Bromelain Production: Current Trends and Perspective

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Abstract
Bromelain is a mixture of proteolytic enzymes derived from the stems and juice of pineapples. It has been extensively used in food industry; for meat tenderization, baking processes, and in prevention of browning of apple juice. Stem bromelain is a highly accepted phytotherapeutic agent. It has anti-tumor and anti-inflammatory effects, use in wound healing, as digestive aids, etc. This review aims at presenting a detailed account on the level of achievement on bromelain production from the conventional methods. Specifically, the isolation, purification and the biochemical properties of bromelain are discussed. The study also focused on the current trends and the progress made in the production of recombinant stem bromelain in *Escherichia coli*. This involved cloning of stem bromelain gene and its expression, optimization of lab-scale fermentation conditions for *E. coli* harboring recombinant bromelain as well as the enzyme thermal and storage stability measured using differential scanning calorimetry. It is hoped that this review would provide relevant information to contemporary researchers who are active in the field of bromelain and other related proteases.

Keywords: Bromelain, Cloning, *Escherichia coli*, Fermentation, Phytotherapeutic

1. Introduction

The numerous industrial and therapeutic applications of enzymes necessitated their production. Proteases are enzymes considered to be the most significant of all industrial enzymes with annual sale of about US$3 billion (Leary *et al*., 2009). In fact, they represent about 60% of all commercial enzymes worldwide. They are widely used in food, pharmaceutical and detergent industries (Feijoo-Siota and Villa, 2010). Plant proteases have been gaining unique attention in the field of biotechnology and medicine due to their exploitable properties. The most recognized plant proteases with greater commercial values are papain from *Carica papaya*, ficin from *Ficus spp*. and bromelain from pineapple plant (*Ananas comosus*) (Dubey *et al*., 2007).

Bromelain is one of the protease enzymes found in the pineapple plant (*Ananas comosus*). Stem bromelain (EC 3.4.22.32) is the major protease present in extracts of pineapple stem while fruit bromelain (EC 3.4.22.3) is the major enzyme fraction present in the juice of the pineapple fruit (Kelly, 1996). Some other minor cysteine endopeptidases (ananain, comosain) are also present in the pineapple stem bromelain (SBM). Although the fruit bromelain (FBM) was discovered much earlier than stem bromelain, the biochemical characterization of the latter enzyme has been described in more detail (Harrach *et al.*, 1998). SBM is widely used in industry and medicine, but FBM is not commercially available, even if it could be easily obtained from pineapple juice by simple ultrafiltration (Larocca *et al*., 2010). Similarly, SBM preparation contains a complex mixture of different thiol-endopeptidases and other partially characterized components such as phosphates, glucosidases, peroxidases, cellulases, glycoproteins and carbohydrates,

among others (Maurer, 2001). The entire extract of SBM has been shown to exhibit its activity over a wide pH range of 5.5 to 8.0 (Yoshioka et al., 1991).

Stem bromelain has abundant therapeutic, industrial and other applications. The Food and Drug Administration, USA, had categorized bromelain as a food additive and is among the substances that are generally accepted as safe (Food and Drug administration, 2006). It has been extensively used in food industry; for meat tenderization, baking processes, protein hydrolysate production, beer clarification, as food supplement and in prevention of browning of apple juice (Tochi *et al*., 2008). In addition, it is also used as active ingredient to provide gentle peeling effects in cosmetic industries (Aehle, 2007). Moreover, it has also been used in leather industries for skin pre-tanning, softening and bating (Walsh, 2002). In textile industries, bromelain is used for improving the dyeing properties of protein fibers, decomposing or partially solubilizing protein fiber from silk and wool (Koh *et a*l., 2006). The use of bromelain as hydrolyzing agent for the release of antimicrobial peptides of leather jacket's insoluble proteins had been reported (Salampessy *et al*., 2010).

Similarly, SBM is a highly accepted phytotherapeutic agent (Maurer, 2001). In fact, it had obtained universal acceptability as therapeutic drug. This is owing to its history of effectiveness and safety (Bhattacharyya, 2008). It was firstly introduced as a therapeutic compound in 1957 (Kelly, 1996). Bromelain is used for acute inflammation and sports injuries. It is not a licensed medical product, thus, it, is freely available to the general public in health food stores and pharmacies in the USA and Europe (Brien *et al*., 2004). Additional clinical applications of bromelain include modulation of tumour growth, third degree burns and improvement of antibiotic action. It is also used as a drug for the oral systemic treatment of inflammatory, blood-coagulation-related diseases and some malignant diseases (Maurer, 2001; Tochi *et al*., 2008). Furthermore, bromelain is involved in the reversible inhibition of platelet aggregation, sinusitis, bronchitis, angina pectoris, thrombophlebitis, surgical traumas, pyelonephritis and improved absorption of drugs especially antibiotics (Maurer, 2001). Bromelain has also been successfully used as digestive enzyme in many intestinal disorders. It has been shown that the enzyme serves as adequate replacement of pepsin and trypsin in case of deficiency. It also heals gastric ulcers in experimental animals (Orsini, 2006). Very recently, it was reported that bromelain ameliorated the wound microenvironment and improved the healing of firearm wound (Wu *et al*., 2012).

As a result of this wide range of applications of bromelain, there is a high demand of the enzyme. Thus, highly purified commercial bromelain is not cheap; costing up to 2,400 USD per kilogram (Ketnawa *et al*., 2012). This led to the development of various strategies in order to reduce the cost of stem bromelain production. This review is aimed at presenting a detailed account on the level of achievements made on stem bromelain production using the conventional methods. The extraction, purification and biochemical properties of bromelain are considered. It also focused on the current trends and the progress made so far in the production of recombinant stem bromelain in *E. coli*. It is expected that the review will be helpful to the researchers in this field and other related areas.

2. Extraction and purification strategies for bromelain

Heinecke and Gotner (1957) reported that bromelain concentration was very high in pineapple stems and hence leading to its extraction and utilization as phytomedicinal compound. Unlike the pineapple fruit which is normally used as food, the stems are waste by-product and thus, very cheap source of bromelain (Tochi *et al*., 2008). Apart from the stem and fruit, it had also been discovered that other parts of the pineapple plant contain bromelain. For instance, bromelain was extracted from the peel, core, stem and crown of wastes from two pineapple cultivars (Ketnawa et al., 2012). The highest protein contents and proteolytic activity were obtained from the extracts of the crowns while lowest values were recorded from the stem of both cultivars.

