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Review Article

A review of clinical uses of Bromelain and concerned purification methods to obtain its pharmacological effects efficiently

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ABSTRACT
Bromelain comprises a group of proteolytic enzymes obtained from the tissue of Pineapple, Ananas comosus, belonging to the Bromeliaceae plant family. Different studies indicated its antithrombotic, fibrinolytic, antimicrobial, anticancer, and anti-inflammatory properties. Bromelain has significant applications in clinical, industrial, and cosmetic sectors. Therefore, researchers have always explored suitable extraction and purification methods for the final application. In this paper, we have mainly focused on the potential pharmacological activity of bromelain and future applications. We have identified, compared, and evaluated the different extraction and purification methods of bromelain for utilizing its pharmacological activities. We have also discussed the pharmacokinetics, toxicity profile, and health effects of bromelain. The study concluded with findings of a huge area of their clinical uses and proper methods to obtain the purest form of bromelain.

Keywords: Ananas comosus, Anti-Inflammatory Effect, Clinical use of Bromelain, Extraction, Pineapple, Purification

INTRODUCTION

Bromelain is the common term used for some commonly associated proteolytic enzymes or endopeptidases present in the tissue of the Bromeliaceae plant family (Heinicke,1953; Benucci et al., 2011). Pineapple, Ananas comosus, is the best-known plant of this family. Bromelain was first chemically recognized in 1876 (Tochi et al. 2008), it was first separated and described in 1891 by the Vicente Marcano, a Venezuelan chemist (Upadhyay et al. 2010). Since 1894, the study of bromelain and its isolation had been explored (Neta et al. 2012). This plant, in addition, holds a small amount of other proteinases enzymes like ananain as well as comosain. Bromelain is the major and most broadly explored of these enzymes ((Larocca et al. 2010; Nadzirah et al. 2013; Amini et al. 2016). Physiologically, in pineapple plants, bromelain is found in the stem and fruit part in a higher amount. Heinecke discovered in 1957 (Heinicke et al., 1972) that its stem includes substantially more bromelain than its fruit. Later studies have shown that it may also be extracted from the other parts such as root, leaves, peel, etc. in limited quantities ((Hennrich, 1969; Murachi,1970; Doko, 1991; Hebbar et al., 2008; Hossain et al., 2015).

Bromelain isolated from the pineapple fruit is assigned the number EC 3.4.22.33 and is called fruit bromelain (FBM). Similarly, bromelain found in the pineapple stem is referred to as stem bromelain (SBM) and assigned the number EC 3.4.22.32 (Smith-Marshall and Golden, 2012). The higher content of SBM guided to the enzyme production commercially, and its use as a useful phytomedical agent (Novaes et al., 2015). Researchers have concentrated on the use of pineapple waste mainly as a source of bromelain by isolation (Upadhyay et al. 2010, Seguí & Maupoey, 2017).

Structural Chemistry of Bromelain

A proteolytic enzyme is the most important ingredient in crude bromelain. It is glycosylated therefore making it a glycoprotein. Stem bromelain is further classified as a cysteine proteinases, belonging to the common group of sulfhydryl proteolytic enzymes. Bromelain is a thiol endopeptidase and thus reported to remain as a combination of substances e.g. carbohydrates,
glucosidase, peroxidases, cellulases, phosphatases, glycoproteins, and a number of protease inhibitors (Bhattacharyya, 2008). The activity of all these enzymes depends on the thiol group (SH) of a cysteine residue. Bromelain is a

glycoprotein that has only a single oligosaccharide moiety per molecule (Benucci et al., 2011). Stem bromelain is composed of a glycosylated, 24.5 kDa single-chain moiety with a pI of 9.55 and A_1%, 280 of 20.1, (Murachi, 1970), with seven cysteines (Harrach et al., 1995) and hence most definitely three disulfide bonds. Stem bromelain has a fairly stable secondary structure and shows activity in the pH range 7-10 with its activity being irreversibly lost beyond pH 10 (Dave et al., 2010). A characteristic molten globule structure of stem bromelain is achieved at alkaline pH and may exhibit a charge heterogeneity which generates numerous chromatographic variants, for unknown reasons, although they are all immunologically similar (Rowan 1988). Bromelain is reported to be stable for an extended period if stored under -20 ^{0}C (Rowan et al. 1990).

