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Potential Role of Bromelain in Wound Healing Application: A Review

Celine Ng[1], Mohd Syahir Anwar Hamzah[1], Nadirul Hasraf Mat Nayan[1,2*]

[1]Faculty of Engineering Technology,
Universiti Tun Hussein Onn Malaysia, Pagoh Higher Education Hub, Jalan Panchor, Pagoh, 84600, MALAYSIA

[2]Oasis Integrated Group, Institute of Integrated Engineering,
Universiti Tun Hussein Onn Malaysia, Parit Raja, 86400, MALAYSIA

*Corresponding Author

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Abstract: Bromelain is a proteolytic enzyme derived from the pineapple plant (*Ananas comosus*). Bromelain can be extracted from pineapple stems and fruits. Additionally, it can be derived from pineapple wastes such as the core, crown, and peel. Various extraction and purification methods such as reverse micellar system, aqueous two-phase system, chromatographic techniques, and membrane filtration have been used in order to produce high-quality bromelain. Bromelain has been used clinically since 1876 and was first introduced as a therapeutic agent in 1957. Bromelain has gained increasing acceptance and compliance among patients as a phytotherapeutic drug due to its safety and lack of undesirable side effects. Bromelain is regarded as a nutrient that promotes wound healing due to the presence of several closely related proteinases that exhibit anti-inflammatory, fibrinolytic, and debridement properties.

Keywords: Bromelain, wound, anti-inflammatory, fibrinolytic, debridement

1. Introduction

Pineapple or Ananas comosus is grown in several tropical and subtropical countries, including India, China, Kenya, Hawaii, South Africa, Malaysia, Philippines and Thailand [1]. It has been used as a medicinal plant in several native cultures. Crude pineapple extract belongs to a group of proteolytic enzymes (proteases) and is under the classification of cysteine proteases [2]. There are at least 4 distinct cysteine proteases identified from the crude extract of pineapple: stem bromelain, fruit bromelain, ananain and comosain. Ananain and comosain only be found in the pineapple stem [3]. The major proteases that are present in pineapple stem and fruit are bromelain. Ananain is the second most abundant cysteine enzyme found in the pineapple stem where 5% of total protein is present, and the exact amount of comosain that can be found in the pineapple stem is still unidentified. Figure 1 below shows the parts of the pineapple plant.

Bromelain was chemically known since 1876 and was introduced as a therapeutic compound in 1957 when Heinicke found it in high concentrations in the pineapple stem [4, 5]. Bromelain from pineapple fruit is called fruit bromelain EC 3.4.22.33, whereas bromelain extracted from pineapple stem is called stem bromelain (EC 3.4.22.32) [6]. Besides the fruit and stem, bromelain can also be isolated in small amounts from pineapple wastes such as core, leaves, crown, and peel [7]. Bromelain concentration is high in stem compared to fruit and is considered an inexpensive source of bromelain.

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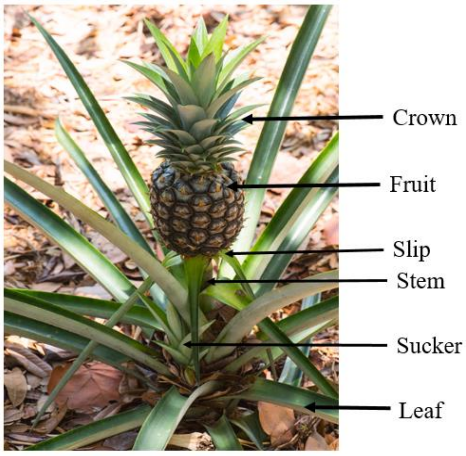

Fig 1 - Parts of the pineapple plant

2. Biochemistry of Bromelain

Bromelain is a mixture of different thiol endopeptidases and other components peroxidases, phosphatases, cellulases, carbohydrates, several protease inhibitors and organically bound calcium [8,9]. Fruit bromelain and stem bromelain possess different biochemical properties and compositions when compared. As mentioned, along with stem bromelain and fruit bromelain, two other cysteine proteases are present in the pineapple, which are ananain and comosain. The extracellular matrix turnover, antigen presentation, processing events, digestion, immunological invasion, haemoglobin hydrolysis, parasite invasion, parasite egress, and processing surface proteins are just a few of the cysteine roles protease play. It is important to differentiate the enzymes according to their types to maximize the role of pineapple cysteine proteases. Therefore, to differentiate and characterize these 4 proteases, a few tests, including SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), mass spectroscopy, N-terminal amino acid sequence analysis and monosaccharide composition analysis, had been conducted. To date, 8 basic proteolytically active components have been detected in the stem bromelain. The two main components have been labelled as F4 and F5. The proteinase considered the most active fraction had been designated as F9, which comprises about 2% of the total proteins. These components were fractionated and isolated from the crude extract of pineapple stem by using cation exchange chromatography and further purified by affinity chromatography [10]. A small amount of comosain was isolated; however, the exact percentage remains unidentified. SDS-PAGE and mass spectroscopy were used to determine the molecular weight of each component. The molecular weight of F4, F5, F9 and comosain is 24.4 kDa, 24.5 kDa, 23.4 kDa and 24.5 kDa, respectively [2, 10, 11].

Figure 2 presents the first 20 amino acid sequences of F4, F5, F9 and comosain. All these components demonstrated a similar sequence; however, their differences can be distinguished in positions 9, 10 and 20. Since F4 and F5 were the main active components found, their protein sequence was considered the reference for comparison. In F4 and F5, about 70% of the major sequence was started with valine (V), and about 30% of the minor sequence started with an additional alanine (A) [12]. Compared with F9, the sequence differs at positions 10 and 20. At position 10, tyrosine (Y) was substituted by serine (S), asparagine (N) was substituted by glycine (G) at position 20. The amino acid sequence of comosain differs at positions 9 and 20. Aspartic acid (D) at position 9 was substituted by asparagine, while at position 20, glycine substituted asparagine amino acid [11]. The 20 amino acids are listed in Table 1

Component / Fraction	Amino acids sequences																			
	1																			20
F4 & F5	V	P	Q	S	I	D	W	R	D	Y	G	A	V	T	S	V	K	N	Q	N
F9	V	P	Q	S	I	D	W	R	D	S	G	A	V	T	S	V	K	N	Q	G
Comosain	V	P	Q	S	I	D	W	R	N	Y	G	A	V	T	S	V	K	N	Q	G
Fruit bromelain	A	V	P	Q	S	I	D	W	R	D	Y	G	A							