The commercially available product is most often made from stem bromelain, whereby the extract is removed from cooled pineapple juice through centrifugation, ultra filtration and lyophilization (Corzo *et al*., 2011). After the extraction processes, the crude extract containing the enzyme of interest is then subjected to various purification operations in order to remove contaminants that may interfere with the application of bromelain as well as to increase the specific activity of the enzyme. In the case of industrial enzymes, yield of the enzyme recovery rather than purity is the major concern. However, for specialty enzymes, purity is the main priority over the yield (Illanes, 2008). In addition, enzyme purification is vital for investigating the 3-D structure and the structure–function relationships of enzymes. Downstream processing may account up to 60–90% of the total enzyme production cost (Lightfoot, 1990), particularly for therapeutic and diagnostic enzymes. Thus, the purification strategies to be employed should be cost-effective, rapid, high-yielding and robust. Moreover, they need to have the potentials for continuous

product recovery, with a moderately high capacity and selectivity for the desired products (Gupta *et al*., 2004).

The conventional procedures employed for bromelain extraction and purification are often tedious, and frequently result in low yields of the enzyme. Thus, novel purification techniques are highly needed to enhance the overall enzyme yields and at the same time to lessen the number of steps involved in the downstream processing of bromelain.

Considering the various applications of bromelain, some novel purification strategies have been recently employed for the enzyme extraction and purification. These comprise of aqueous two phase systems (Babu *et al*., 2008; Ketnawa *et al*., 2010; Ketnawa *et al*., 2011a, Ketnawa *et al*., 2011b Ferreira *et al*., 2011; Rabelo *et al*., 2004), reversed micellar systems (Hebbar *et al*., 2008; Hebbar *et al*., 2012; Kumar *et al*., 2011) membrane processes (Doko *et al*., 2005) precipitation (Doko *et al*., 2005; Silvestre *et al*., 2012; Gautam, *et al*., 2010) and different chromatographic techniques (Gautam *et al*., 2010; Yin *et al*., 2011; Devakate *et al*., 2009). In this review, a brief description of some of these novel methods is discussed. The various strategies employed for extraction and purification of bromelain are summarized in Figure 1.

2.1 Isolation using reverse micellar system

Reverse micellar system (RMS) is a promising liquid-liquid extraction technique for downstream processing of biomolecules from dilute solutions. In addition, RMS has been greatly used for the enhancement of protein separations. It had been shown to provide outstanding conditions for protein separation. Some of the advantages obtained in using the RMS include higher sample loading capacity, simple operation and continuous preparation (Dong et al., 1999).

There are many reports from the literature on the use on RMS for bromelain extraction from pineapple plant. For instance, reverse micellar systems were employed for the extraction and purification of bromelain from crude aqueous extract of pineapple wastes (core, peel, crown and extended stem) (Hebbar *et al*., 2008). The pineapple core yielded highest bromelain activity recovery of 106 % and 5.2 purification-fold. The elevated enzyme recovery achieved might have been due to the modification of bromelain active site by the concanavalin A ligand used. In addition, RMS was reported to have been used for bromelain extraction from pineapple juice (Hemavathi *et al*., 2011). The extraction technique yielded 97.56% activity recovery and 4.54 purification fold for bromelain.

Besides, an attempt was made on the scale-up studies for phase transfer mode of reverse micellar extraction for the separation and purification of bromelain from pineapple waste (Hebbar *et al*., 2011). The scale-up process yielded purification fold of 2.43 with an activity recovery of 81.3%. Similarly, an integrated approach was used for coupling RMS with ultrafiltration to enhance the overall efficiency of extraction and purification of bromelain from aqueous extract of pineapple core (Hebbar *et al*., 2012). The RMS resulted in an activity recovery of 95.8 % and purification factor of 5.9-fold. In addition, the purification of bromelain was enhanced to 8.9-fold after ultrafiltration.

Equally, optimization of the extraction of bromelain from pineapple fruit by reversed micelles had been reported (Fileti *et al*., 2007). The process yielded maximum purification factor of 3 for the backward extraction. On the other hand, optimal points for the continuous extractor generated purification factor of 4.96. In addition to this, the batch and continuous extraction of bromelain from pineapple juice by reversed micelles under optimized conditions was also studied (Fileti *et al*., 2009). The study reported the same results as above.

In the same vein, a neural modeling for bromelain extraction from pineapple juice by reversed micelles was developed (Fileti *et al*., 2010). The results obtained indicated that purification factor of 4.96 and productivity of 1.29 ml/min were obtained at the optimal operating points. In the same way, a study was conducted on the use of affinity based reverse micellar extraction and separation technique to extract and purify bromelain from pineapple waste (Kumar *et al*., 2011). The results obtained yielded purification of 12.32 fold with an activity recovery of 185.6 %. The higher bromelain activity recovery might be due to the alteration of the enzyme active sites by the cationic surfactant used in the purification process.

2.2 Bromelain extraction using aqueous two phase system (ATPS)

The aqueous two-phase systems have been widely used in bioseparation. It is involved in the partitioning of proteins in aqueous two phase systems, depending on their physico-chemical properties. The great advantages of aqueous two phase extraction are based on volume reduction, rapid separations, high capacity, and mildness of the process. The technique can be used in the early purification stages and is relatively easier to scale-up. The ATPS technique is highly suitable for the extraction and purification of proteins that may prove difficult to purify with other existing techniques (Gupta *et al*., 1999). Many reports from the literature had also indicated the application of ATPS for bromelain separation and purification.

For instance, ATPS was employed for the separation and purification of mixture of bromelain and polyphenol oxidase from the pineapple (Babu *et al.*, 2008). The technique yielded about 228% activity recovery and 4.0-fold increase in purity for bromelain.

In addition, an attempt had been made on optimizing the extraction of bromelain from pineapple peel using ATPS (Ketnawa *et al*., 2010). The process generated higher enzyme activity recovery of 113.54% and purification fold of 2.23. In another study, the extraction of bromelain from pineapple peels using ATPS was investigated (Ketnawa *et al*., 2011a). The purification factor and activity recovery were found to be 3.44-fold and 206 %, respectively. Moreover, Ketnawa et al., (2011b) had extracted bromelain from pineapple peels using distilled water (DI), DI containing cysteine and ethylene diamine tetra acetic acid (EDTA) (DI-CE), sodium phosphate buffer pH 7.0 (PB) and PB containing cysteine and EDTA (PB-CE) as extractants. Results for the study had revealed that highest bromelain activity (1032 units) was obtained when it was extracted with PB-CE.

Response surface methodology had also been employed for optimizing bromelain extraction from pineapple fruit in ATPS (Navapara *et al*., 2011). The results of the model predicted a maximum enzyme yield of 90.33% with a purification factor of 2.4. In the same vein, Ferreira *et al*., (2011) had explored the use of thermodynamic equilibrium and applying of ATPS on the purification of bromelain extracted from pineapple. The purification folds obtained for the enzyme were within the range of 25-62 folds. In addition, a study had been carried out on thermoseparation of bromelain by poly(ethylene oxide) (PEO)–poly (propylene oxide) (PPO)–poly(ethylene oxide) (PEO) block copolymers aqueous solutions (Rabelo *et al*., 2004). The enzyme activity recovery attained was about 79.5%, with purification factor around 1.25. The bromelain activity recovery could be seen to be very high (> 100%) in some of the conditions studied above. This is most likely due to the structural modification of the enzyme active sites in the presence of PEG used. It may is also be due to removal of natural inhibitor(s) to the bromelain during downstream processing and/or activation of zymogen to mature bromelain enzyme.