Comparison of Stem Bromelain and Fruit Bromelain

Stem bromelain has higher protease activity compared with bromelain derived from the fruit. Both types of bromelain are single-chain glycosylated monomeric enzymes. They have dissimilar characteristics. Stem bromelain has lower proteolytic action and its specificity is lower for peptide bonds than fruit bromelain. The reported optimum pH range for stem bromelain has shown by various researchers to be in the range of 6-7, and its most favorable temperature range for showing activity is at 50-60 ºC (Gautam et al. 2010). The optimum pH range for fruit bromelain is 3-8, (Lopes et al. 2009), and the suitable temperature range is 37-70 ºC (Silvestre et al. 2012). Although both enzymes are monomeric in nature, the molecular weight range for stem bromelain is 26-37kDa and that of fruit bromelain is 24.5-32kDa (Lopes et al. 2009; Gautam et al. 2010; Kumar et al. 2011). Stem bromelain possesses diverse biochemical characteristics and differs in composition as in comparison to bromelain obtained from other parts of the plant (Pavan et al. 2012). Stem bromelain contains diverse thiol-endopeptidases.

Importance of bromelain

Bromelain has shown to have a variety of beneficial uses as a phytomedical compound. Bromelain has been reported to improve healing after surgery and drug overdose (Graf, 2000 ; Bresolin, 2013). In addition to its medicinal uses (Ketnawal et al. 2009), bromelain has been utilized by many other industries such as the food and brewery industries (Maurer 2001), and the clothing, cosmetics, and pharmaceutical sectors (Babu et al. 2008). Bromelain has therapeutic benefits in mediating inflammation, allergy and autoimmune disorders (Miller et al., 1992; Harrach et al., 1995; Báez, 2007; Dave et al., 2010; Singer et al., 2010; Chobotova et al, 2010; Hu et al., 2011). Through applying traditional and new methods, such as controlling pH, hydrophilicity, and temperature in conjunction with the exclusive characteristic s of bromelain, researchers worldwide have focused their attention on purification strategies (Arshad et al., 2014). The quantity of production would vary depending on their anticipated industrial use. In recent years, breakthroughs in recombinant DNA technology have enabled potential large-scale recombinant bromelain development and purification for innovative technologies (Soares et al., 2012).

Pharmacokinetics of bromelain

Absorption and Bioavailability of Bromelain

Bromelain is highly absorbed in the body and thus has a high bioavailability (Shiew et al., 2010). It can bind to the two antiproteinases in the blood namely, alpha 2-macroglobulin, and alpha1-antichymotrypsin. It has been focused that almost 12 gm/day of bromelain can be taken with no adverse impacts (Castell et al., 1997). Bromelain is absorbed across the gut epithelium in its active form; about 40% of the ingested amount is absorbed from the intestine (Seifert et al., 1979). Castell et al. (1997) found that bromelain also has activity in plasma.

Toxicity profile of bromelain

Bromelain has been reported to have almost no toxic effects in clinical and preclinical studies. Taussig et al., 1988 concluded with the results that bromelain in mice, rodents, and rabbits has low toxicity. Preclinical studies also indicated no toxicity in six months with a growing intensity of bromelain up to 750 mg/kg upon daily administration. 1500 mg/kg per day, if given to rats caused no carcinoma. Eckert et al. (1999), in their study, administered bromelain (3000 FIP unit/day) to the human body over a period of 10 days and came up with no noteworthy alterations in blood coagulation factors.

Clinical Application

Bromelain is clinically important as a number of studies show (Ahmad et al. 2007, Bhattacharya et al., 2009; Rathnavelu, 2016). Bromelain improves bioavailability and lowers the adverse impacts of different antibiotics (Ahmad et al. 2007, Bhattacharya et al., 2009; Pavan et al., 2012). Bromelain also functions as an immunomodulator, being antimetastatic, anti-edematous, antithrombotic, and anti-inflammatory (Harrach et al., 1995; Dave et al., 2010; Báez,2007) discovered the primary

therapeutic application for the treatment of burns as infectious, in vaccine preparation, antitumor, and skin debrider. It has been shown that most of the bromelain's biochemical function could not be attributed to a particular proteolytic component, and the intended actions of bromelain are possibly related to several factors like the method of isolation, purification and so on (Ahmad et al. 2007, Bhattacharya et al., 2009).

