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A review on chemistry, therapeutic applications, extraction & purification of bromelain

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Abstract

This review focused on the usage of bromelain in numerous applications, as well as the most recent literature on the chemistry, extraction, and purification of bromelain from pineapple (*Ananas comosus)*. Bromelain is a crude, aqueous extract obtained from the stems and fruits of pineapple, leading edible member of the family Bromeliaceae. Bromelain contains a mixture of different thiol endopeptidases as well as phosphatases, glucosidases, peroxidases, cellulases, & glycoproteins. It exerts its enzymatic activity over a P^H range 4.5 – 9.5. The isolation & purification of bromelain has been done by various methods includes aqueous two-phase extraction, reverse micellar extraction, ultrafiltration & precipitation & chromatographic techniques. Bromelain, a cysteine protease, has found commercial use in a variety of industries including food, beverage, tenderization, cosmetic, pharmaceutical, and textile. Bromelain act as an immunomodulatory, anti-inflammatory agent, anti-oedematous, anti-thrombotic & anti-metastatic. Although the precise mechanism of action of bromelain has not been established, bromelain has received widespread acceptance as a phytotherapeutic agent due to its long history of safety and absence of adverse effects.

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Introduction

Pineapple is an edible fruit bearing plant and is the most economically significant member in the family of Bromeliaceae [1]. It is endemic to the Philippines, Thailand, Indonesia, Malaysia, Kenya, India, and China, among other tropical and subtropical nations [2]. Pineapple's extensive popularity as a medical food is due to the presence of the enzyme bromelain. Bromelain refers to a group of proteolytic enzymes or proteases found in pineapple plant tissues such as the stem, fruit, and leaves [3]. Apart from insoluble components such as colour pigments, organic acids, minerals, protease inhibitors, organic solvents, and excipients utilised for enzyme recovery, the major element in crude bromelain is a proteolytic enzyme called glycoprotein [4]. Stem bromelain and fruit bromelain are the two main sources of bromelain, with the former containing cysteine proteinase and the latter glucoprotein proteinase [5]. Commercially, stem bromelain is more widely used than fruit bromelain [6]. Bromelain's potential therapeutic value stems from its biochemical and pharmacological properties. In food industry, It is utilised in meat tenderization, clarification of beer, baking, and the synthesis of protein hydrolysates, among other things. Tanning for the leather and textile industries, hair removal, wool, skin softening, and detergent formulations are some of the other uses. Bromelain has also been

used as a folk remedy, wound healer, anti-inflammatory, anti-diarrhea, and digestive aid [7].

Chemistry

Bromelain is made up of various thiol endopeptidases as well as other components such as phosphatases, glucosidases, peroxidases, cellulases, glycoproteins, carbohydrates, and protease inhibitors [8]. Stem bromelain is the main protease found in pineapple stem extracts, while fruit bromelain is the main enzyme fraction found in pineapple juice. Studies on stem bromelain have been well described than fruit bromelain. The molecular weight range of stem bromelain was discovered to be 26-37, with an ideal pH range of 6-7 and a temperature range of 50-60 ^{0}C. Fruit bromelain has a molecular weight range of 24.5-32.5, a pH optimal range of 3-8, and a temperature optimum range of 37-70^{0}C [9]. Other minor cysteine endopeptidases in pineapple stem are ananain & comosain[10]. The activity of bromelain extract has been demonstrated over a pH range of 5.5 to 8.0[11]. Bromelain extracts from stem in their natural state contain eight basic proteolytically active components. The most essential components are F4, F5, and F 9, with F 9 being the most active protinease fraction.F9 makes up roughly 2% of the total protein content. F4 and F5 (stem bromelain) proteins are predicted to be glycosylated with fucose, N-acetyl glucosamine, xylose, and mannose to 50%, whereas F9 (ananain) proteins are discovered to be unglycosylated. The sequences of the F4, F5 (212 amino acids), and F9 (216 amino acids) fractions have been completed. The pH of the F4 and F5 fractions is 4.0 to 4.5, while the pH of the F9 fraction is near to neutral. The amino acid sequence of F9 differs from the main components F4 and F5 in two places: tyrosine at position 10 is replaced by serine, and asparagine at position 20 is replaced by glycine. F4 and F5 have molecular masses of 24,397 and 24,472, respectively. Protein F9 has a lower molecular mass of 23,427. Approximately 70% of F 4 and F5 aminoacid sequences begin with valine, while 30% begin with additional alanine [12].