Fig 2 - N-terminal amino acid sequences of cysteine proteases of the pineapple plant [11]

Table 1 - List of 20 amino acids [18]

Name	Abbreviation	
	Three letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Hydroxyproline	Hyp	O
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Pyroglutamatic	Glp	U
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

The results from monosaccharide composition analysis showed the different nature of F4, F5, F9 and comosain. Fraction of F4 and F5 contained fucose, N-acetylglucosamine, xylose and mannose at the ration of 1:2:1:2 and 1.1:2:1:2, respectively. It is estimated that 50% of the proteins in F4 and F5 contain a carbohydrate chain. In addition, comosain was found to have similar carbohydrate composition. F9, on the other hand, showed no monosaccharide was detected. From the results of monosaccharide composition analysis, it can conclude that F4, F5 and comosain were glycosylated whereas F9 was not glycosylated, whereas F9 was found to be unglycosylated [2, 10, 11, and 12]. Through characterizations' results of each fraction, it can be concluded that F4 and F5 represented stem bromelain, while F9 represented ananain. The optimal pH for the F4 and F5 fractions is between 4.0 and 4.5, and for F9 close to a neutral pH. The entire extract of bromelain has exhibited activity over a pH range of 4.5 to 9.8 [10]. F4 and F5 has isoelectric point (pI) of 9.55, F9 is more than 10 [2, 14, 15].

For fruit bromelain, similar testing as stem pineapple's protein fractions was conducted for characterization. Fruit bromelain has a molecular weight range of 28-31 kDa and not a glycoprotein, meaning it is not glycosylated. N-terminal amino sequence analysis showed that fruit bromelain amino sequence started with alanine (A) [16], as shown in Figure 2. The pI of fruit bromelain is 4.6 [17]. Table 2 summarizes the biochemistry properties of cysteine proteases from the pineapple plant.

Table 2 - Summary of biochemistry properties of cysteine proteinase of the pineapple plant

Name	**Fraction/ Abbreviation**	Molecular **weight (kDa)**	**Isoelectric point (pI)**	**Glycosylation**
Stem bromelain	F4 & F5	24.4 & 24.5	9.55	Glycosylated
Ananain	F9	23.4	>10	Unglycosylated
Comosain	-	24.5	-	Glycosylated
Fruit bromelain	-	28-31	4.6	Unglycosylated

3. Bioavailability

The degree to which the targeted biological destination fully absorbs a medicine or substance is known as bioavailability. The rate and percentage of a drug's initial dose that effectively reaches either its biological target or the body fluid realm, where its intended targets have unrestricted access, is more appropriately referred to as bioavailability. In bromelain cases, it is normally absorbed through the gastrointestinal tract before spreading throughout the body. The highest concentration, up to 40% of the high molecular weight substances of bromelain, is detected in the blood after 1-hour oral administration. In a study by Castell et al., (1997), the human body can absorb a significant amount of bromelain. The result of the study shows that about 12 g/day of bromelain can be consumed without causing any major side effects on the body [19]. In 2010, a study demonstrated that 3.66 mg/mL and 2.44 mg/mL of bromelain were stable and remained in artificial stomach juice and artificial blood after 4 hours of reaction [20].

4. Toxicity and Side Effects

Bromelain is low toxicity, with LD50 greater than 10 g/kg. LD50 refers to the amount of a substance that will kill 50% (one-half) of a set of test animals when administered all at once. No immediate toxic reaction was observed when bromelain was administrated in mice and rats at 37 mg/kg and 85 mg/kg, respectively, through intraperitoneal injection. The result is similar for intravenous administration of 30 mg/kg bromelain to mice and 20 mg/kg bromelain to rabbits [21]. Toxicity tests conducted on rats with 500 mg/kg/day of bromelain oral administered daily showed no toxic effect and alteration towards food intake, growth, histology of heart, kidney and spleen. No carcinogenic and teratogenic effects were observed in rats after administered dosages of 1500 mg/kg/day of bromelain [22]. Normal doses of 3000 FIP units/day given to humans over 10 days did not significantly affect blood coagulation parameters [23]. Table 3 summarizes the toxicity test conducted for bromelain.

Table 3 - Summary of toxicity test for bromelain

Experimental Target	Amount, Administration method	Observation	Ref.
Mice	37 mg/kg, intraperitoneal injection	No immediate toxic reaction	21.
Rats	85 mg/kg intraperitoneal injection	No immediate toxic reaction	21
Rabbits	20 mg/kg, intravenous administration	No immediate toxic reaction	21
Mice	30 mg/kg, intravenous administration	No immediate toxic reaction	21.
Rats	500 mg/kg/day, oral administration	No side effect and changes towards food intake, growth, histology of heart, kidney and spleen	22.
Rats	1500 mg/kg/day	No carcinogenic and teratogenic effects	22
Human	3000 FIP units/day	Does not affect blood coagulation parameters after 10 days being administrated	23

5. Bromelain Extraction and Purification Techniques

Fruit bromelain can be easily extracted from the juice of pineapple through ultrafiltration [24], whereas stem bromelain can be extracted through centrifugation, ultrafiltration, lyophilization [25] and two-step Fast Protein Liquid Chromatography [26]. Generally, parts of the pineapple plant intended for bromelain extraction are collected and sent for preliminary treatment: cleaning, peeling and size reduction. Then, they were homogenized or crushed for cell disruption before removing debris through centrifugation or filtration. Once the extraction process is done, the crude mixture consists of bromelain enzyme and then undergoes a purification process to eradicate impurities that may interfere with bromelain that can hinder its application and reduce the specific activity of the enzyme [27]. The method used for bromelain purification includes reverse micellar system (RMS), aqueous two-phase system (ATPS), chromatographic techniques and membrane filtration. The overview of the extraction and purification process of bromelain is shown in Figure 3.

