MINI-REVIEW

Bromelain: an overview of industrial application and purification strategies

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Abstract This review highlights the use of bromelain in various applications with up-to-date literature on the purification of bromelain from pineapple fruit and waste such as peel, core, crown, and leaves. Bromelain, a cysteine protease, has been exploited commercially in many applications in the food, beverage, tenderization, cosmetic, pharmaceutical, and textile industries. Researchers worldwide have been directing their interest to purification strategies by applying conventional and modern approaches, such as manipulating the pH, affinity, hydrophobicity, and temperature conditions in accord with the unique properties of bromelain. The amount of downstream processing will depend on its intended application in industries. The breakthrough of recombinant DNA technology has facilitated the large-scale production and purification of recombinant bromelain for novel applications in the future.

Keywords Bromelain · Cysteine protease · Purification · Recombinant bromelain

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Introduction

Modern technological advancements have raised the protease production industrially worldwide. Today, proteases dominate with approximately 60 % market share of the total enzyme market worldwide where the major producers are Novo Industries, Gist-Brocades, Genencor International, and Miles Laboratories (Feijoo-Siota and Villa 2011). This growth is entrenched with the rising general awareness of the need to protect the environment from the impact of chemical industrialization. Recently, bromelain has drawn attention in diverse industrial applications owing to its properties and higher commercial values (Heinicke and Gortner 1957). Bromelain is a protease derived from the stem and fruit of pineapples (*Ananas comosus*). Stem bromelain (EC 3.4.22.32), ananain (EC 3.4.22.31), and comosain are extracted from pineapple stems, while fruit bromelain (EC 3.4.22.33) is mainly from fruit juice (Rowan et al. 1990). Similar proteases are also present in pineapple peel, core, crown, and leaves, with the highest proteolytic activity and protein contents detected in the extract of pineapple crown (Ketnawa et al. 2012).

Stem bromelain can be isolated from stem juices by means of precipitation and centrifugation (Devakate et al. 2009; Heinicke and Gortner 1957). Moreover, using chromatographic methods, other basic proteolytic (ananain, comosain, F4, F5, and F9) and acidic components of stem bromelain fraction A (SBA/a) and fraction B (SBA/b) have been partially and fully purified (Feijoo-Siota and Villa 2011; Harrach et al. 1995, 1998; Murachi et al. 1964; Napper et al. 1994; Rowan et al. 1990; Wharton 1974). The molecular weight of purified stem bromelain is 23.40–35.73 kDa, fruit bromelain 31.00 kDa, ananain 23.43–23.42 kDa, and comosain 23.56–24.51 kDa. The isoelectric point of stem and fruit bromelain is at pH 9.55 and 4.6, respectively (Murachi et al. 1964; Yamada et al. 1976). There is minimal difference in the amino acid composition between stem bromelain, ananain, and comosain

as shown in Table 1. Stem bromelain contains 285 amino acids where the most abundant amino acids are alanine and glycine, while histidine and methionine are present in the lowest amounts (Murachi 1964; Ota et al. 1964). It appeared to differ significantly in the number of lysine, arginine, and isoleucine compared to ananain and comosain (Napper et al. 1994). Ananain possesses more hydrophobicity in the region near histidine-157 and sequence insert between 170 and 174 residues which cannot be found in stem bromelain (Lee et al. 1997). Stem bromelain also contains four hexosamines, and 2.1 % carbohydrate, meaning it is a glycoprotein (Murachi 1964; Murachi et al. 1964). This observation was corroborated by the findings of Ota et al. (1964), who detected 1.5 % carbohydrate, including six glucosamines, in purified stem bromelain. Later, Murachi et al. (1967) isolated 30 mg of glycopeptides from 1 g of stem bromelain using gel filtration in an attempt to confirm the presence of a glycoprotein. Murachi et al. (1967) and Yasuda et al. (1970) reported that the carbohydrate composition in stem bromelain consists of mannose, fucose, xylose, and glucosamine in the ratio of 3:1:1:4 using gas chromatography analysis, whereas Scocca and Lee (1969) obtained the same oligosaccharide group with a different ratio of 2:1:1:2 using automated borate chromatography.