The two main acidic proteinase forms present in bromelain crude extract were SBA/a and SBA/b with molecular mass of 23,550 and 23,560, respectively. Both are highly glycosylated and found to be non-typical cysteine proteinases of the papain superfamily [13].

Bromelain enzymatic activity is measured using a variety of substrates, including casein, gelatine digestion units, and chromogenic tripeptides [14]. Bromelain can be made more stable and active by both chemical and covalent modifications. Chemical modification of lysine residues in stem bromelain with anhydride groups brought about enhancement of thermal stability and the resistance to alkali and the surfactant [15]. Treatment with 30 % trifluoroethanol resulted in transformation of the pre-molten globule to a molten globule at protein's isoelectric point caused by the rearrangement of hydrophobic interactions [16]. PEG treatment resulted in the formation of a molten globule and the unfolding of acid unfolded state [17].

Therapeutic Applications

Anti-inflammatory action of bromelain

Inflammation is a crucial factor in the progression of cancer. Infections, persistent irritation, and inflammation are all factors that contribute to the development of cancer [18]. Most inflammatory agents mediate their effects through the activation of NF-kB(nuclear factor-kappa B). [19]. Cyclooxygenase-2 (COX-2) expression is regulated by NF kB, and it is involved in the manufacture of prostaglandin E2 (PGE2), the only prostanoid that has been shown to play a major role in tumour genesis, progression, and metastasis. [20][21]. COX 2 facilitates the conversion of arachidonic acid to PGE2. Bromelain inhibits COX-2 and PGE2 expression in murine microglial cells and human monocytic leukaemia cell lines, according to research [11].

In addition to the blockage of arachidonic acid cascade at the enzyme cyclooxygenase, bromelain may selectively decrease thromboxane generation and shift the ratio of thromboxane/ prostacyclin (PGI2) in favor of prostacyclin [22].At inflammation site, a dose dependent reduction of bradykinin and prekallikrein levels were seen after administration of bromelain. Bromelain thereby prevents the production of oedema and decreases the severity of existing oedema [23].

Bromelain suppresses ERK-2 activation in T Helper cells activated by the TCR or with a combination of PMA and calcium ionophore. Bromelain also reduces PMA induced accumulation of IL-2, IFN-gamma, and IL-4 mRNA through modulating Extracellular Regulated Kinase 2 (ERK-2) signalling [24]. Bromelain has also been demonstrated to suppress CD4 T cells in animals, which are the primary effectors of chronic inflammation [25].

Bromelain stimulates mononuclear blood leukocytes to make significant amounts of TNF-alpha, IL-1beta, and IL-6 after oral administration of a polyenzyme medication including bromelain, notably in monocytes. Granulocytes reacted to bromelain by producing reactive oxygen

radicals, which have been shown to have antibacterial and anticancer properties [26].

Bromelain treatment in inflamed tissues from patients with inflammatory bowel disease showed a reduction in TNF-alpha, IFN gamma, G-CSF, granulocyte-macrophage colony stimulating factor (GM-CSF) secretion [27]. On T cells, bromelain has the ability to remove surface T cell CD44 molecules [28]. It increased IFN-γ-mediated nitric oxide and TNF-α production by macrophages as well as IL-2- and IL-12-mediated IFN-γ production by Natural Killer cells [29]. Bromelain also reduced TGF-Beta expression in patients with rheumatoid arthritis who had high TGF-beta levels [30]. The process could be owing to a direct proteolytic impact on TGF-beta or its precursor, as well as converting the protease inhibitor alpha 2-macroglobulin from slow to fast form,which binds with TGF-beta and inactivates irreversibly [31].