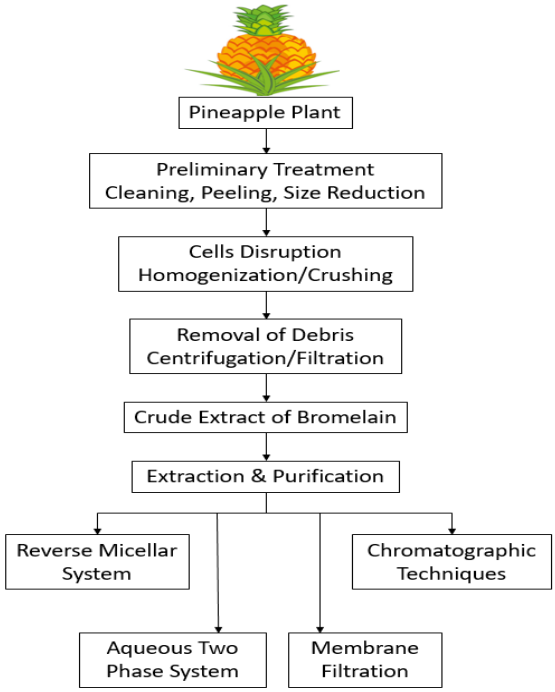

Fig 3 - Overview of extraction and purification process of bromelain [24]

5.1 Reverse Micellar System

A reverse micellar system (RMS) is an interesting and promising liquid-liquid extraction technique for the downstream processing of biomolecules [28, 29]. Micelle is an aggregate of molecules possessing both polar and non-polar regions. Reverse micelles are thermodynamically stable surfactant water droplets dispersed in organic solvents [30]. This system provides simple, easily scalable, energy efficient and mild separation conditions for enzyme recovery in active form. Only protein of interest will be entrapped in the core of the reverse micelle, whereas the impurities will remain in the organic phase. In most cases, electrostatic and hydrophobic interactions between protein and reverse micelles are considered the driving forces for the diffusion of solutes into the core of the reverse micelle [31]. RMS consists of 2 stages: forward extraction and back extraction. Forward extraction is the process that involves the diffusion of protein of interest from the aqueous phase into the reverse micelles in the organic phase, and the protein of interest is then diffused back into the new aqueous phase from the reverse micelles during back extraction. Figure 4 shows the overview of forwarding and back extraction of RMS. 2 types of surfactant can be used in forwarding extraction: cetytrimethylammonium bromide (CTAB) and sodium bis(ethylhexyl) sulfosuccinate (AOT). CTAB is a type of cationic surfactant, whereas AOT is an anionic surfactant [29]. During forward extraction, in the organic phase, AOT and CTOB needed isooctane as their solvent and co-solvent (n-butanol and n-hexanol) for CTOB. Due to CTAB forming small micelles, co-solvents are needed to help the recovery action resulting from the extraction process [32]. AOT prefers forward extraction at the pH lower than the protein's isoelectric point. It is reported that AOT formed a complex with bromelain, and white precipitates were observed at the aqueous-organic interphase at a pH lower than 4.2 [33]. Hence, AOT is not suggested to act as a surfactant in forwarding extraction. CTAB, on the other hand, works better in the forward extraction of fruit bromelain since it has relatively low pI (4.6) with a wide range of pH stability above pI.

In a study of comparison between AOT and CTAB surfactants in forwarding extraction of bromelain by Hemavathi et al., (2007) CTAB was found to be the most suitable for the extraction of fruit bromelain concerning activity recovery of 97.56% and 4.54 fold of degree of purification when employed as a 150mmolL^{-1} CTAB/isooctane/5% (v/v) hexanol/15% (v/v) butanol system [28]. RMS was applied to extract bromelain from pineapple wastes such as core, crown, peel and extended stem. Bromelain extracted from pineapple core with a fairly good activity recovery, 106% and 5.2 fold of purification was obtained using CTAB. Pineapple peel, extended stem and crown yielded purification folds of 2.1, 3.5 and 1.7, respectively, with RMS extraction by a cationic surfactant (CTAB) [29]. Several modifications in RMS have been studied to improve protein yield and purification fold. The affinity-based reverse micellar extraction and separation technique to extract bromelain from pineapple wastes yielded an activity recovery of 185.6% with 12.32-fold purification [15]. Ultrafiltration coupled with RMS to upgrade the efficacy of RMS resulted in a purification fold of 8.9 and activity recovery of 95.8% for bromelain [34, 35].

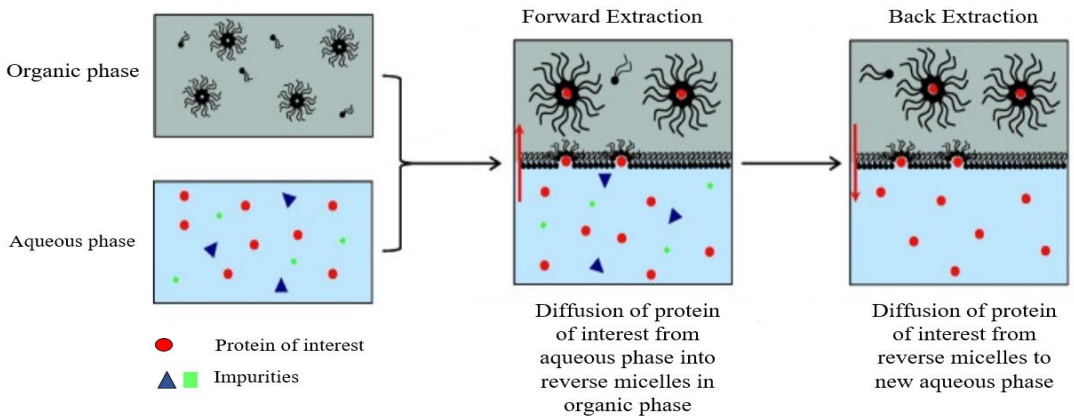

Fig 4 - Overview of the reverse micellar system [29]

5.2 Aqueous Two-Phase System

An aqueous two-phase system (ATPS) is a liquid-liquid extraction method. It is based on incompatible and immiscible two aqueous solutions. ATPS provides advantages such as low operational cost, highly selective, scalable, non-toxic, reusable polymers and can withstand high biomass load [36, 37, 38, 39]. The most common biphasic system is formed by two polymers (polymer/polymer) or a polymer with a salt (polymer/salt). Other types include alcohol with salt (alcohol/salt) and polymer with ionic liquid (polymer/ionic liquid) [40, 41, 42]. In the polymer/polymer system, the common material used is polyethylene glycol (PEG) and dextran. In the polymer/salt system, PEG was normally paired with either phosphate, sulfate or citrate salt. An illustration of ATPS is shown in Figure 5. Water was utilized as both phases' main component or solvent because it can form a gentle environment for biomolecules to separate and stabilize polymers' structure and biological activities [43]. The phase-forming compounds must be solubilized above a critical concentration in an aqueous solution to form 2 separate phases. In polymer/salt of ATPS, salts contain ions of different hydrophobicity, and the hydrophobic ions force the portioning of counter ions to phase with higher hydrophobicity and vice versa. The salting out effect moves the biomolecules (protein of interest) from the salt-rich phase to the polymer-rich phase [44]. High recovery of enzymes can be achieved with ATPS due to the presence of polymer, especially PEG, which caused the alteration in the structure of active sites of the enzymes [45]. ATPS had been applied to extract and purify bromelain of crude extract of pineapple fruit, stem and its wastes.