2.3 Chromatography techniques

Chromatographic techniques have been widely and continuously developed for separation of bromelain from pineapple. Such techniques include ion exchange chromatography, affinity membrane chromatography, gel filtration chromatography and capillary electrochromatography (Babu *et al*., 2008;

Devakate *et a*l., 2009; Cheng and Huang, 2004; Chen *et al*., 2008). All of these methods have been used successfully to separate bromelain from pineapple. However, some of them are characterized with low separation efficiency and recovery, low sample loading capacity and may involve several separation steps (Yin *et a*l., 2011). Consequently, the development of new methods with high efficiency and large sample loading capacity is greatly needed for separation of bromelain from pineapple. For instance, two main basic glycosylated proteases were isolated from crude bromelain (Harrach *et al*., 1995; Rowan *et al*., 1988). Further purification of these protease by using different cation-exchange chromatography techniques produced a minor basic and non-glycosylated proteases referred to as ananain (Rowan *et al.*, 1988), or bromelain F9 (Harrach *et al*., 1995). In another study, fruit bromelain had been isolated using an acetone fraction obtained from pineapple fruit juice by cation-exchange and affinity chromatography (using Sepharose-Gly-Phe-glycinaldehyde semicarbazone as a resin) (Rowan *et al*., 1990).

Similarly, two forms of an acidic bromelain protease had been isolated and purified from crude extracts of pineapple stem (Harrach *et al*., 1998). This was achieved using a two-step FPLC purification technique. The basic main components were isolated by cation exchange chromatography while the breakthrough fraction was resolved further into 15 protein fractions by using anion exchange chromatography. However, only two of the fractions with acidic bromelain proteases were found to have proteolytic activity. In an attempt to develop newer methods of chromatography for better bromelain separation, fruit bromelain had been purified using high-speed counter-current chromatography (HSCCC) (Yin *et al*., 2011). The technique yielded about 3.01 g of bromelain from 5.00 g of the crude extract.

In the same way, adsorption of bromelain from an aqueous solution by Polyacrylacid (PAA)-bound iron oxide magnetic nanoparticles had been investigated (Cheng and Huang, 2004). The process generated about 87.4 % of bromelain activity. In another study, 7-mer peptides were covalently immobilized on novel nylon membranes as ligands for bromelain purification directly from pineapple waste (Cheng *et al*., 2008). Significant amount of the adsorbed bromelain (94.8%) was eluted from peptide–nylon membrane. Similarly, adsorption of bromelain in expanded bed conditions gave 13-fold purification factor while the total protein was reduced by 4 fold (Silveira *et al*., 2009).

In another study, immobilized metal affinity membrane (IMAM) was used for the separation and purification of mixture bromelain and polyphenol oxidase from the pineapple (Nie *et al*., 2008). Bromelain

purification produced a 15.4-fold purification factor with specific activity recovery of about 94.6%. In addition, a comparative study on the extraction, purification and evaluation of bromelain from stem and fruit of pineapple plant was carried out (Gautam *et al*., 2010). The purification of the enzyme preparations was achieved by the combinations of centrifugation, salt precipitation technique, dialysis and then ion-exchange chromatography. The results revealed that chromatographic technique had greatly maintained the structural integrity of the purified bromelain. The use of precipitation and chromatographic techniques for the purification of the bromelain from pineapple fruit yielded purification fold almost 3.3 times of that from precipitation (Devakate *et al*., 2009).

2.4 Membrane filtration and other techniques

The use of microfiltration and ultrafiltration as membrane technology, has been recently gaining popularity in bioseparation industries. The method is employed to separate the components of a solution based on molecular size differences. The technology is highly required for liquid foods separation, in juice processing, as well as protein isolation and concentrate production (Cassano *et al*., 2003). There are many reports on the use of membrane technology for bromelain extraction and purification. Doko *et al*., (2005) had employed the use of membrane filtration to extract and purify bromelain from pineapple. In their study, purified bromelain extracts were obtained using sequential batch membrane processing systems that involved microfiltration and ultrafiltration and then followed by ammonium sulfate extraction and ultracentrifugation. The results obtained showed 50 % recovery of the extracts yield with 98 % protein. Similarly, the extraction of bromelain from pineapple pulp by the combination of microfiltration and ultrafiltration had been studied by Lopes *et al*., (2009). About 85% of bromelain activity was recovered during microfiltration while ultrafiltration retained 100% of proteolytic activity and 10 fold concentrated bromelain extract. Moreover, 64.7% yield and 5.3 fold purification of stem bromelain were achieved by adsorption on nano-TiO_2 and ultrafiltration (Chao *et al.*, 2012). The results obtained indicated that the activity recovery yield was 64.7% with 5.3 fold purification. Additionally, Li and Li (2009) studied the extraction of bromelain from pineapple using ultrafiltration, kaolin adsorption and tannin precipitation methods. They concluded that ultrafiltration method yielded bromelain of higher proteolytic activities as compared to the other two methods.

A pilot scale bromelain extraction process from pineapple fruit core by using homogenization afforded protein content of 9.33 mg/ml with the proteolytic activity of 1400 GDU/ml (Loh *et al*., 2005). Besides, extraction and biochemical analysis of bromelain from different pineapple varieties had been carried out by Abilio *et al*., (2009). The results revealed that the crude extract of the imperial cultivar had the highest proteolytic activity and protein content in the pulp as well as in the rind. In another development, bromelain had been extracted from pineapple peel by using ethanol, isoelectric and ammonium sulfate precipitations (Silvestre *et al*., 2012). The ethanolic extract was found to have higher bromelain recovery yield as compared to the precipitation methods.

The recent purification strategies employed for bromelain are summarized in Table 1. It could be seen that the bromelain recovery yield ranged from 50 % to 228 %. On the other hand, the purification fold for bromelain ranged from 1.25 to 62 fold.

3. Biochemical properties of bromelain

The biochemical properties of bromelain have been studied comprehensively in order to enhance its various industrial and therapeutic applications. The various properties of bromelain (viz. stability, pH and temperature optima, molecular weight, immobilization, modification, effects of some chemicals) are summarized in Table 2, 3, 4 and 5. Conversely, concise accounts of some of the biochemical properties are discussed in the following section. Additionally, these biochemical properties are summarized in Figure 2.

