (2.07 fold).[49] The organic solvents need to be used at subzero temperature for obtaining desired concentration of protein and avoiding denaturation.[50] To overcome the problem of temperature control, organic polymers like polyethylene glycol (PEG) has been used to precipitate the proteins.[51]

Aqueous Two Phase Systems

The process makes use of two immiscible phases formed by mixing a polymer and salts or two incompatible polymers like PEG and dextran in an aqueous system. Temperature, molecular weight of the polymer, pH, ionic strength and the type of salt dictates the distribution of proteins between the two phases. For instance in the case of PEG-dextran system, the protein under favorable conditions tends to separate into the top or PEG phase which is less polar, less dense and hydrophobic, while the contaminants are found in the lower or dextran phase which are more dense, more polar and hydrophilic in nature.[47] The proteins can be separated by centrifugation. Several works has been carried out to extract bromelain from different parts of pineapple using PEG-potassium phosphate [52], PEG-magnesium sulfate [53] and PEG-ammonium sulfate systems.[54] All these studies suggested that the maximum recovery of bromelain was possible at the highest concentrations of PEG and salts. However, studies also suggest that the partitioning of bromelain in the PEG (top) phase decreases due to structural alterations of enzyme sites in presence of high molecular weight PEG. [55]

Adsorption

This process involves binding of a dissolved solute to a solid surface, known as the adsorbent. There is

large number of instances employing various adsorbents for the proper extraction of bromelain. In the last two decades, there has been an increased use of nanoparticles as an adsorbent in the purification process. Use of magnetic nano adsorbents using iron oxide nano particles successfully produced 87.4% bromelain after adsorption at pH 3-5 and followed by desorption at pH 7.[56] Similarly, use of lectin concanavalin A (Con A) precoupled sepharose matrix resulted in separation of 60% bromelain at pH 11.[57] Use of reactive red 120 (Red 120) dye as a ligand immobilized onto magnetic nano particles coated with chitosan by Song et al. (2011), achieved bromelain adsorption of 88.2%.[58]

Reverse micelle extraction

The process involves a liquid-liquid extraction technique for separation of biomolecules in downstream processing. The system consists of an organic phase separated from droplets of aqueous phase by surfactants present in the interface of the two phases. The polar head of the surfactant is hydrophilic in nature and is located inside the micelle whereas the non-polar tail (hydrophobic) remains in contact with the solvent to the outside of the micelle. Ionic strength, concentration of the surfactant and the pH are certain factor that induces the movement of the protein to inside and outside of the micelle.[59] Recent study by Chaurasiya and Umesh Hebbar show successful application of the technique in separation and purification of the enzyme bromelain.[60] The process of extraction can be of two types – the forward extraction process and the back extraction process. The extraction rate of protein increases in the forward reaction process when low ionic strength salts and high concentrations of surfactants are applied whereas, in the back extraction process the high ionic concentration of salt may reduce enzyme exclusion from the interior surface of reverse micelle in the back extraction process.[61] Conditions optimized with 150 mM of cetrimonium bromide (CTAB) and 0.1 M sodium chloride in the forward extraction process and 0.5M potassium bromide in the back extraction process showed highest efficiency in extraction of bromelain.[7] The optimized conditions were used coupled with the ultrafiltration process obtaining bromelain activity recovery of 95.8% with a purification fold of 8.9.[52] Kumar et al. 2011, recently used “affinity based reverse micellar extraction and separation” for purification of bromelain extracted from core of the pineapple. A purification fold of 12.32 and enzyme activity of 185.6% was achieved and was higher than the previous mentioned findings. The purification was achieved by affinity interaction of bromelain and a ligand namely concanavalin A which was coupled into the reverse micelle system.[62]

Column chromatography

Column chromatography is a technique which is used to separate biomolecules like proteins from other contaminants. The migration of the protein through a column generally depends on molecular size, charge of the protein, affinity towards a particular ligand and as well as the nature of both stationary and the mobile phase. The separation of proteins can be categorized into basically two mechanisms: adsorption and non-adsorption.[50] The most commonly used method to isolate bromelain from different parts of pineapple is the ion exchange chromatography. Anion exchange chromatography followed by ammonium sulfate precipitation yielded bromelain with a specific activity of 10U/mg from the fruit of pineapple.[63] On the other hand work has been carried out wherein ammonium sulfate precipitation and dialysis has been used prior to ion exchange chromatography for the purification of fruit and stem bromelain.[64] Purification of fruit and stem bromelain by gel filtration and ion exchange chromatography followed by successive use of Sephadex G-75 column and Carboxymethyl-Sephadex and diethylaminoethyl-Sephacel ion exchange chromatography was successful in separating stem bromelain into six proteolytic components namely SBA and SBB1-SBB5. It also separated fruit bromelain into two active components – Fruit bromelain fraction A and B.[65] Cation exchange and affinity chromatography purification of stem bromelain have been reported to yield ananain, a non-glycosylated component. [66]

Bromelain a plant protease has wide therapeutic and industrial applications. As such, proper extraction, purification and recovery steps are needed to be followed for the commercialization of the enzyme. The purification techniques discussed in this paper

are widely used in the current scenario but each of the technique has its own pros and cons. The ultrafiltration is although helpful to concentrate the protein and efficient in removing impurities but still the process is time consuming and have the risks of clogging of the membrane pores. Similarly, the process of precipitation is efficient, easy to scale up, needs low energy and simple equipment's, it needs further purification step like dialysis to remove excess of salt. The aqueous two phase system is equally efficient in removing trace contaminants, involves low cost, short separation time and is easy to scale up producing higher yield. However, it faces difficulty in complete recovery of target protein. One of the highly efficient methods is the reverse micelle system which can be easily scaled up and can be operated in a continuous manner making the purification process a continuous affair. There is no loss of protein or its activity in this process; still it faces the same problem as aqueous two phase system and the recovery of target protein from surfactant containing organic solvent is quite difficult. The highest level of purification can be obtained through column chromatography which is highly specific and can be scaled up. Such type of purification levels are desired for pharmaceutical applications or products. The problem with this technique is that the separation process is very costly and slow. Besides, the sample loading capacity is quite small and involves multiple steps. [67]

Keeping in the pros and cons possessed by various techniques, the aqueous two phase system and the reverse micelle extraction system appears to be promising. Despite having few drawbacks, both of the processes are easy to scale up and require low investment for set up and are able to achieve high purification levels within a short span of time. Commercialization mainly depends on low time, high quality and low cost of investment and these two strategies satisfy these criteria to the fullest.

CONCLUSION

Bromelain being highly important as a commercial enzyme has been studied extensively for extraction and to attain high purification. Among all the techniques employed, the aqueous two phase system and the reverse micelle system stand out to be the best in the business. The study suggests the use of these two techniques widely for purification of the protease. Further research needs to be carried out in this field so as to overcome the limitations posed by the techniques. A combination of two or more of the discussed techniques can be used followed by proper optimization to maximize the output and minimize wastage of the precious enzyme in the process.

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