A study investigated the optimum PEG and magnesium sulfate salt (MgSO4) concentration for bromelain extraction from pineapple peels. It had been determined that 18% PEG with a molecular weight of 6000 / 17% MgSO4 is the optimum concentration that resulted in a high purification fold (3.44) and activity recovery (206%) [45]. ATPS of PEG 1500/ potassium phosphate was employed to extract and purify fruit bromelain. The research results show that 18% PEG 1500 with 14% potassium phosphate yielded 228% activity recovery and 4-fold purity for fruit bromelain extraction [46]. Another research composed of 14% PEG 1500/ 17.66 % potassium phosphate ATPS resulted in 89.65% fruit bromelain activity recovery and 2.8-fold purification [47]. A study by Coelho et al., (2012) focused on stem bromelain extraction and purification with ATPS of polymer/salt method. Using 10.86% PEG 4000 and 36.21% saturated ammonium sulphate yielded stem bromelain with 11.8-fold purification and 66.38% activity recovery [48].

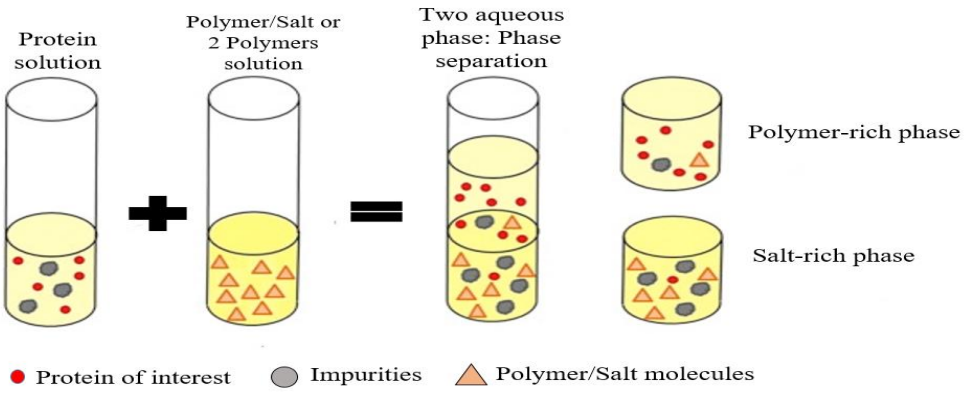

Fig 5 - Illustration of the aqueous two-phase system [43]

5.3 Chromatography Techniques

Chromatography is a separation technique to extract and purify the desired biomolecules. The biomolecules mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture travel at different speeds, causing them to separate [49]. The biomolecules' separation speed is affected by their molecular characteristics related to adsorption, partition and affinity among their molecular weights. The purpose of applying chromatography is to achieve a satisfactory separation within a suitable time interval and used as a quantitative analysis method [50]. Chromatography is a relatively simple method where precise separation and purification are possible, a low sample volume is required, and it works on a wide range of samples, including drugs, tissue extracts and food particles. Chromatography extraction and purification techniques were used to extract bromelain from pineapples. These techniques include ion exchange chromatography, gel filtration chromatography, affinity chromatography and high-speed counter-current chromatography [34].

Two liquid chromatography steps for purified stem bromelains are ion exchange chromatography and gel filtration chromatography. In the first step of the liquid chromatography ion exchange method, a glass column was packed with caboxymethyl-celullose as a stationary phase. The crude extract of pineapple was allowed to move along the carboxymethyl-cellulose resin. Then the experiment proceeded with gel filtration chromatography. Aliquots from ion exchange chromatography were collected and submitted to a glass column filled with Sephadex G-50®. High recovery of enzymatic activity (89%) and a purification factor of 16.93 were obtained from the two steps of liquid chromatography [51]. High-speed counter-current chromatography (HSCCC) is a liquid-liquid extraction technique based on hydrodynamic equilibration of the two-phase solvent system in the separation column. It is also recognized as a hybrid technique of liquid-liquid counter current distribution and liquid chromatography [52]. In a study by Yin et al., (2013), HSCCC was coupled with the RMS consisting of 0.10 g/mL CTAB with isooctane and hexanol as solvent and co-solvent, respectively. This study recovered a total of 3.01 g of fruit bromelain from 5.00 g of crude pineapple extract in 200-minute run [53]. The immobilized metal affinity membrane (IMAM) was used to separate and purify the mixture of bromelain and polyphenol oxidase. A microfiltration nylon membrane obtained activated membranes for covalent immobilization of hydroxyethyl cellulose (HEC) with formaldehyde and zinc ions loaded on the membranes. IMAM method successfully yielded 94.6 % bromelain activity recovery with 15.4-fold purification [54].

5.4 Membrane Filtration

Membrane filtration uses semi-permeable membranes to separate or purify the desired molecules based on size differences [34]. The desired molecules will be diffused through the semi-permeable membrane. There are a few types of membrane filtration: microfiltration, ultrafiltration, nanofiltration and reverse osmosis. Figure 6 below illustrates the membrane filtration process. This purification method had been utilized to extract and purify bromelain from crude pineapple extract. Membrane-based technology offers an alternative to producing high-quality purified bromelain in a more efficient and sustainable process [55]. Membrane filtration of bromelain was applied either with two-step filtration or a combination of membrane-based filtration with other purification techniques.