3.1 Molecular weight, pH and temperature optima for bromelain

The commercial proteolytic enzyme preparations are evaluated according to their proteolytic activity, which should be measured within the optimal conditions of enzymatic reactions. Bromelain activity can be determined under optimal pH and temperature conditions, with various substrates including casein, gelatin and synthetic substrates. From the several studies conducted, SBM was found have molecular weight range (26-37), pH optimum range (6-7) and optimum temperature range of 50-60 º C (Harrach *et al*., 1998; Kumar *et al*., 2011; Suh *et al*., 1992; Xue *et a*l., 2010; Liang *et al*., 2011). On the other hand, FBM had been shown to have molecular weight range (24.5-32.5), pH optimum range (3-8) and optimum temperature range of 37-70 º C (Corzo *et al*., 2011; Ketnawa *et al.*, 2010, 2011a &2011b, Kumar *et al*., 2011; Lopes *et al*., 2009; Suh *et al.*, 1992; Jutamongkon and Charoenrein, 2010; Liang *et al.*, 2011).

The molecular weight, pH and temperature optima for bromelain are summarized in Table 2. It can be seen from the table that the molecular weight of bromelains ranged from 24.5-37 kDa, pH optima 3-9 while for temperature, the range is about 37-70 ºC.

3.2 Effects of some chemicals and structural modification of bromelain

There are numerous chemical methods that have been employed to modify the activity and stability of enzymes. These include cross-linking of the enzyme with chemicals, modification of the amino acid side-chains of enzymes, the introduction of hydrophilic and hydrophobic groups, etc. (Xue et al., 2010). Chemical modification of proteins is extensively used as a device for studying localization of individual amino acids, their contribution in the maintenance of the native conformation and stabilization of protein. The influence of salts and alcohols on the conformation of partially folded intermediate of stem bromelain at low pH had been investigated (Haq *et al*., 2005). The results of indicated that the alcohol-induced state showed a cooperative thermal transition while the salt-induced state exhibited non-cooperative thermal denaturation of bromelain. In another development, Gupta *et al*., (2003b) discovered that binding of antibromelain monomeric FabV improved the stability of stem bromelain against inactivation. The results showed that complexing of bromelain with the FabV resulted in significant stabilization of bromelain against thermal inactivation and alkaline pH.

Bhattacharya and Bhattacharya (2009a) studied the resistance of bromelain to SDS binding. The results obtained indicated that SDS acted as a partial non-competitive inhibitor to bromelain. Hence bromelain is resistant to SDS binding and denaturation which is common for β-sheet rich kinetically stable proteins. Besides, induction of 'molten globule' like state in acid denatured state of unmodified preparation of stem bromelain had been studied (Gupta *et al*., 2003b). Results of the study indicated significant accumulation of secondary structure when the acid-denatured bromelain is subjected to 70% (v/v) trifluoro ethanol (TFE). It was concluded that there was the existence of a molten globule state in acid-denatured bromelain between 60 and 70% (v/v) TFE. Moreover, Rasheed *et al*., (2003) explored the denaturation of both glycosylated and deglycosylated stem bromelain by using guanidine hydrochloride. The deglycosylated bromelain was found to be more susceptible towards guanidine hydrochloride denaturation. In the same vein, an investigation was conducted on the effect of different size of poly (ethylene glycol) on the structure of the acid unfolded state of unmodified stem bromelain (Ahmad *et al*., 2006). It was conclude that PEG-400–

induced state has characteristics of molten globule, and higher molecular weight PEGs led to the unfolding of the tertiary structure of bromelain. Furthermore, Ahmad and Khan (2003) had conducted a study on the acid unfolded and molten globule states of stem bromelain. The results obtained indicated that bromelain at pH 2.0 is maximally unfolded and characterized by significant loss of secondary structure (~80%) and almost complete loss of tertiary contacts. However, on further decreasing the pH to 0.8, a molten globule state was observed, which is characterized with secondary structure content identical to that of native protein but without tertiary structure.

Similarly, the specific molten globule conformation of stem bromelain at alkaline pH had been studied (Dave *et al*., 2010a). There was an irreversible loss of secondary and tertiary structure above pH 10. The study had established the existence of a distinct conformational rearrangement in SBM, at the protein's isoelectric point. Moreover, use of hexafluoroisopropanol (HFI) to induce the helix–sheet transition of stem bromelain was studied (Dave et al., 2010b). The results showed that treatment of bromelain with HFI (10–30%) induced the partially folded intermediate to adopt much of the native protein's secondary structure. Moreover, addition of slightly higher concentrations of HFI caused transformation from an α-helix to a β-sheet and induced formation of a compact non native structure. In the same vein, use of TFE to stabilize the molten globule state and induce non-amyloidic turbidity in stem bromelain near its isoelectric point was considered (Dave *et al*., 2011). The results obtained showed that the protein retained its native secondary structure at TFE concentrations of up to 30%. However, at slightly higher TFE concentrations (≥40%), a 2.5-fold induction of helical feature and a time-dependent increase in non-amyloidic turbidity were observed.

Similarly, investigation had been conducted on the chemical modification of lysine residues in stem bromelain using pyromellitic anhydride acid and poly(maleic anhydride) (Xue *et al*., 2010). The results showed that almost 60% and 57% of the residues in bromelain were found to be modified by pyromellitic anhydride and poly(maleic anhydride), respectively. Moreover, the modified bromelain had improved thermal stability and the resistance to alkali and the surfactant. In the same vein, a study was carried out on the effects of dynamic high-pressure microfluidization treatment on activity, stability and conformation of stem bromelain (Zhaoqin *et al*., 2010). The results indicated that bromelain activity was inhibited by the treatment although the residual activity did not decrease with increasing pressure. Equally, the secondary or

tertiary structure of bromelain was also disturbed. More research in this field is needed in order to enhance the stability of bromelain.

The effects of the chemicals and covalent modification of bromelain are summarized in Table 3. It can be observed that some of the chemicals decrease the activity of bromelain while others enhance its activity. In addition to this, covalent chemical modifications of bromelain could lead to its partial denaturation. On the other hand, it could improve the stability of bromelain to heat, in different pH conditions, or increase its shelf-life.

3.3 Immobilization of bromelain

Several applications of proteases necessitated the immobilization of the enzymes on different media. This is because immobilized enzymes are more resistant to denaturation, more easily manipulated and can be reused. Above all, the enzymatic by-products are not contaminated by these enzymes (Feijoo-Siota and Villa, 2010). The performance of immobilized enzymes can be improved by uniformly orienting them favorably on solid supports. Many strategies are being employed for this purpose including the use of monoclonal antibody supports, binding or coupling through lone ligands, among others. There are numerous reports on immobilization of bromelain. A study was undertaken in which bromelain was covalently immobilized onto the surface of porous chitosan beads with and without alkyl chain spacers of different lengths (Seo *et al*., 1998). The findings indicated that the pH, thermal, and storage stabilities of the immobilized bromelain were higher than those of the free bromelain. In the same vein, research was carried out on immobilization of bromelain onto porous copoly(c-methyl-L glutamate/L-leucine) beads (Yodoya *et al*., 2003). The results of the study indicated that the immobilized bromelain was found to have higher thermal stability, apparent K_m and lower V_{max} values as compared to free bromelain.