Impacts of Bromelain on Blood Coagulation

Bromelain has been reported to have anticoagulant effects and it is available at an average price of 40-80 US dollar/ kg depending on the source and quality. It affects blood clotting by mounting the fibrinolytic capability of the serum, and by inhibiting blood-clotting protein, fibrin synthesis (Lotz-Winter, 1990). A rat study by Taussig et al., (1988) showed a dose-dependent relationship of its activity on blood clotting. Plasma prekallikrein is a proenzyme. It has to be converted to the kallikrein, the active form of the enzyme which helps in coagulation, and Bradykinin is a compound released in the blood and causes smooth muscle contraction and blood vessel dilation helping in coagulation. It was also observed to decrease prekallikrein(Bryant et al.,2009; Kaur et al. 2014).

Effects of Bromelain on Cardiovascular Disease

Bromelain helps fight against cardiovascular diseases and has a lower cost than any cardiovascular drug. According to amazon.com, marketed bromelain supplements of 500 mg capsules of 1200-1800 tablets are available in the price range of 14-30 dollars depending on sources and companies. bromelain's anticancer property is mainly attributed to its capability to inhibit blood platelet aggregation (Heinicke et al., 1972). The blood coagulation factors, fibrin, and thrombus formation are important in blood coagulation(Bryant et al.,2009). Fibrinolysis and inhibition of thrombus formation impede blood clotting in the blood. Pavan et al. (2012) reported the fibrinolytic activity as well as inhibition of thrombus development and platelet aggregation lessening activity of Bromelain. Thrombophlebitis is an inflammatory process which helps to a blood clot to form and block one or more veins, especially in legs. Bromelain assists to decrease the potential threats of thrombophlebitis and helps its management. Studies reported bromelain to be effective in curing acute thrombophlebitis by reducing patient walking difficulty and inflammatory symptoms, including skin temperature, tenderness, oedema, and discomfort. Bromelain can also inhibit the angina attacks and result in symptomatic relief in hypertension(Maurer et al., 2001). Bromelain has also been reported to protect against ischemic injury (Neumayer et al., 2006). It increases blood flow and oxygen transport (Shibayama,1986). In experimental animals, bromelain was found to play an anti-hypertensive role for a prolonged period of administration. Bromelain supplements can, therefore, reduce risk factors for cardiovascular disease.

Effect of Bromelain on Cancer Cells

Several studies have shown that bromelain has anticancer effects (Eckert et al., 1999; Báez et al., 2007; Chobotova et al., 2010). Cell growth and proliferation are usually regulated, and cell cycle disparities can result in abandoned cell growth and transformation into cancer cells. There are various mechanisms inside cells to defend their DNA from harm from toxins and genomic instability (Chobotova et al., 2010). Tumor cells when losing checkpoint controls become responsible for normal cell cycle regulation (Báez et al., 2007). Juhasz et al., (2008) observed that bromelain action in mouse tumor cell lines caused inhibition of cell growth. Bromelain therapy greatly reduced the development of Kato-III cell lines in gastric carcinoma. Bromelain slows down the growth inhibitory response of MCF-7 cells in mammary carcinoma cells and stimulates the autophagy cycle. It promotes the monocytic cytotoxicity in women with breast cancer when administered orally (Eckert et al., 1999).

5-Fluorouracil (5-FU) is a drug used to treat cancers of the breast, colon, stomach, rectum, and pancreas. The antitumoral activity of stem bromelain was found to be greater than that of 5-FU, the survival index of which was around 263 percent relative to the untreated dose. When observed with 5-Fluorouracil, bromelain greatly decreased the number of lung metastases caused by LLC transplants. The bromelain has antitumor activity against tumor models such as S-37 and EAT. Its activity and the unaffected tumor development in the metastatic model mean that the antimetastatic action comes from a process not depending on the main antitumoral effect (Báez, 2007).

Antimicrobial Activity of Bromelain

Bromelain inhibits the growth of intestinal bacteria, such as Vibrio cholera and Escherichia Coli. Bromelain can also inhibit enterotoxigenic Escherichia coli (ETEC) bacteria. It can protect against Escherichia coli based diarrhea. Therefore, bromelain shows potential use as prophylaxis against ETEC infection (Mynott, 1991).