Bromelain has also been reported to be useful in ulcerative colitis; causing endoscopic remission following oral treatment [32].Treatment with bromelain relieves physical symptoms and improves general well-being in healthy adults suffering from mild knee pain [33]. In patients with knee osteoarthritis, an evaluation of an oral bromelain enzyme combination vs diclofenac revealed equivalent efficacy [34].

Anticancer action of bromelain

Bromelain was discovered to trigger apoptosis in mouse skin papillomas by upregulating p53 and Bax, followed by caspase activation and a reduction in the anti-apoptotic protein Bcl-2[35]. Apoptosis proteins like apoptotic induction factor, Endo G, and caspases 3, 8, and 9 were found in high amounts in Bromelain-treated Colon Rectal Cancer (CRC) cells. Bromelain also induced high levels of ROS (Reactive Oxygen Species) and Superoxide production in CRC cells thus enabling cell death of tumor cells. Bromelain decreased cell proliferation in colon rectal cancer cells via triggering autophagy. The levels of autophagy-related proteins ATG5/12, beclin, and p62 were all raised after bromelain therapy [36]. Bromelain's in vivo antitumor activity was similar to that seen in vitro, implying that it works by inducing leucocyte differentiation or by a direct proteolytic effect on tumour antigen – antibody complexes, triggering the action of cytotoxic T cells [37].

Bromelain reduced the number of lung metastasis in an animal model of Lewis lung carcinoma. The antimetastatic effect of bromelain was suggested by its inhibitory effect on platelet aggregation by endothelial cells and downregulation of tPA(tissue Plasminogen Activator) receptors[37]. In an in vitro invasion assay, bromelain reduced the migration of tumor cells through Extra Cellular Matrix. Bromelain also reduces uPAR (urokinase plasminogen activator) expression and prevents plasmin from degrading ECM. Bromelain decreases matrix metalloproteinases (MMP-9) expression and prevents the malignant cells to invade through the ECM. The cleavage of CD44 by bromelain inhibits lymphocyte adhesion to blood vessels and limits lymphocyte migration to tumour sites [26]. It also has anti-angiogenic properties by regulating pro-angiogenic genes and signalling pathways such as VEGF, basic fibroblast growth factor (bFGF), angiopoetin-1 and 2, COX-2, MMP-9, AP-1, and NF-kB [38].

Action on blood coagulation and fibrinolysis

Bromelain is a fibrinolytic agent that works both in vitro and in vivo. Bromelain injection resulted in a dose-dependent decrease in serum fibrinogen levels in rats. Both prothrombin time (PT) and activated partial thromboplastin time (APTT) are considerably prolonged when larger quantities of bromelain are given [23].

Bromelain stimulates the conversion of plasminogen to plasmin and slows the clotting process by destroying fibrin [26]. Kallikrein, which is produced from its precursor prekallikrein, is involved in the contact activation phase of coagulation and generates bradykinin in the kallikrein-kinin system [39]. Bromelain inhibits prekallikrein synthesis and prevents the formation of bradykinin, relieving pain and inflammation while improving blood circulation at the site of infection [40].

Action on cardiovascular system and thrombus formation

Bromelain has been shown to prevent platelet aggregation and thrombus formation both in vivo and in vitro, indicating that it could be used to treat a variety of cardiovascular diseases, including angina pectoris, ischemic attacks, coronary heart disease (heart attacks), cerebrovascular disease (stroke), raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, heart failure, and congenital heart disease[40]. Bromelain improved cardioprotection against ischemia-reperfusion injury in rat myocardium via the Akt/FOXO pathway.Bromelain enhanced aortic flow while reducing infracts size and degree of apoptosis [41]. Bromelain reduced apoptosis rate and endothelial damage while reducing AST levels, according to reperfusion tests [42]. It has been demonstrated to be effective in the prevention

and treatment of thrombophlebitis [38]. Bromelain supplements have the potential to eradicate all cardiovascular disease risk factors and successfully regulate symptoms of diabetes, hypertension, and hypercholesteremia[11].