Gupta and Saleemuddin (2006) undertook a study on the bioaffinity based oriented immobilization of stem bromelain. They discovered that the immobilized bromelain was more resistant to thermal inactivation and exhibited a broader pH activity as compared to the native form. Similarly, another work was done on the oriented immobilization of stem bromelain via the lone histidine on a metal affinity support (Gupta *et al*., 2007). The results obtained indicated that immobilized bromelain was more resistant to thermal inactivation and exhibited a broader pH activity profile in acidic range, as compared to the native enzyme. In addition, the use of calcined layered double hydroxide (LDO) as the novel biomolecular vessel for the

immobilization, storage and release of bromelain had been studied (Shi *et al*., 2007). It was concluded that there were significant improvements on the stability of immobilized bromelain on heat and storage time as compared with those of free bromelain.

Besides, Mahmood and Saleemuddin (2007) had investigated the stabilization of stem bromelain coupled to a thermosensitive polymer by uniform orientation and using polyclonal antibodies. Their findings indicated that the polymer-coupled preparations of bromelain exhibited broader pH-activity profiles while the optimum temperature of the oriented preparation also rose to 70 ºC. At the same time, the binding of antibromelain antibodies improved the resistance to inactivation of the polymer-coupled preparations. Moreover, Rong (2008) had studied the immobilization of bromelain on attapulgite modified by 3-aminopropyltriethoxysilane. The results obtained showed that the stability and utilization rate of the immobilized bromelain were significantly enhanced. Furthermore, the effects of cross-linking stem bromelain with glutaraldehyde were investigated (Anwar *et al*., 2007). It was concluded that the cross-linked bromelain preparations were found to be more stable against urea, guanidine hydrochloride and temperature-induced inactivation. They also exhibited slightly better storage stability as compared to the unmodified bromelain.

In another development, a study was conducted on the isotherm, kinetic and thermodynamic analysis of bromelain adsorption on reactive blue 4 immobilized composite membranes (Su *et al*., 2009). The results indicated that Freundlich isotherm was able to effectively describe the adsorption of bromelain on dye affinity membranes. In addition, the pseudo-first-order kinetic model described the bromelain adsorption process with a good fitting. In addition, the immobilization of bromelain embedded in sodium alginate had been explored (Zhang *et al*., 2009). The results obtained indicated that the optimum pH of the immobilized enzyme was shifted from 6.8 to 7.6 while the optimum reaction temperature for bromelain was 50 ºC. Similarly, a study was done on the affinity adsorption of bromelain on reactive red 120 immobilized magnetic composite particles (Song *et al*., 2011). It was concluded that the affinity super paramagnetic particles were suitable for bromelain adsorption. Likewise, Tan et al., (2008) investigated the effects of linoleic-acid modified carboxymethyl chitosan on bromelain immobilization onto self-assembled nanoparticles. The results obtained indicated that the stability of bromelain for heat and storage was enhanced after immobilization on nanoparticles.

The various immobilization strategies employed and their effects on bromelain activity are presented in Table 4. It could be concluded that immobilization greatly enhanced the stability of bromelain on high temperature, extreme pH conditions. It also significantly increases the shelf life of the immobilized bromelain and its affinity to substrates.

3.4 Drying and Stabilization of bromelain

The main limitation for the industrial, therapeutic and analytical applications of enzymes is their liability to various forms of inactivation (Gupta *et al*., 2003b). This stimulated research towards the development of strategies for the enhancement of enzymes stability. As a result of this, several studies have been carried out on the stability of bromelain.

After the purification of enzymes to the desired level, the preparations have to be formulated in accordance to their intended applications. Spray drying and Freeze-drying are the most widely used techniques in preparing bromelain powder. The freeze drying of bromelain preparations had been investigated (Doko *et al*., 2005). A low-moisture freeze-dried and light-colored bromelain extracts of 50% yield recovery was generated. The bromelain proteolytic activity was almost 100% recovered, at completion of the process. In the same vein, the freeze drying and spray drying of bromelain had been reported (Devakate *et al*., 2009). The bromelain yield obtained were 50–70% and 96%, for spray drying and freeze drying, respectively. Similarly, investigations were carried out on the effects of outlet temperature and spray pressure of spray-drying on the activity yield and water content of the bromelain juice (Cao *et al*., 2007). The results obtained under optimized conditions generated bromelain product with activity yield and water content of 47.1 % and 5.83 % respectively. Correspondingly, Cabral *et al*., (2009) investigated the effects of drying parameters on the retention of the enzymatic activity and on the physical properties of spray-dried pineapple stem extract. They concluded that high processing temperatures yielded a bromelain product with a smaller moisture content, particle size, and lower agglomerating tendency. Moreover, a product with insignificant losses of the proteolytic activity (≈ 10%) and low moisture content (less than 6.5%) was produced at selected spray drying conditions.

In addition to this, the proteinase activity and stability of natural bromelain preparations had been studied (Hale *et al*., 2005a). The stem bromelain had better proteinase stability followed by fruit bromelain and then ananain. Moreover, the proteolytic activity of concentrated bromelain solutions was found to remain

relatively stable for at least 1 week at room temperature. Moreover, a study was conducted on the stability of bromelain in pineapple juice that was complexed with polyphenol compounds from fresh apple juice (Abdulmajid *et al*., 2009). It was concluded that the bromelain-polyphenol complex enhanced the bromelain thermal stability and the shelf life of juice when stored at lower temperature.

Similarly, Bhattacharya and Bhattacharya (2009b) investigated the preservation of natural stability of fruit bromelain. The authors reported that storage of fresh pineapple fruit without preservatives at -4 ºC led to retention of about 75 % proteolytic activity after 180 days and there was no microbial contamination. Moreover, investigation was done on the effects of $Ca^{2}+$ on thermostability and secondary structure of bromelain (Wang *et al*., 2009). The results showed that calcium ions had evidently promoted the bromelain activity and stability at 60 ºC. In the same vein, Zhao *et al*., (2011) studied the effects of additives on bromelain activity. Fructose was found to be the most powerful affecting factor followed by glucose and soluble starch. Besides, metabisulfite was revealed to be the best activator on bromelain activity as compared to other activators studied. Moreover, Zhang and Huang (2009) investigated effects of ultrasonic wave on the thermostability and secondary structure of bromelain. They concluded that the activity of bromelain increased to 119.05% under 240W ultrasound for 5min. Thus, the treatment significantly improved the thermostability of bromelain. More studies on stability and formulation of bromelain are highly required in order to increase the enzyme shell life and stability.