Bromelain has also been reported to exert antihelminthic activity against Trichurismuris, Trichoderma viride, and

Heligmosomoidespolygyrus. These are gastrointestinal nematodes (Stepeket al., 2006). So, it has the capability to counter particular intestinal pathogens. Besides, bromelain has the synergism effect when administered with antibiotics. These two mechanisms can be explored for the benefits of bromelain against specific infections. Further, Bromelain has been reported to act as an anti-fungal agent (Brakebusch et al., 2001). Pityriasislichenoideschronica is a skin disease. Bromelain has been shown to completely cure the disease caused by Pityriasislichenoides (Tinozzi et al., 1978; Massimiliano et al., 2007; Massimiliano et al., 2007).

Application of bromelain in Debridement Burns

Quick debridement and/or elimination of eschar is really important in the healing of intense partial and full-thickness burns (Singer et al., 2010). It aims at reducing wound bioburden and enables early healing of wounds by careful care or skin grafting (Hirche et al., 2017). Effective removal of the eschar within 72h is considered to boost the outcome of burn wound treatment (Krieger et al., 2017). 35 percent bromelain in a lipid base, when used as a cream, helps in necrotic tissue debridement and accelerates healing due to the presence of escharase in bromelain (Rosenberg et al., 2012; Krieger et al., 2017). It cannot hydrolyze regular protein substrates or glycosaminoglycan multiple substrates (Pavan et al., 2012). As bromelain facilitates the debridement mechanism and provides better and faster healing and efficient reepithelialization, scientists suggest bromelain for treating postoperative wounds and relieving discomfort and swelling, etc.

Anti-inflammatory activity of bromelain

Though bromelain has many therapeutic activities, the most prominent effect is its anti-inflammatory type action. Bromelain was prescribed as an adjunctive treatment strategy for chronic inflammatory, malignant, and autoimmune disorders and reported to increase the treatment efficiency of the diseases (Pavan et al., 2012; Kargutkar et al.,2017). Mast cell proteases help in the treatment of asthma and allergic disorders by inhibiting the concerned proteins and globulins (Engwerda et al., 2001a; Caughey G.H., 2011). Bromelain, as a protease, is assumed to produce similar effects like other proteases in the treatment of asthma and allergies (Secor et al., 2005). Bromelain, in conjunction with the quick reaction to cellular stress, effectively stimulates the stronger immune system. Bromelain decreases the release of interleukins and tumor necrosis factors (Neumayer et al., 2006). Bromelain, when taken orally, has been shown to induce both analgesic and anti-inflammatory results in rheumatoid arthritis (Leipner et al., 2001). It triggers the expression of TGF-β, one of the main inflammatory regulators in people having osteomyelofibrosis and rheumatoid arthritis (Bierie, 2006; Leipner et al., 2001). Many studies reported the immunomodulatory effect of bromelain (Oh-ishi et al., 1979). Stopper et al., (2003) found in their research that bromelain in inflammatory bowel disease would decrease the expression of INF-ÿ and TNF-α. Bromelain administration prior to surgery will promote early recovery from discomfort from pain and postoperative surgery. Its analgesic properties mean that in women with episiotomy, bromelain can be useful in reducing swelling, bleeding, and discomfort.

Bromelain has been reported to be beneficial for sinusitis and it can be used for the treatment (Rathnavelu et al., 2016). Sinusitis patients reported complete relief from breathing complications and inflammation of the nasal mucosa (Ahle et al., 1987). So, bromelain has a wide range of actions against inflammation.