Stem bromelain is an endoprotease that breaks peptide bonds within the protein molecule. Stem bromelain is under the papain family which resembles papain unequivocally in the alignment of amino acid sequence. A prominent similarity is observed in their mechanism of action as shown in Fig. 1. Initially, noncovalent bonding involves the free enzyme (structure a) and substrate to form the complex (structure b).

Table 1 Amino acid composition of stem bromelain, ananain, and comosain (Napper et al. 1994)

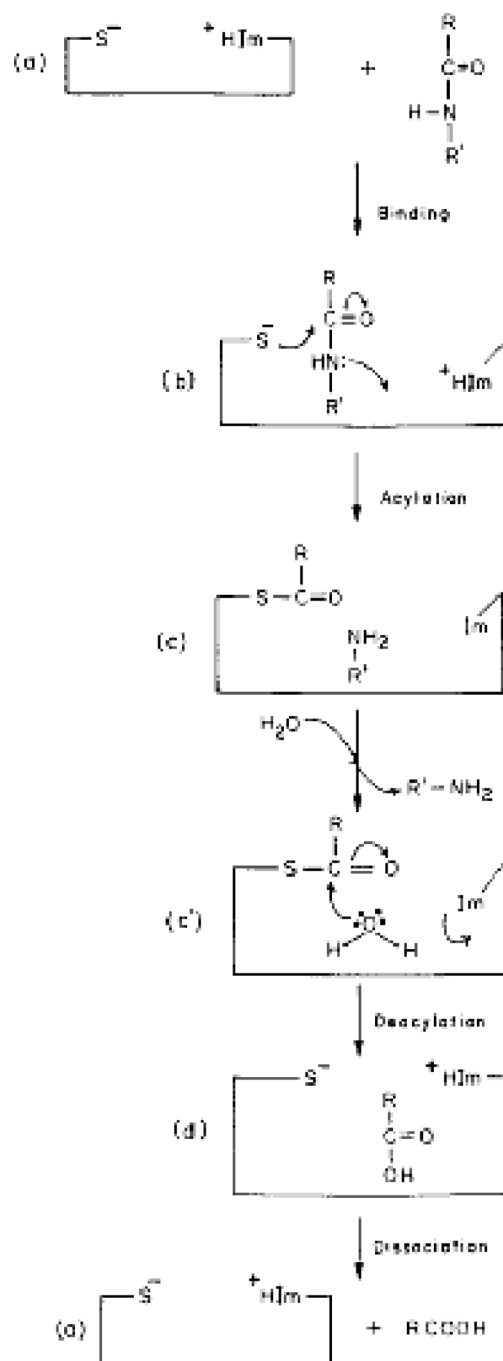| Amino acid	Stem bromelain	Ananain	Comosain
Asp	18	19	18
Thr	9	8	7
Ser	17	18	17
Glu	16	13	13
Gly	22	24	25
Ala	25	20	20
Val	14	14	13
Met	3	2	3
Ile	17	14	12
Leu	6	9	9
Tyr	14	12	12
Phe	6	5	7
His	1	2	2
Lys	15	11	10
Arg	6	10	11
Cys	7	7	7

Fig. 1 Catalytic mechanism of cysteine proteinase action. Im and $^+$HIm referred to imidazole and protonated imidazole (Rao et al. 1998)

Then, the next step is the acylation of the enzyme (structure c) which produces amine, with R'-NH_2 as the first product. Finally, the deacylation reaction step takes place between the acyl-enzyme and water to release the carboxylic acid, RCOOH, and free enzyme (Rao et al. 1998). The presence of asparagine-175 hydrogen-bonded with histidine-159 favors the catalytic function in papain. Stem bromelain is distinguished from papain by its lower value of second-order rate constant of inactivation by iodoacetate and iodoacetamide due

to the absence of asparagine-175 and two adjacent residues as well as the mutation of serine-176 to lysine in its structure (Ritonja et al. 1989). It is noteworthy that stem bromelain exhibits a weak inhibition by L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido (4-guanidino) butane (E64) about 678 M^{-1} s^{-1} in contrast to papain which is about 638,000 M^{-1} s^{-1} (Barrett et al. 1982; Rowan et al. 1990).