Simultaneous use of microfiltration and ultrafiltration to separate bromelain from pineapple pulp yielded 85% activity recovery through microfiltration, and 10-fold purification was obtained by ultrafiltration [56]. Two-step ultrafiltration with ceramic membrane was used to purify bromelain from pineapple's crude waste mixture. The ultrafiltration was performed with 75 kDa and 10 kDa tubular ceramic membranes. In the first stage of ultrafiltration, 96.8% of enzyme recovery was achieved, and the purity of bromelain increased up to 2.5 fold in the second stage of ultrafiltration [57]. The effect of diafiltration on two-stage ultrafiltration of bromelain from pineapple crude waste mixture (crown. peel, and core) was investigated. The purpose of diafiltration was to dilute the bromelain in a diluent followed by concentrating. 75 kDa and 10 kDa of tubular zirconium oxide were used as the membrane in the ultrafiltration set-up. The diafiltration was introduced during the second stage of ultrafiltration. This combination of methods resulted in 46 % of bromelain recovery, and the purity increased to 4.4-fold [58]. An integrated approach by coupling RMS with ultrafiltration was studied to improve fruit bromelain extraction and purification. The RMS of cationic surfactant 150 mM CTAB/80 % isooctane/ 5% n-hexanol/ 15% n-butanol (v/v) used for bromelain extraction resulted in an activity recovery of 95.8% and purification of 5.9-fold. The purification of fruit bromelain increased to 8.9-fold after ultrafiltration with cellulose acetate membrane [35].

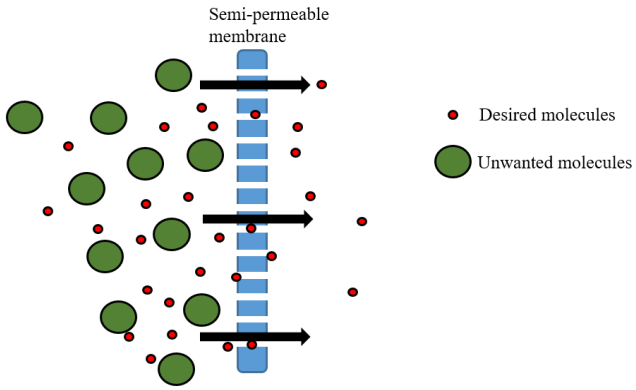

Fig 6 - Illustration of membrane filtration

6. Injury-Induced Wound and Wound Healing Properties

Injury to the skin provides a unique challenge as wound healing is a complex process. This process's main objective or goal is to completely restore the skin structure and functions. The wound healing process consists of three interrelated phases: hemostasis and inflammation, proliferation and tissue remodelling. The first stage, hemostasis and inflammation, occurs soon after the skin is damaged or injured. Hemostasis involves coagulation which causes bleeding to stop and clotting of blood. Fibrinogen, one of the major components of the skin's connective tissues, leads to coagulation of exudates and, together with the formation of a fibrin network, produces a clot in the wound, which stops the bleeding [59]. Inflammation takes place simultaneously with hemostasis. Inflammation is also called a cleansing phase, where some inflammatory cells involved in wound cleansing and defending infections enter the wound medium and penetrate inside the dead cells [60, 61].

Proliferation is the second stage of the wound healing process. Proliferation aims to provide fast vascular regrowth and closure to the wound by the generation of new tissue. Granulation tissues are formed and comprise a type III collagen network that acts as a quick fix to close the wound and prevent infection [62]. This granulation tissue is subjected to regression and is gradually replaced by stronger, long-strand type I collagen in the form of scar tissue after its function is fulfilled. The final stage would be the remodelling of tissue. At this stage, fibroblasts completely cover the surface of the wound as a new layer of the skin, and there is no evidence of the wound.

Nutrient deficiencies can impede wound healing, and several nutritional factors required for wound healing may improve healing time and wound outcome [63]. Nutrients such as Vitamin A [64], Vitamin C [65], Vitamin E [66], zinc [67], protein [68, 69], bromelain [64] and many more are essential in wound healing process.

7. Wound Healing Application of Bromelain

Over the years, various research and studies have been done to investigate the potential of bromelain as an alternative enzyme in medicinal use and as nutritional support in wound treatment. In terms of wound healing application, bromelain has proved itself as a useful proteolytic enzyme as it exhibits anti-inflammatory, fibrinolytic and debridement activities. These will be discussed in further detail in the following section.

7.1 Anti-Inflammatory Agent

Inflammation is defined as the body's attempt at self-protection or the immune system's response to harmful stimuli, such as pathogens, damaged cells, toxic compounds, or irradiation [70], and acts by removing harmful stimuli and initiating the healing process [71]. Generally, there are 2 types of inflammation: acute inflammation and chronic inflammation. Acute inflammation is a short-term process which starts rapidly in response to tissue injury or damage due to trauma, microbial invasion or noxious compounds [72]. Chronic inflammation refers to a prolonged inflammation that involves a progressive change in the type of cells present at the site of inflammation [73]. The prolonged inflammatory response may result in deregulated differentiation and activation of keratinocytes, hindering progress through normal stages of wound healing [74]. Wound healing disorders present as hypertrophic scars or non-healing chronic wounds, also known as ulcers. Ulcers are considered the most prevalent wound healing problem in humans. Most non-healing wounds fail to progress through the normal phases of wound repair and remain in a chronic inflammatory state (prolonged inflammatory response). Thus, the transition from acute to chronic inflammation must be avoided for the injury site to be treated in normal stages of wound healing without causing the wound to become more severe and lead to ulcers.

Bromelain is had been clinically used as an anti-inflammatory agent in soft tissue injuries, chronic pain and surgical wound care. The effectiveness of bromelain is studied in a clinical trial with a group of 60 patients that undergo surgery for fixation of long bone fractures. 30 of them were treated with 90 mg of bromelain/tablet whereas

the other 30 patients were treated with standard anti-inflammatory drugs. The volume of the operated limb was measured, and the starting volume value on the 1st post-operative day was 100%. On the 14th post-operative day, the average volume of the operated limb for the patients treated with bromelain tablet reduced by 12% compared to the other 30 patients with only a 9% reduction in average volume. A significant reduction in pain and swelling with accelerated healing was observed in the bromelain-treated patients [75]. Rhinoplasty is a surgery that changes the shape of the nose for appearance changes or breath, or both purposes. In a random and placebo-controlled study, it has been proved that orally administered bromelain minimized edema, pain and swelling after the surgery [76]. In a clinical trial on patients undergoing cataract surgery, it was demonstrated that bromelain was orally administered 2 days prior to surgery and 5 days post-operatively, resulting in significant inflammation and pain reduction [77]. The anti-inflammatory activity of bromelain is closely related to the Kinin system. Bromelain reduces High Molecular Weight Kinin (plasma kininogen), thus inhibiting the production of bradykinin, an agent that induces inflammation, pain and swelling [78]. Administration of bromelain before surgery can reduce the average days for the complete disappearance of pain and post-surgery inflammation [79]. Nowadays, bromelain is used to treat post-surgical wounds and help lessen the pain and swelling.