Isolation And Purification Methods of Bromelain

Bromelain is one of the few plant proteases which can be obtained from different parts of the pineapple plant, such as fruit pulp, stem, peel, and the leaves. Stem is one of the most accessible sources of bromelain. Other parts outside the stem are also researched for the existence of bromelain (Ketnawa et al. 2012) like peel, core, and crown, etc. Researchers strive to search for different more efficient methods to attain extremely pure bromelain in fewer stages and lower costs. Scientists continue to study recombinant DNA technology for bromelain extraction and purification to achieve novel utilization in future ((Tochi et al. 2008; Arshad et al., 2014)

Preparation of crude Bromelain extract

Marketable and cost-effective bromelain is usually obtained from the fruit stem through lyophilization or centrifugation and ultrafiltration method (Corzo et al. 2011). In another study, the crude extract was prepared and the peeled stems were washed to remove soil from the surface of the stems. They were then cut into one-centimeter cube thickness. Then known amounts of waste are crushed along with extraction buffer at 1:1 ratio for 10 minutes and filtered (Umesh et al., 2008). The filtrate is then centrifuged at 10,000g for 15 min. Then the supernatant, i.e., crude enzyme extract is collected. The supernatants are

refrigerated at 4^0C after the addition of 0.05% sodium azide.

Purification of Bromelain from Crude Extracts

Various conventional and latest purification techniques are explored to obtain bromelain of the utmost purified variety at low expenditure (Arshad et al., 2014). After extraction, the crude mixture containing the desired enzyme and its isophores are subjected to various purification processes to remove impurities that inhibit may bromelain activity and impede its application and final enzyme activity (Illanes, 2008). Due to the increased interest in bromelain, researchers have investigated numerous innovative purification methods for its withdrawal and refinement. These include Precipitation, Purification through membrane filtration, Aqueous two-phase system (ATPS), Different Chromatographic processes and Reverse micellar systems (Manzoor et al., 2016; Rathnavelu et al., 2016).

Precipitation

Precipitation is considered the most reliable process for the huge amount of purification of proteins (Silva et al., 2006). Precipitation is commonly initiated by adding organic solvent, a non-ionic polymer, salt, a metal, or by altering the pH. Ammonium sulfate ($(NH4)_2SO4$) can be utilized for the purification of bromelain by precipitation method. In this method, the crude plant extract is treated with ammonium sulfate in varying concentrations with mild stirring for 30 minutes to get saturation of 0–20%, 20–40%, 40–60%, and 60–80%. Then they are centrifuged at 10,000 rpm for 15 minutes at 4°C, and the precipitates are obtained (Devakate et al., 2009) and constitute the concentrated bromelain extract.

Purification through membrane filtration

Membrane filtration is the method for the purification of molecules based on their size variation. Membrane-based technology is an efficient and sustainable procedure for the production of high quality purified bromelain (Nor et al., 2017). Concurrent involvement of microfiltration and ultrafiltration provides a yield of 85% using microfiltration and 10 times concentrated bromelain by ultrafiltration (Lopes et al., 2009). Enzymatic pretreatment and diafiltration operation can be used for bromelain production for better flux performance and bromelain separation (Nor et al., 2018).

Aqueous two-phase system (ATPS)

Aqueous Double Phase System (ATPS) is an efficient and commercially feasible process for extracting and purifying protein and enzyme mixtures. The ATPS consists of either two incompatible polymers like PEG or dextran or one polymer and one salt in an aqueous solution like PEG and phosphate salt. The two phases differentiate as these phase-forming materials are solubilized in an aqueous solution over a critical concentration. This will get rid of unwanted byproducts at hand in the system which decreases enzyme action (Ketnawa et al., 2011). In a study by Rabelo (2004), bromelain was cleaned up by PEO-PPO-PEO. The outcome was the best. Ketnawa et al. (2010) found that lower molecular weight PEG showed better performance in the separation of bromelain with the help of PEG and MgSO4. This study showed a partition coefficient of 12.6 and a purification factor of 2.4 with a 90% yield.

Different Chromatographic processes

Scientists have explored different types of chromatography techniques for bromelain purification. Bromelain yields of 87.4% were obtained when the aqueous bromelain extract solution was passed through Poly Acryl Acid (PAA)-bound Ferric oxide magnetic nanoparticles packed column to adsorb bromelain (Chen and Huang 2004). Immobilized metal affinity membrane (IMAM) was used for bromelain purification, which yielded 15.4 times purification factor with 94.6% active yield (Nie et al., 2008). Thus through chromatography, a higher yield is obtained.