Anti-microbial action of bromelain

Bromelain is thought to be an effective antidiarrheal medication because it inhibits intestinal secretion by secretagogues that act via cAMP, cGMP, and calcium-dependent pathways [43]. Bromelain has antihelminthic efficacy against the gastrointestinal nematodes Haemonchus contortus, according to in vitro studies [44]. Stem bromelain treatment reduces M.tuberculosis load in murine and human macrophages since bromelain can reduce the foamy phenotype of infected macrophages [45]. Antibiotic therapy combined with bromelain supplementation has been shown to be more effective than antibiotic therapy alone in treating conditions such as pneumonia, cellulitis, pyelonephritis, perirectal and rectal abscesses, sinusitis, and urinary tract infections [46].

Bromelain formulations were found to be effective against P. acnes, S. aureus, C. diphtheria, and E. coli. It can be used clinically for treating acne [47]. SARS-CoV-2 is prevented from entering cells when bromelain and curcumin combination were used .Thus, it can be administered as an immune-boosting nutraceutical to help prevent COVID-19 infection [48].

Action on skin

Bromelain cream (35 percent bromelain in a lipid base) can aid in the removal of burn debris and the speeding up of the healing process. This effect is thought to be caused by escharase, a non-proteolytic component. It exhibits no hydrolytic enzyme activity in the presence of normal protein or glycosaminoglycan substrates. Its activity differs depending on the preparation [46]. Chitosan nanofibers containing 2 % w/v bromelain has excellent wound healing activity, and it could be used as an effective natural topical burn wound healing treatment [49]. In a pig model, enzymatic debridement investigations using bromelain-based agents indicated rapid dissolution of burn eschar while causing no harm to normal tissues [50]. Enzyme debridegment with bromelain is a superior alternative to surgical debridegment as the latter is painful, non-selective, and exposes patients to the risk of anaesthesia.

Extraction & Purification

Bromelain is commercially produced from pineapple through a series of procedures that include extraction, purification, drying, and powder packaging. Isolation and purification need the most expensive materials and activities, or are extremely time-consuming [51]. In this context, modern approaches such as precipitation, membrane filtration, reverse micellar systems, aqueous two phase extraction, and chromatographic procedures have showed promise [52]. In chromatographic purification, elution efficiency was found to be in the 80–90% range. When chromatography was used instead of precipitation, the fold purity obtained was 3.3 times higher [53].

Precipitation

Protein precipitation is accomplished through the incorporation of salts, polar solvents, nonpolar solvents, and organic polymers into cell extracts, as well as by altering the temperature or pH. Precipitation occurs when the solubility of proteins in solution is lowered by increasing the concentration of precipitating agents [54].

Bromelain was precipitated from clarified pineapple fruit juice (20 ml) by gentle addition of ammonium sulphate [(NH4)2SO4] at 4degree Celsius with continual stirring. Initially, 2.27 g (NH4)2SO4 was added to achieve a saturation range of 0–20 %. After adding all of the salt, the stirring was continued for 30 minutes to allow the dissolved and aggregated proteins to equilibrate. Additional processes, such as centrifugation and dialysis, are required to eliminate contaminating proteins in order to retrieve the protein of interest[55].Centrifugation was done at 10,000 × g for 15 mins[56]. In protein precipitation, organic solvents such as ethanol, ketones, metha nol, propanol, and other alcohols have been widely used. Because some of the water has been replaced by organic solvents, the addition of organic solvents reduces the activity of water and causes the proteins to precipitate. Bromelain was recovered using ethanol at concentrations of 30% and 70%, yielding a purification factor of 2.28 times and more than 98 percent of total enzymatic activity [57]. After dialysis, ammonium sulphate precipitate fractions and supernatants were collected at 20% saturation and tested for protein content and specific protease activity [58].

Ion Exchange Chromatography (IEC)

Ion exchange chromatography, capillary electro chromatography, Affinity chromatography are used for the separation of bromelain from pineapple. Among this ion exchange chromatography is a well-known approach for separating charged molecules [58].