7.2 Role of Fibrinolysis

Fibrinolysis is a process to prevent fibrin clots from growing and allows the body to clear fragments of clots safely. Fibrinolysis occurs by converting plasminogen to plasmin to degrade fibrin into soluble fibrin degradation products (FDP). Plasminogen is activated by either two primary serine proteases, which are tissue plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA) [80, 81]. tPA is synthesized and released by endothelial cells, whereas uPA is produced by monocytes, macrophages and urinary epithelium. Due to high concentrations of specific inhibitors such as plasminogen activator inhibitor 1 (PAI-1), both tPA and uPA have short half-lives in circulation, about 4-8 minutes. Since tPA and plasminogen bind at the same binding site of fibrin (ogen), the zymogen and its activator are brought into close proximity, resulting in efficient local generation of plasmin [82]. The uPA, on the other hand, has a lower affinity for plasminogen in which it does not require fibrin as a cofactor, and under normal conditions, uPA appears to act mainly in extravascular locations. Circulating serine protease inhibitors or serpins neutralize plasminogen and plasmin activators in excess concentration [83]. Serpins that are important in fibrinolysis are plasminogen activator inhibitor-1, plasminogen activator inhibitor-2 (PAI-2) and α2-antiplasmin (A2AP) [84, 85, 86]. PAI-1 released into the circulation from endothelial cells, platelets, and other cells rapidly inhibit tPA and uPA. Plasmin and A2AP bind with a stoichiometry of 1:1, at which point both become inactive. Plasmin is protected from A2AP inhibition when bound to fibrin, allowing fibrinolysis to proceed [87]. Fibrinolytic activities are inhibited by thrombin activated inhibitor (TAFI), a non-serpin inhibitor activated by thrombomodulin-associated thrombin. TAFI removes C-terminal lysine and arginine residues on fibrin, thus decreasing the number of available plasminogen binding sites, slowing down the plasmin generation and stabilizing the clots [88, 89]. Figure 7 shows the illustration of the fibrinolysis process.

Bromelain is an effective fibrinolytic agent and prevents blood from coagulation. It influences blood coagulation by exaggerating the transformation of plasminogen to plasmin, inhibiting fibrin synthesis, a protein involved in blood clotting [34, 90]. In vitro and in vivo studies have suggested that bromelain is an effective fibrinolytic agent as it stimulates the conversion of plasminogen to plasmin, resulting in increased fibrinolysis by degrading fibrin [91]. Bromelain increased the fibrinolytic activity in a dose-dependent manner in a study on an inflammatory animal model [92]. It is found that bromelain prolonged prothrombin and partial thromboplastin time and decreased the adenosine phosphate (ADP) induced platelet aggregation in a dose-dependent manner [93]. The fibrinolytic activity of bromelain has been attributed to enhancing the conversion of plasminogen to plasmin, which degrade the fibrin and limits the spread of the clotting process.

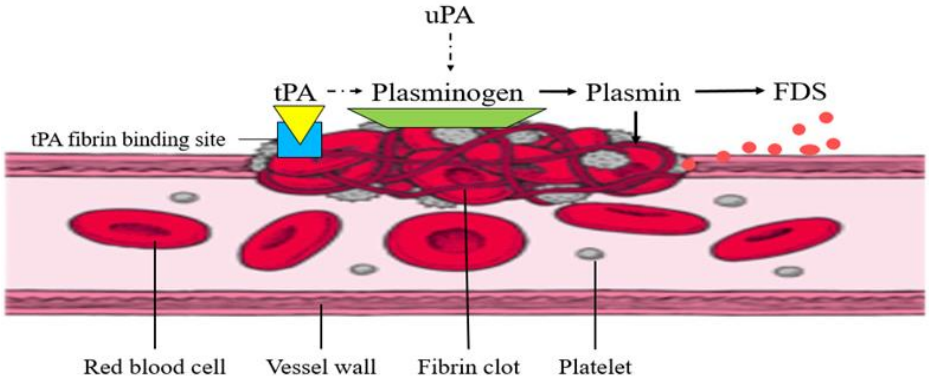

Fig 7 - Illustration of fibrinolysis process [89]

7.3 Debridement Agent

Debridement is the procedure involved in removing devitalized tissue such as necrotic tissue. Debridement is considered a major component of wound management to prepare the wound bed for reepithelization [94]. The purpose of debridement is to transform a chronic wound into an acute wound and initiate the healing process. Devitalized tissue, such as necrotic tissue, serves as a source of nutrients for bacteria that can cause infection in the wound, making the wound more severe [95]. Devitalized tissue also acts as a barrier for reepithelization, preventing applied topical compounds from directly contacting the wound bed to provide their beneficial properties [96]. Several types of debridement can be applied for the removal of devitalized tissue. These include autolytic debridement, biological debridement, mechanical debridement, surgical debridement and enzymatic debridement.

Mechanical debridement is a non-selective type of debridement, which means that it will remove both devitalized tissue and debris as well as viable tissue. This method involved mechanical force such as wet-to-dry dressing, wound irrigation, and wound scrubbing [97]. The wet-to-dry dressing and wound scrubbing require using a sponge, brush or gauze to remove devitalized tissues from the wound bed. Mechanical debridement will have a higher risk for bleeding and peri-procedural pain. Mechanical debridement is applied on acute and chronic wounds with moderate to large amounts of necrotic tissue, regardless of the presence of an active infection.

Surgical debridement uses a sharp instrument such as a scalpel and curettes to remove devitalized tissue on the wound. It is the most rapid and effective method but is considered the most aggressive method [98]. Surgical debridement can be performed at the bedside, wound care centre or operating room depending on the adequacy of anaesthesia and the ability to control perioperative complications. This debridement should be done by skilled, trained, qualified and licensed healthcare professionals. Surgical debridement is similar to mechanical debridement, where it will have a higher risk of bleeding and possible general complication from the anaesthesia [99].