Specificity of stem bromelain was markedly examined using different substrates, such as gelatin (gelatin digestion unit), casein (casein digestion unit), azocasein and azoalbumin, hemoglobin, sodium caseinate, Z-Arg-Arg-NH-Mec, and Z-Arg-Arg-ρNa (Corzo et al. 2012; Gautam et al. 2010; Iversen and Jørgensen 1995; Napper et al. 1994; Rowan et al. 1990). Its proteolytic ability efficiently had hydrolyzed glucagon at arginine-alanine and alanine-glutamic acid bonds where it shows preference for glutamic acid, aspartic acid, lysine, or arginine in the P1 site yet leaves intact the arginine-arginine and lysine-tyrosine bonds. The release of DNP (2,4-dinitrophenyl)-alanine and DNP-glutamic acid after FDB (fluorodinitrobenzene) treatment becomes an evidence of the successful cleavage at its preference bonds (Murachi and Neurath 1960; Napper et al. 1994). Profiling of substrate specificity for stem bromelain using positional scanning synthetic combinatorial libraries (PS-SCLs) had displayed a cleavage site at arginine (Choe et al. 2006). Generally, stem bromelain is in full activity under the presence of ethylenediaminetetraacetic acid (EDTA) and cysteine, and the addition of mercury (II) chloride (HgCl$_2$) causes inhibition of its activity (Murachi and Neurath 1960). The optimum pH and temperature for stem bromelain activity are in the range of pH 6.5–8.0 and 55–60 °C, respectively, for most of the substrates which are presented in Table 2. Studies of the stability of pineapple fruit in storage without preservative at −4 °C indicate that bromelain retains 75±5 % of its activity, and no microbial growth is detected after 180 days (Bhattacharya and Bhattacharyya 2009). The bromelain degradation profile in aqueous solution at various temperatures follows first-order kinetics, and its activation energy is 41.7 kcal/mol (Yoshioka et al. 1991).

While the biochemical properties such as molecular weight, optimum pH and temperature as well as carbohydrate composition in this enzyme display varying degrees of homology from various researchers, judgment on the variation of bromelain resource and purification process has to be scrutinized extensively before awaiting for further industrial development. Thanks to the major breakthrough of modern biotechnology, new analytical technologies to characterize and ensure the safety of the source and end product application have been developed (Staub et al. 2011). Today, escalating growth of commercial manufacturers of stem bromelain (retrieved from http://www.chemicalbook.com) worldwide has swiftly found usage in a wide array of industrial applications such as food, textile, brewing, cosmetic, and dairy products (Polaina and MacCabe 2007). Thus, this review will start with an overview on various applications of bromelain in tenderization, baking industry, protein hydrolysate, enzymatic browning inhibition agent, animal feed, textile industry, tooth whitening as well as cosmetic industry. Next, an in-depth summary on recent purification strategies of bromelain in the last 10 years which include ultrafiltration, precipitation, aqueous two-phase system (ATPS), adsorption, reverse micelle extraction (RME), and chromatography will be provided.

Industrial applications of bromelain

Bromelain is a plant protease isolated from pineapple. Its strong proteolytic activity has created a wide interest in numerous applications, mainly in tenderization, foods, detergents, and the textile industry. Bromelain has also been envisaged to have extensive applications as an active ingredient in tooth-whitening dentifrices and skin products. In this part of the review, the major applications of bromelain are summarized (see Table 3).