Anion exchange chromatography (AEC), Cation exchange chromatography (CEC)

Are the two types of IEC [59]. Protein purification usually employs anion, cation, and mixed (anion & cation) columns, as proteins are complex ampholytes with both positive and negative charges. The CEC column is used to separate positively charged compounds [58]. The cation exchange resins were packed into a glass column with a 25 mm internal diameter and 300 mm length, having negatively charged acidic functional groups [56]. Through ionic interaction, these columns can bind cationic substances such as protonated bases. The stationary phase in AEC column contains positively charged basic functional groups capable of binding anions [58].

Ultra Filtration (UF)

Ultra filtration is an appealing technology for protein separation and concentration that has been widely employed on both laboratory and commercial sizes [60]. The molecular weight threshold for the ultrafiltration membrane ranges from 3 to 100 kDa[54]. The raffinate containing the protein of interest is kept behind the membrane and regenerated until the appropriate concentration is reached [59]. Bromelain purification necessitates a membrane pressure differential of 0.5 to 4 bar, a temperature range of 10 to 30oC, and a pH range of 4 to 8.5, with no corrosive substances or chemicals [62]. Complex carbohydrates in pineapple extract, such as polysaccharides, impact membrane function due to aggregation of these polymers with proteins under diverse physicochemical circumstances, resulting in severe fouling and significant flow decrease [59].

Aqueous two-phase extraction (ATPE)

Aqueous two- phase extraction results in the development of two immiscible phases in an aqueous solution by the mixing of polymer and salts or by the combination of two incompatible polymers [61]. Liquid-liquid extraction gained a sparked attention as a step that replaces more expensive separation methods or reduces the number of steps necessary for the process [67]. It allows for the efficient fractionation of protein-containing mixtures, removing undesired by-products such as polysaccharides, colors, and interfering proteins that reduce an enzyme's activity [54]. Under ideal conditions, the target protein is partitioned into the top phase, which is less dense and less polar, whereas contaminating proteins are located in the denser, more polar, and hydrophilic phase and may be removed using centrifugation[59]. The ionic strength, pH, temperature, polymer molecular weight, and salt type are the factors that influence the distribution of protein samples between these two phases [58]. The high active recovery of enzymes during ATPE is owing to the presence of PEG, which causes a change in the structure of the enzyme's active sites. As the molecular weight of PEG increases, the partition of bromelain on the top phase decreases [63].

Reverse Micellar Extraction (RME)

Reverse micelles are small, thermodynamically stable surfactants that have an organic phase surrounding them and hold water in their centre [64]. Only the protein of interest is captured in a micelle, while the remainder of the contaminants is left in the organic phase [65]. Protein mobility inside and outside the micelle can be generated by adjusting the pH, aqueous phase ionic strength, and surfactant concentration [54]. Bromelain's forward extraction efficiency, purification fold, and specific activity are best at pH 7.0– 8.0[66]. Forward extraction with the anionic surfactant AOT was performed by combining 10 ml of the organic phase (solvent/surfactant) with an equal volume of the aqueous phase (enzyme extract/salt).The organic phase of the cationic CTAB/isooctane system includes a mixture of co-solvents (5% (v/v) hexanol and 15% (v/v) butanol)[66]. Back extraction was also carried out using 0.5M potassium bromide. The phases were well mixed for 1 hour & centrifuged at 4000g for 15 minutes. The aqueous phase obtained after forward and back extractions were checked for bromelain activity and total protein content [68].

Conclusion

Bromelain, as a plant extract, contains various components such as peroxidases, cellulases, glucosidases, and glycoproteins, which all combine to make bromelain a proteolytic enzyme and keep it in a safe zone as a traditional folk medicine for the treatment of various health diseases, as well as a nutrient supplement with few side effects. Bromelain is well absorbed in the body upon oral treatment and has been recommended as an anti-inflammatory, anti-oedematous, anti-metastatic, and anti-thrombotic drug. All of the information presented in this study indicates that bromelain is safe and non-toxic and

that it can be used to cure ailments as well as a health supplement.

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