Autolytic debridement is the lysis or breakdown of damaged tissue at a wound site by the body's natural defence system, in which endogenous phagocytic cells and proteolytic enzymes digest specific components of body tissues or cells [100]. This type of debridement requires a moist environment and a functional immune system. Therefore, moisture retentive dressings such as hydrocolloids, hydrogels, alginates and transparent films are encouraged to support the maintenance of moisture and provide optimal conditions for the body's natural enzymes to activate wound debridement [101]. Autolytic debridement induces softening of necrotic tissue and eventual separation from the wound bed. It will take a few days for the tissue to be removed. The effectiveness is mandated by the amount of devitalized tissue removed and the actual wound size.

Biological debridement uses sterile larvae of *Lucilia sericata* (species of green bottle fly) or maggot therapy to remove the devitalized tissue. It is more suitable to be applied on the large wound where a painless removal of devitalized tissue is needed. The larvae or maggots release proteolytic enzymes that contain secretions and excretions that dissolve necrotic tissue from the wound bed [102]. A study has shown that complete debridement by free-range maggots therapy took 14 days, whereas autolytic hydrogel debridement took 72 days to complete debridement.

Enzymatic debridement is described as a selective method of removing devitalized tissue using exogenous proteolytic enzyme, fibrinolytic enzyme and collagenase [97]. Enzymatic debridement provides faster than autolytic debridement, but slower when compared to mechanical and surgical methods [103]. Nowadays, many enzymes are commercially available and being promoted as an alternative to surgical methods, and bromelain is one of them and is the most employed [6, 104]. Table 4 summarizes the advantages and disadvantages of the debridement method mentioned above.

Bromelain shows debridement properties as evidenced by the hydrolysis of devitalized wound tissue in studies in vitro and in vivo without apparent effects on the surrounding normal tissue [78]. Burns are characterized by forming an eschar, which is made up of burned and traumatized tissue. Eschar also serves as a medium for bacterial growth, resulting in infection to the injury site and the neighbouring undamaged tissues [56]. Burns are common injuries associated with significant morbidity and mortality, often leading to disfigurement and dysfunction due to scarring. Tropical bromelain (35% in a lipid base) has complete debridement on experimental burns on rats in 2 days, compared to collagenase, which required 10 days, with no side effects and damage to adjacent burned tissue [94]. Bromelain contains escharase, which is responsible for this tremendous effect. Escharase is non-proteolytic and has no hydrolytic enzyme activity against normal protein substrates or glycosaminoglycan substrates [91]. The efficacy of enzymatic debridement of deeply burned hands with bromelain-containing gel, Debrase® is being studied. Debrase® is in the form of lyophilized dry powder, can be activated by a hydrating vehicle gel or saline, applied and covered the wound with an occlusive dressing for 4 hours. A total of 69 patients with deep burned wound hand was being assessed and needed surgical procedure for skin grafting before enzymatic debridement. After application of Debrase®, only 36.2% (25 out of 69 patients) require skin grafting for the treated wounded area. Moreover, the actual burn area that required skin grafting was 1.0 ±0.7% total body surface area, a decrease of 40% from the area initially estimated (1.4 ± 0.8% total body surface area) as deep and requiring surgery. This study showed that the enzymatic debridement reduced the number of patients with burns who needed an operative procedure and skin grafting for the wounded area. [105]. In a study by Bavata et al., (2019), the efficiency of bromelain-loaded chitosan nanofibers for burn wound repair was investigated in an animal model. The burn healing effect of chitosan-2% w/v bromelain nanofiber was studied in the

induced burn wounds in rats for 21 days. The results showed that chitosan-2% w/v bromelain nanofiber was more efficient to heal burned skin than chitosan nanofiber alone in the animal model tested [106].

Table 4 - Summary of 5 debridement methods

Debridement method	Mechanism of action	Advantages	Disadvantages
Mechanical	Uses mechanical force such as wet-to-dry dressing, wound irrigation and wound scrubbing	Fast method	Painful High risk of bleeding Non-selective as it removed both devitalized and viable tissue
Surgical	Uses scalpel and curettes to devitalized tissue on the wound, can be done at the bedside, wound care centre or in the operating room	Fast Effective Selective	Aggressive method Painful High risk of bleeding High cost
Autolytic	Uses the body's enzymes and moisture to rehydrate, soften and remove devitalized tissue	Low risk of side effects	Slow process
Biological	Application of green bottle fly larvae and maggots to remove damaged tissue	Selective and fast methods Suitable for large wound	Not suitable for all patients Higher cost than autolytic debridement
Enzymatic	Removing devitalized tissue by using exogenous proteolytic enzyme, fibrinolytic enzyme and collagenase	Selective Commercially available Painless and minimal blood loss	High cost Slower than mechanical and surgical method

8. Kinin System and Clotting Cascade

The Kinin system is a plasma and tissue proteolytic system which consists of blood proteins that play a role in inflammation, blood pressure control, coagulation and pain [107]. The Kinin system can be said to be involved in the wound healing process. Blood coagulation is a process where a clot is formed to stop the bleeding at the injury area. The clotting process is broken into 2 stages: primary and secondary haemostasis [108, 109]. Haemostasis is defined as the arrest of bleeding, comes from Greek, haemo meaning blood and stasis meaning to stop [110]. The formation of a weak platelet plug is achieved in primary haemostasis. A platelet plug is formed to temporarily protect from haemorrhage until further stabilization of fibrinogen to fibrin via thrombin occurs in secondary hemostasis. Secondary hemostasis involves the clotting factors acting in a cascade to stabilize the weak platelet plug. Platelets alone are not enough to secure the damage in the vessel wall. A clot must be formed at the site of injury [111]. The formation of a clot depends upon several substances called clotting factors. Roman numerals I designate these factors through XIII, which activate each other, known as the clotting cascade. This cascade results in fibrinogen, a soluble plasma protein, cleaving into fibrin, a non-soluble plasma protein. The fibrin proteins aggregate to form a clot [107].