Table 2 Optimum pH and temperature for bromelain with different substrates

Type of bromelain	Optimum pH	Optimum temperature (°C)	Substrates	References
Fruit	6.5	55	Azocasein	Corzo et al. (2012), Bhattacharya and Bhattacharyya (2009)
	7.5	55	Azoalbumin	
	7.7	59	Casein	
	6.5	59	Sodium caseinate	
	2.9	37	Hemoglobin	
Peel	8	60	Casein	Ketnawa and Rawdkuen (2011)
Recombinant	4.6	45	N$^\alpha$-CBZ-L-lysine *p*-nitrophenyl ester (LNPE)	Amid et al. (2011)
Stem	7	40	Casein	Khan et al. (2003)
	8	–	Bz-Phe-Val-Arg-pNA	Benucci et al. (2011)

Table 3 Industrial applications of bromelain

Applications	Reasons	References
Baking industry	– Improve dough relaxation and allow the dough to rise evenly – Produce hypoallergenic flour	Kong et al. (2007), Tanabe et al. (1996), Watanabe et al. (2000)
Tenderization	– Hydrolyze meat myofibril proteins – Hydrolyzing agent for meat, oyster, chicken, and squid	Hage et al. (2012), Sullivan and Calkins (2010), Ketnawa and Rawdkuen (2011), Chuapoehuk and Raksakulthai (1992)
Fish protein hydrolysate	– Hydrolyze fish protein to generate fish protein hydrolysate	Elavarasan et al. (2013), Ren et al. (2008), Tanuja et al. (2012)
Antibrowning agent	– Inhibit browning of fruits and phenol oxidation	Lozano-De-Gonzalez et al. (1993), Srinath et al. (2012)
Alcohol production	– Enhance protein stability of beers – Prevent haze formation	Benucci et al. (2011)
Animal feed	– Estimate protein degradation in ruminant feed	Tománková and Kopečný (1995)
Textile industry	– Minimize softening time in cocoon cooking – Remove scale and impurities of wool and silk fibers – Enhance dyeing properties of protein fibers	Koh et al. (2006), Devi (2012), Singh et al. (2003)
Tooth whitening	– Remove stains, plaque, and food debris on the outer surface of teeth	Chakravarthy and Acharya (2012), Kalyana et al. (2011)
Cosmetic industry	– Treat acne, wrinkles, and dry skin – Reduce post-injection bruising and swelling	Ozlen et al. (1995), Levy and Emer (2012)

Tenderization

Tenderness is an important characteristic of meat. The overwhelming demand for guaranteed tender meat has attracted players in the meat industry to provide an acceptable quality of product. In the USA, certified Angus beef was introduced in the late 1970s. Consumers have found it consistently flavorful, juicy, and tender (Koohmaraie and Geesink 2006). Many approaches have been employed to improve postmortem tenderness, including blade tenderization (Pietrasik et al. 2010; Pietrasik and Shand 2011), moisture enhancement technology (Streiter et al. 2012), and enzymatic treatment (Gerelt et al. 2000; Pietrasik and Shand 2011). In a traditional tenderization method, meat is kept cool for up to 10 days to allow postmortem proteolysis by proteolytic enzymes: cathepsins and calpains (Nowak 2011).

The potential of proteolytic enzymes such as bromelain, papain, and ficin has been recognized, as shown by their long history of use in meat tenderization. Compared with papain and ficin, bromelain is commercially available in the market under name brands such as McCormick and Knorr. Commercial papain, bromelain, and zingibain efficiently hydrolyze a few meat myofibril proteins, such as actomysin, titin, and nebulin, as revealed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Hage et al. 2012). Sullivan and Calkins (2010) reported that bromelain can improve the sensory evaluation rating and tenderness of meat comparable to other exogenous enzymes. The addition of bromelain can also produce a tenderizing effect on myosin and other myofibrillar proteins of coarse dry sausage. A number of methods of tenderization using bromelain have been reported. Lizuka and Aishima (1999) successfully applied continuous monitoring using attenuated total reflectance infrared spectroscopy (ATR-IR) to observe the structural changes in meat protein after treatment with pineapple juice. Melendo et al. (1997) studied the effect of pH, temperature, cutting method, and cooking time during marinating with bromelain. Ketnawa and Rawdkuen (2011) demonstrated that treatment of bromelain at up to 20 % (w/w) leads to extensive proteolysis on beef, chicken, and squid. Besides, bromelain can be a good substitute for hydrochloric acid as a hydrolysis agent for oyster meat and, at the same time, receives higher acceptability scores in sensory evaluation in oyster sauce production (Chuapoehuk and Raksakulthai 1992).