In research by Devakate et al. (2009), three times the purification of bromelain was achieved by ion-exchange chromatography than it was found in the process of precipitation with ammonium sulfate. Researchers also found 10-fold purification and 84.5% enzyme recovery. Silveira et al. (2009) observed that bromelain purification by extended bed adsorption is reasonable with Amberlite IRA 410 as an adsorbent, demonstrating strong performance for the extract retrieved and a 13 fold purification factor. A higher purification factor resulted in improved flow velocity and other variables such as bed extension, bed voidage, and axial dispersion. Gautam et al. (2010) used ion-exchange chromatography with DEAE anion exchanger and compared bromelain concentration and activity in pineapple stem and fruit. It was observed in the study that the centrifugal fraction shows greater enzymatic activity than crude extract, the increase was small. The recovered enzyme retained structural integrity according to these findings and displayed greater activity than extraction and centrifugation.

Bresolin et al. (2013) used this method on DEAE-Sephafore to increase the bromelain specific action. The highest level of action was obtained at pH 7.0, in which the enzyme is most stable. This

method yielded an 89% enzymatic action upturn and purification factor of 16.93.

Reverse micellar systems

Reverse micellar extraction offers unique features such as large interfacial region, less energy requirement, single-stage and continuous mode processing, low-cost factors, and fast scale-up (Krishna et al., 2002). The reverse micellar system has also been utilized for the withdrawal and refinement of bromelain from pineapple stem and waste (Novaes et al., 2015). With the use of the Reverse micellar system, Hebbar et al. (2008) found a yield of 106% upturn and 5.2 purification fold. Modifications in Reverse micellar systems can be done to increase the output and purification fold. For larger scale purification, scale-up studies using a reverse micellar system gave purification of 2.43 fold with an active recovery of 81.3% (Hebbar et al., 2011). RMS if used along with ultrafiltration provides a better result. Hebbar et al., (2012) found that RMS and ultrafiltration together yield an activity recovery of 95.8 % but higher purification factor of 8.9-fold, which means to yield compound with higher purity rather than RMS alone. The continuous extraction method was preferable to the batch extraction method. Kumar et al. (2011) used affinity-based reverse micellar extraction and isolation method to isolate and clean bromelain from pineapple stems, with the even better result including 12.32 fold cleansing and 185.6 percent yield.

Comparison of the processes

Based on different published data, we have summarized and compared the advantages and limitations of this process. ATPE showed the highest purification factor of 16.3 (Wu et al., 2017), although the active recovery of bromelain was 55.6%. Limitations associated with this method are the high salt concentration, difficult to recover and recycle, and low level of purity of the product. Precipitation method had a purification factor of 4.9, and the active recovery of bromelain was 85.97%. Precipitation yields in high precipitant content, so it becomes inconvenient to separate bromelain. But this is mostly used for commercial applications due to the high active recovery together with low cost (Chaurasiya& Hebbar,2013). Chromatography is the most costly method for little separation efficiency, little recovery, and little sample loading capability. Ion Exchange Chromatography showed a purification factor of 10, and the active recovery of bromelain was 84.5%. (Devakate et al., 2009). A combined method of IEC and gel filtration chromatography reported yielding at a high level of purification (Costa et al., 2014). Membrane filtration showed a purification factor of 10, and the active recovery of bromelain was 90%. The advantage of this method is it offers high specificity (Lopes et al., 2009), so it is more applicable for research purposes. Yin et al. (2011) showed that HSCCC when combined with RMS was supposed to give the highest purification level. But the cost involved in chromatography is higher than other processes. So, it is better for analytical research purposes.

CONCLUSION

Owing to its remarkable uses in numerous fields, Bromelain is one of the thoroughly researched proteolytic enzymes. Researchers have long studied to employ strategies to yield the most purified bromelain in fewer stages efficiently. Different traditional and new purification approaches as well as their combinations are being explored and proved to be effective in this regard. Ion exchange chromatography is an expensive method, precipitation, and aqueous two-phase extraction involve high salt concentration streams. We found that chromatography is the best method for obtaining Bromelain for its clinical use. Our research indicates that bromelain is a safe and versatile therapeutic agent and is being used for many therapeutic purposes e.g., bronchitis, sinusitis, arthritis, and inflammation. It has also been reported to have anti-cancer and antimicrobial effects. After oral administration, bromelain is finely absorbed in the body. Even after prolonged use, it does not have a major side effect. All these pieces of evidence reviewed in this article propose that bromelain is a potential and effective in the treatment of many diseases.

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