The various strategies used in drying and stabilization of bromelain are summarized in Table 5. From the Table, it could be deduced that the techniques had greatly enhanced the shelf life of bromelain. Moreover, stabilization of bromelain enhances its activity and resistance to heat and pH conditions.

4. Novel developments in the field of bromelain

4.1 Production of recombinant bromelain

The recent advances made in biotechnology have been making microbial enzymes to replace those from other sources and might now account for almost 90% of the total market (Illanes, 2008). This is owing to the fact that microbial cells are excellent systems for enzyme production. Actually, they are metabolically vigorous, very versatile, easy to propagate and simple to manipulate. Besides, they have simple nutritional requirements and their supply is independent of season. Consequently, research works on recombinant proteins have been comprehensively inspired. However, from a biotechnological point of view, plant

cysteine proteases are inadequately explored. They were initially used as crude extracts, then in semi-purified or fully purified forms and their chemical modifications have only started very recently (Feijoo-Siota and Villa, 2010). Some of the recombinant cysteine proteases include papain expressed in *Pichia pastoris* (Dufour, *et al.*, 2005) proteinase IV (glycyl endopeptidases) produced in *E. coli* (Taylor *et al*., 1995), and fruit bromelain whose gene was expressed in transgenic plant (Jung *et al*., 2008).

In the field of bromelain research, the production of stem recombinant bromelain is one of the most recent novel developments. Only few studies have been conducted involving the cloning of stem bromelain gene and its expression in *E. coli.* Recently, for the first time, stem bromelain had been expressed as recombinant enzyme in *E. coli* by Amid *et al*., (2011). In the study, the gene encoding stem bromelain from *Ananas comosus* was cloned into pENTR/TEV/D-TOPO vector before being sub-cloned into pDEST17 expression vector. This was then transformed into *E. coli* BL21-AI, in which enzymatically active bromelain was produced from the recombinant construct. Very highly purified, 43 kDa recombinant bromelain was obtained using a single step Ni-NTA spin column purification method. Besides, the enzyme was found to have optimum activity at pH 4.6 and 45 ºC coupled with higher enzymatic activity as compared to commercial bromelain. In the same vein, Bala *et al*., (2011) had studied the expression of recombinant bromelain as soluble (active) and insoluble (inactive) enzyme in *E. coli* BL21- A1. The results obtained indicated that the purification folds for both (soluble) and (insoluble) bromelains were 3.7 and 2.7, with the corresponding yields of 64% and 60.5%, respectively. Similarly, Muntari *et al*., (2012) had carried a study on optimization of recombinant bromelain production under different cultivation conditions (post-induction temperature, L-arabinose concentration and post-induction period) in shake flasks culture. In addition, validated experiments for the model yielded bromelain activity of 9.6 ± 0.02 U/mg at 0.15% (w/v) L-arabinose, 8 hr and 27 ºC. Thus, cultivation conditions for better production of recombinant bromelain in shake flask culture had been developed. In another study, conducted by Ismail and Amid (2012) recombinant bromelain thermal and storage stability were measured and compared to the commercial bromelain using Differential Scanning Calorimetry (DSC). The results obtained showed that recombinant bromelain was more stable than commercial bromelain at higher temperature. However, the stability of the enzyme decreased after 7 days of storage at 4 ºC. This signified that recombinant bromelain had better structural conformation as compared to commercial one.

5. Conclusion

The various applications and biochemical properties of bromelain have rendered it as an invaluable asset that will continue to be exploited for a long period of time. The significance of the enzymes has been booming in different industrial and pharmaceutical markets. To ensure abundant availability of bromelain, it is vital to develop various production and downstream processes that are simple, rapid, cost-effective and robust. In order to meet the demand of bromelain applications, more strategies need to be devised on improving the existing biochemical properties of the enzyme. The biotechnological developments observed in the growing enzymes markets have to be equally applied to bromelain production and standardization. The use of protein engineering involving mutagenesis and direct evolution to enhance some of the enzymes biochemical properties to the desired goals, are highly required. It is hoped that this review would provide pertinent information to contemporary researchers who are dynamically involved in bromelain and other related enzymes works.

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Table 1: Extraction and purification of bromelain

Purification technique	Yield (%)	Purification fold	Reference
Reverse micelle systems	97.56	4.54	Hemavathi *et al*., 2007
Reverse micelle systems	106	5.2	Hebbar *et al*., 2008
Optimization, neural modeling, batch & continuous extraction by reverse micelle system	_	4.96	Fileti *et a*l., 2007, 2009 & 2010
Optimization reverse micelle systems	89.6	2.8	Navapara *et a*l., 2011
Reverse micelle system, ultrafiltration, aqueous two phase system, ammonium sulfate	95.8	5.9-8.9	Hebbar *et a*l., 2012
Affinity-based reverse micelle system	185.6	12.32	Kumar *et al*.,2011
PEG/K_2So_4 aqueous two phase system	228	4.0	Babu *et al*., 2008
PEG/$MgSo_4$ aqueous two phase system	113.54	2.23	Ketnawa *et al.,* 2010
PEG/$MgSo_4$ aqueous two phase system	206	3.44	Ketnawa *et al*., 2011a
4 different aqueous two phase system	_	_	Ketnawa *et al*., 2011b
PEG/$MgSo_4$ aqueous two phase system	_	25-62	Ferreira *et al*., 2011
Block copolymers aqueous two phase system	79.5	1.25	Rabelo *et al*., 2004
Microfiltration, ammonium sulfate precipitation, ultracentrifugation	50	_	Doko *et al*., 2005
Microfiltration & ultrafiltration	85-100%	10	Lopes *et al.,* 2009
Nano-$TiO_{2,}$ ultrafiltration	64.75	5.3	Chao *et al*., 2009
Ultrafiltration, kaolin adsorption & tannin precipitation	_	_	Li and Li, 2009
Homogenization	_	_	Loh *et al.*, 2005
Centrifugation, salt precipitation, dialysis, ion-exchange chromatography	_	_	Gautam *et al*., 2010
Ethanolic extraction, isoelectric point precipitation, ammonium sulfate precipitation	_	_	Silvestre *et al.,* 2012
Precipitation, chromatography	_	_	Devakate *et al*., 2009
High speed counter-current chromatography, reverse micelle system	_	_	Yin *et al*., 2011
Polyacrylacid-bound iron oxide	87.4	_	Chen *et al*., 2004
7-merpeptide bound to nylon membrane	94.8	_	Chen *et al*., 2008
Pack-bed adsorption	_	13	Silveira *et al*., 2009
Immobilized metal affinity membrane	94.6	15.4	Nie *et al*., 2008

Various extraction and purification strategies for bromelain as employed by different researchers