Baking industry

Gluten is a functional component of wheat food products, such as flour. It consists of two major proteins, gliadin and glutenin. Gluten becomes insoluble and forms lattice-like structures when it is hydrated. Therefore, gluten must be degraded to avoid resistance to dough stretching (Walsh 2002). The use of proteolytic enzymes such as bromelain can improve dough relaxation, enhance solubility, and prevent dough shrinkage. This will allow the dough to rise evenly during the baking process (Kong et al. 2007; Polaina and MacCabe 2007). Bromelain also has been used to produce hypoallergenic flour that is suitable for wheat-allergic patients. The immunoglobulin E (IgE)-binding epitope, Gln-Gln-Gln-Pro-Pro, is a major allergen in flour. Thus, the addition of bromelain can help to hydrolyze peptide bonds near proline (Pro) residues, which degrades the epitope structure (Tanabe et al. 1996; Watanabe et al. 2000).

Protein hydrolysate

Protein hydrolysate has a long history of use as a nitrogen source for growth media for microbial, plant, and animal cell culture at the laboratory and industrial scales. It is widely used as a nutritional supplement, pharmaceutical ingredient, and flavor enhancer and in cosmetics and beverages. Current practice in the manufacturing of protein hydrolysate involves acid, alkali, and enzyme hydrolysis. However, enzymatic hydrolysis is preferable due to the ease in controlling the degree of hydrolysis as well as shortening the hydrolysis time. The significant parameters for enzymatic hydrolysis are temperature, hydrolysis time, pH, and degree of hydrolysis (DH) (Pasupuleti and Braun 2010). The addition of proteolytic enzymes can make the enzymatic hydrolysis process efficient and reproducible (Chalamaiah et al. 2012). There has been considerable research on the flavor profile (sweet, salty, sour, bitter, bouillon, and umami taste) and functional properties (hypoantigenic, antioxidant, and antimicrobial) of protein hydrolysate isolated from fish and plant sources using commercial bromelain.

Protein hydrolysate derived from food processing byproducts can be used as precursors in the production of meat, savory food, and chicken flavor substitutes. For example, the hydrolysis of mungbean protein by bromelain has released 16 free amino acids which give a meaty flavor characteristic such as bouillon, sweet smell, green, and fatty (Sonklin et al. 2011). Besides, after bromelain hydrolysis, several amino acids such arginine, lysine, and leucine were found from the protein by-product hydrolysis of *Gracilaria fisheri* (red seaweeds) which characterize seafood flavoring (Laohakunjit et al. 2014). Sangjindavong et al. (2009) reported on the production of fish sauce from the fermentation of surimi waste accelerated with pineapple core and peel.

In spite of the fact that protein hydrolysate can be used as a flavor agent, the hydrolysis process is often accompanied by bitterness effect due to the reaction of endoprotease with the protein substrate during the hydrolysis. The bitter intensity can be expressed as the taste dilution (TD) factors. Partial hydrolyzation of soy protein isolate (SPI) by bromelain showed lower (TD) factors of 4 and 16 in 10 and 15 % DH, respectively, compared to alcalase and neutrase. This phenomenon could be due to the remaining nonpolar amino acid residues at the C-terminus of the peptide after bromelain hydrolyzes the hydrophobic amino acid residues (Seo et al. 2008). Cheung and Li-Chan (2014) found that after 8 h of hydrolysis, shrimp hydrolysate by bromelain demonstrated less bitter and high umami taste in the instrumental taste sensing system rather than alcalase and protamex hydrolysate. The bitterness characteristic results from enzymatic hydrolysis and is due to the presence of N-terminal peptide residues which are glycine, leucine, isoleucine, phenylalanine, and valine (Hevia and Olcott 1977).