The clotting cascade is triggered through 2 major pathways: intrinsic and extrinsic. The intrinsic pathway is activated by trauma inside the vascular system and is activated by platelets, exposed endothelium, chemicals or collagen. Clotting factors that are involved in this pathway are Factors XII, XI, IX, VIII [111]. On the other hand, external trauma activates the extrinsic pathway that causes blood to escape from the vascular system. Factor VII is the clotting factor involved in the extrinsic pathway [112, 113]. Both pathways meet and finish the pathway of clot production in what is known as the common pathway. The common pathway involves factors I, II, V, and X. When a surface (wound) is contacted by collagen or platelets, the kinin system and clotting cascade will be activated by stimulating the conversion of Hageman factor to an active protease, Factor XIIa. The presence of Factor XIIa causes the conversion of plasma prekallikrein into kallilrein and continues the intrinsic path of the clotting cascade by converting Factor XI to its active form. In the autocatalytic loop, Kallikrein accelerates the activation of the Hageman factor, which continues to potently activate both the kinin system and clotting cascade. In addition, Kallikrein cleaves High Molecular Weight Kinin (HMWK) to produce bradykinin. Bradykinin is a mediator of inflammation, where it stimulates both pain and vascular permeability, causing them to increase. The clotting cascade will convert fibrinogen to fibrin, a protective matrix around the injury that inhibits tissue drainage, promotes edema, and blocks blood flow [8] as illustrated in Figure 8.

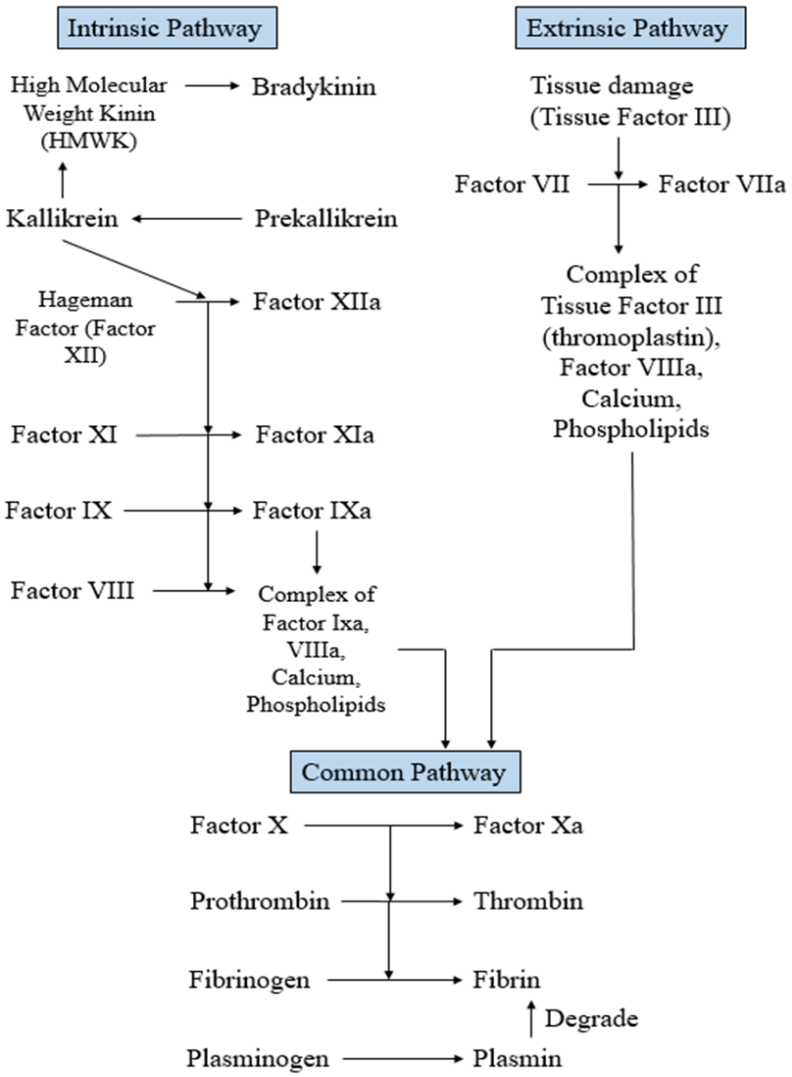

Fig 8 - Overview of Kinin system and clotting cascade [8]

8.1 Effect of Bromelain on Kinin System and Clotting Cascade

Many studies and research have been conducted to investigate the effect of bromelain on the kinin system, especially on plasma kallikrein, bradykinin levels and plasma exudation at the inflammatory site. Most of the studies were done on laboratory rats. 5 mg/kg and 7.5 mg/kg of bromelain caused the dose-dependent decrease of bradykinin levels at inflammatory sites and prekallikrein levels in sera [114]. With a single injection of 10 mg/kg of bromelain, the levels of HMWK and prekallikrein were markedly decreased in rat plasma [115]. Rats treated with bromelain show a reduction in Factor X and prothrombin, which are needed for the activation of fibrinogen to fibrin through a common pathway of the intrinsic and extrinsic cascade. These studies show that bromelain inhibits the generation of bradykinin at the inflammatory site via depletion of the plasma kallikrein system and limiting the formation of fibrin by reducing clotting cascade intermediates [21]. These activities result in a significant reduction in enema and pain and, at the same time, enhance the circulation to the injured site.

Vessel repair begins after a clot is formed, starting with the conversion of plasminogen to plasmin. The function of plasmin is to degrade the fibrin into smaller components which can be removed by monocytes and macrophages [116]. Bromelain had been shown to stimulate the conversion of plasminogen to plasmin, resulting in increased fibrinolysis in rats. Thus, this minimizes venos stasis, facilitates drainage, increases permeability and restores the tissue's biological continuity [21]. Table 5 below summarises bromelain's effect on the selected system and component.

Table 5 - Summary of bromelain's effect on the Kinin System and the clotting cascade

Mediator/Enzyme	Function/Action	Effect of bromelain
Bradykinin	Pain mediator and vascular leakage	Decrease
High Molecular Weight Kinin (HMWK)	Precursor of bradykinin	Decrease
Prekallikrein	Produce Kallikrein	Decrease
Factor X	Clotting factor	Decrease
Prothrombin	Clotting factor, the precursor to thrombin	Decrease
Plasminogen	Precursor to plasmin	Activate

9. Conclusion

Extensive research and studies have established bromelain's efficacy and efficiency in biomedical applications, most notably wound healing. Due to its low toxicity and lack of adverse effects on the body when consumed, it has become a common alternative phytotherapeutic enzyme among patients. Bromelain can also be extracted from pineapple wastes such as the crown, core, peel, and leaf. Researchers are modifying and improving extraction and purification methods to increase yield and purification fold and produce high-quality bromelain at a lower cost. Bromelain extracted from pineapples has been suggested to have a promising role and play a significant role in wound healing due to its anti-inflammatory, fibrinolytic, and debridement properties.

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