Table 2: Molecular weight, optimum pH and temperature for bromelain

Type of bromelain	Optimum pH	Optimum temperature (^{o}C)	Molecular weight (kDa)	Reference
Stem Fruit	7.0 8.0	60 70	37 32.5	Suh *et al*., 1992
Stem	6-7	_	28	Harrach *et al*., 1998
Fruit	7.0	55	_	Ketnawa *et al*., 2010
Fruit	8.0	60	29	Ketnawa *et al.*, 2011a
Fruit	3-9	50-60	_	Ketnawa *et al*., 2011b
Fruit	2.9-7.7	37-59	_	Corzo *et al*., 2011
Stem	_	_	29	Kumar *et al*., 2011
Fruit			24.5	Lopes *et al*., 2009
Stem	_	_	26	Xue *et al*., 2010
Fruit	_	40	_	Jutamongkon and Charoenrein, 2010
Stem	_	_	30	Gautam *et al*., 2010
Fruit	6.0	70	_	Silvestre *et al*., 2012
Stem	_	50-60	_	Liang *et al*., 2011

The molecular weight, pH and temperature optima of bromelain as reported by various research groups

Table 3: Effects of some chemicals and covalent modification of bromelain

Chemical/treatment	Observed Effects on bromelain	Reference
Salts Alcohols	a cooperative thermal transition non-cooperative thermal denaturation	Haq *et al*., 2005
Antibromelain	Significant stabilization of bromelain against thermal inactivation and alkaline pH	Gupta *et al*., 2003b
Trifluoroethanol	Molten globule state in acid-denatured bromelain	Gupta *et al.*, 2003a
Sodium dodecyl sulfate (SDS)	SDS acted as a partial non-competitive inhibitor to bromelain	Bhattacharya and Bhattacharya, 2009a
Guanidine hydrochloride	Deglycosylated bromelain was more susceptible towards guanidine hydrochloride denaturation.	Rasheed *et al*., 2003
Polyethylene glycol (PEG)	induced molten globule and the unfolding of the tertiary structure of bromelain	Ahmad *et al*., 2006
Trifluoroethanol	Induced-molten globule state without tertiary structure of bromelain	Ahmad and Khan, 2006
Trifluoroethanol	Conformational rearrangement in bromelain	Dave *et al*., 2010a
Hexafluoroisopropanol	Transformation from α-helix to β-sheet and induced formation of a compact non native structure.	Dave *et al*., 2010b
Trifluoroethanol	transformation of the pre-molten globule to a molten globule conformation of bromelain	Dave *et al*., 2011
Pyromellitic anhydride acid and poly(maleic anhydride).	Modified bromelain with improved thermal stability and the resistance to alkali and surfactant.	Xue *et al*.,2010
High-pressure microfluidization	Inhibition of bromelain activity and unfolding of secondary structure.	Zhao-qin *et al*., 2010

The different chemicals and strategies used for modification of bromelain in order to enhance the stability of the enzyme

Table 4: Immobilization strategies for bromelain

Type of support/treatment	Observed effects on bromelain	Reference
Porous chitosan beads	Greater stabilities in pH, temperature and increased shelf-life of the immobilized bromelain.	Seo *et al*., 1998
Porous copoly(c-methyl-L glutamate/L-leucine) beads	Higher thermal stability, apparent Km and lower Vmax values.	Yodoya *et al*., 2003
Sepharose	more resistant to thermal inactivation and a broader pH activity	Gupta and Saleemuddin, 2006
Metal affinity support	More resistant to thermal inactivation with a broader pH activity profile in acidic range.	Gupta *et al.,* 2007
Calcined layered double hydroxide	Higher stability of immobilized bromelain on heat and storage time	Shi *et al.,* 2007
Thermosensitive polymer	broader pH-activity profiles, with optimum temperature of 70°C	Mahmood and Saleemuddin, 2007
Attapulgite modified by 3-aminopropyltriethoxysilane	Enhanced stability and utilization rate of the immobilized bromelain	Rong, 2008
Cmposite membranes	Higher resistance to denaturants with slightly better storage stability	Anwar *et al.,* 2007
Composite membranes	Effective adsorption of bromelain on dye affinity membranes.	Su *et al.,* 2009
Sodium alginate	Raised optimum pH from 6.8 to 7.6 with optimum temperature of 50 ℃.	Zhang *et al.*, 2009
Magnetic composite particles.	Highly suitable for bromelain adsorption	Song *et al*., 2011
Linoleic-acid modified carboxymethyl chitosan	enhanced stability and substrate affinity for the immobilized bromelain	Tan *et al.,* 2008

Different immobilization techniques of bromelain for enhancement of its stability and reusability

Table 5: Drying and stabilization of bromelain as conducted by various researchers

Treatment/study	Observed effects	Reference
lyophilization	Almost 100% bromelain activity was recovered.	Doko *et al*., 2005
Freeze drying Spray drying	96 % bromelain activity was recovered. 50–70 % bromelain activity was recovered.	Devakate *et al*., 2009
Spray drying	47.1 % bromelain activity was recovered.	Cao *et al*., 2009
Spray-drying	90 % activity recovery yield for bromelain was obtained.	Cabral *et al.,* 2009
Activity and stability of bromelain preparations	Concentrated bromelain solutions are more resistant to autolysis.	Hale *et al*., 2005a
Stability of bromelain in pineapple juice	Increased thermal stability and shelf life of bromelain.	Abdulmajid *et al*., 2008
Preservation of natural stability of fruit bromelain	*Retention of about 75 % bromelain activities after 180 days.*	Bhattacharya and Bhattacharya, 2009b
Effects of Ca^{2}+ on stability of bromelain	Enhanced bromelain activity and stability at 60 ℃ with longer half life	Wang *et al*., 2009
Effects of additives on bromelain activity	Fructose significantly increased the stability of bromelain.	Zhao et al., 2011
Effects of ultrasonic wave on the stability of bromelain	improved thermostability of bromelain	Zhang and Huang, 2009

The various method of drying bromelain for its improved shelf-life and storage durability