Recent studies have demonstrated that protein hydrolysates from fish source possess antioxidant activities after enzymatic hydrolysis using various proteolytic enzymes such as bromelain, papain, flavorzyme, protamex, neutrase, and alcalase (Lamsal et al. 2007; Ren et al. 2008; Tanuja et al. 2012). Among these enzymes, bromelain hydrolysate from freshwater carp (*Catla catla*) is appearing to give high disappearance rates of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Elavarasan et al. 2013). Antimicrobial peptides from natural sources can be promising candidates to replace synthetic antimicrobial agents in the treatment of a wide spectrum of bacterial infections. Thus, Ghanbari et al. (2012) explored the application of *Actinopyga lecanora* (sea cucumber) in the production of antimicrobial peptide. They found that after 7 h of hydrolysis, hydrolysates prepared by bromelain exhibited the highest antimicrobial activities against *Pseudomonas* sp., *Pseudomonas aeruginosa*, and *Escherichia coli* compared to other hydrolysates generated by papain, alcalase, pepsin, trypsin, and flavorzyme. That study corroborates a previous study performed by Salampessy et al. (2010), which showed the antimicrobial capability of bromelain hydrolysate from leatherjacket (*Meuschenia* sp.) against *Staphylococcus aureus* and *Bacillus cereus*.

Enzymatic browning inhibition agent

The discoloration of fruits into brown color, especially apples and pears, occurs due to the oxidation of phenols to quinones in the presence of oxygen. Further reactions of quinones with other substances, such as phenolic compounds and amino acids, lead to the development of brown pigments. Enzymatic browning may deteriorate the nutritional quality and flavor of the fruits. To control the browning of fresh and canned fruit, ethylene diaminetetraacetic acid (EDTA), ascorbic acid, L-cysteine, citric acid, and phosphoric acid are commonly used (Ozoğlu and Bayındırlı 2002). However, due to health concerns over the side effects caused by chemical-based enzymatic browning inhibition agents, there has been an increasing amount of research on agents derived from plant-based sources. According to Lozano-De-Gonzalez et al. (1993), eluate from cation exchange of the pineapple juice is effective as a sulfite agent to inhibit browning of fresh and dried apple rings. Later, Srinath et al. (2012) extended that study by partitioning the fruit and crown leaf using cation-exchange chromatography after ammonium sulfate precipitation. They concluded that the protease activity and browning inhibition effects were higher in the fruit extract compared to the crown leaf extract. Similarly, Chaisakdanugull et al. (2007) employed pineapple juice that was fractioned by a solid-phase C_{18} cartridge as an inhibitor of enzymatic browning in bananas. The study reported a 100 % inhibitory effect of bromelain toward polyphenol oxidation (PPO). In contrast to earlier findings, however, stem bromelain had the weakest

browning inhibition effect compared with commercial L-cysteine and ascorbic acid (Tochi et al. 2009).

Animal feed

Generally, forages provide 90 % of food energy and nutrients to ruminant animals at low cost. The variation of the nutritional value of forages is highly influenced by the climate, forage species and cultivars, and the preservation method. Therefore, the estimation of soluble nitrogen compounds in rumen is pivotal to assess the forage attributes to maximize its utilization in ruminants diets (Givens et al. 2002). The in sacco (in situ) technique is widely used to analyze the protein degradability of forage. Unfortunately, the technical challenges and lack of standardization limit its practicability (Abdelgadir et al. 2013). The determination of protein degradation in the rumen by the actions of proteases can be an alternative to replace the conventional method, which is highly expensive and time-consuming. The addition of protease to animal feed can increase protein inversion and availability, decrease the cost of animal feed, and increase the source of protein. The use of bromelain in the estimation of protein degradation of cereals, forages, hays, protein concentrates, and silages in ruminants has been successful and produces comparable results with the in sacco method (Polaina and MacCabe 2007; Tománková and Kopečný 1995).