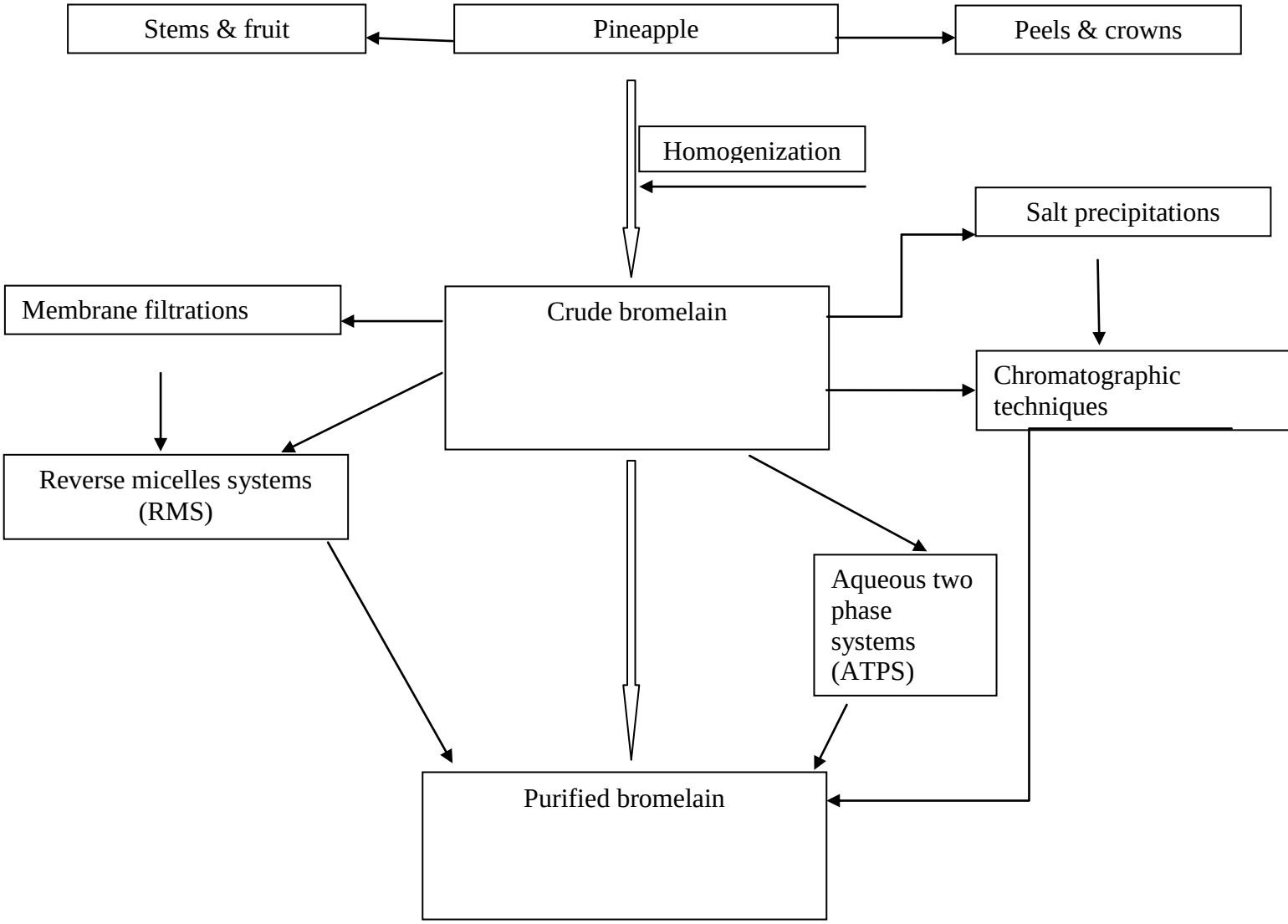

Figure 1: Schematic presentation of extraction and purification strategies of bromelain. Several techniques that are being employed for the extraction and purification of bromelain from different parts of pineapple plant.

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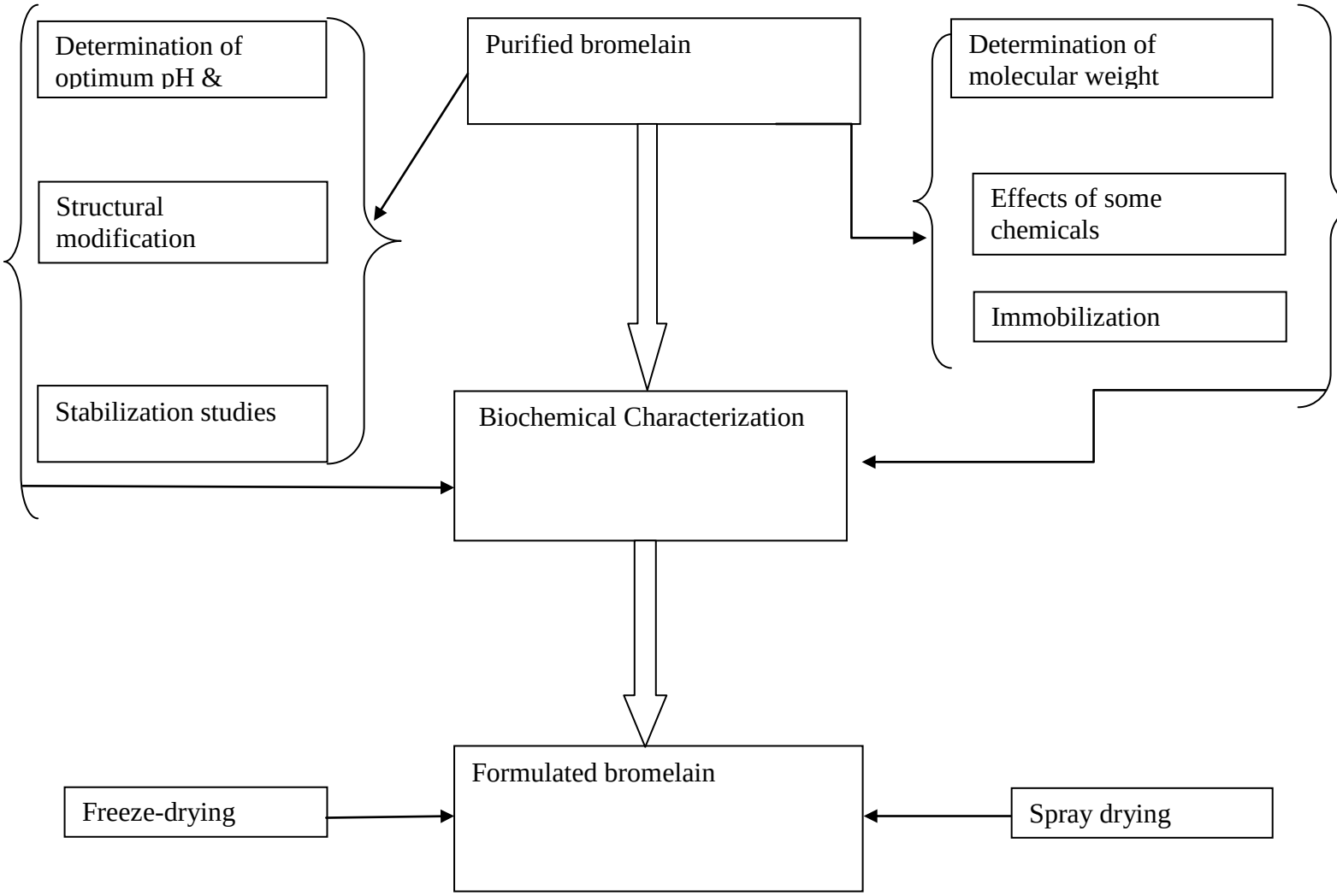

Figure 2: Schematic presentation of biochemical characterization of bromelain.
Different techniques used by researchers for the characterization of bromelain in order to standardized the enzyme.

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