Textile industry

Recently, the use of proteases in the silk industry has expanded enormously, especially the cocoon cooking method. Generally, the cocoon has to be softened by cooking it in the presence of strong alkaline agents and chemicals. However, this conventional technique has a detrimental effect on the quality of the silk thread produced. Thus, the development of an effective cocoon cooking method using enzyme treatment will reduce softening time while increasing production and saving energy. For instance, Singh et al. (2003) reduced the softening time from 20 h to 30 min using pineapple extract incorporated with 9.8 mM of sodium carbonate at 60 °C. Additionally, Koh et al. (2006) reported that the pretreatment of silk and wool with bromelain improved tactile behavior and wettability by removing impurities and scale. These effects increased the dye uptake of the wool and silk fibers and at the same time maintained their tensile properties.

Tooth whitening

Tooth discoloration involves their intrinsic color and extrinsic stains on the surface of the teeth. A number of factors that impart extrinsic discoloration include colored foods and beverages such as tea and coffee, tobacco smoking, and tobacco chewing (Watts and Addy 2001). The stigma attached to discolored teeth has raised the demand for tooth-whitening products. Thus, there is heated competition between manufacturers to develop new technologies and oral care products that overcome tooth discoloration. For tooth-whitening and extrinsic stain removal purposes, toothpaste is usually formulated with abrasive agents that whiten teeth by removing plaque, stains, and food debris accumulated on the tooth surface. However, abrasive agents can accelerate tooth wear, and they become ineffective if their particles are too large, as they cannot be captured by toothbrush bristle (Joiner 2010). Therefore, many types of toothpaste containing different enzymes are marketed to remove extrinsic stains (Hannig et al. 2005, 2010). The novel dentifrice efficiency can be accessed through the lightening value. Recently, an in vitro study by Kalyana et al. (2011) reported on the stain removal efficiency of a dentifrice containing papain and bromelain. The proteolytic enzymes disrupted accumulated protein on the tooth surface, which resulted in higher lightening value after brushing compared to the control dentifrice. Chakravarthy and Acharya (2012) corroborated these results, finding that the application of papain and bromelain extracts in dentifrices had a significant lightening effect in comparison with the control dentifrice (Colgate Regular). However, their formulation had the limitation of a shorter shelf life than the usual dentifrice because bromelain and papain are proteolytic in nature.

Cosmetic industry

The cosmetic products that have papain and bromelain as active ingredients can effectively alleviate skin problems such as wrinkles, acne, and dry skin. These enzymes gently digest the protein of dead cells in the upper layer of the skin, resulting in their replacement by younger skin cells from lower layers (Ozlen and Chatsworth 1995). Bromelain can also reduce bruising and swelling of skin after cosmetic injection treatments (Levy and Emer 2012).

Purification strategies of bromelain

The continuous interest in bromelain in diverse applications has motivated many researchers to extract the enzyme from pineapple juice and waste and characterize it. The mature pineapple stem contains the highest concentration of bromelain (Heinicke and Gortner 1957). However, pineapple wastes (peel, core, crown, and leaves), which are a by-product in the pineapple processing industry, are also rich in bromelain and are still underutilized. Therefore, Ketnawa et al. (2012), Mutalib et al. (2012), and Nadzirah et al. (2012) investigated the physicochemical properties and proteolytic activity of pineapple waste. The selection of extraction buffer and disruption method is crucial and depends on the target

protein and its application. The optimal conditions would maximize the extraction efficiency, minimize oxidation, avoid denaturalization, and increase protein acquisition. Ketnawa et al. (2012) investigated the extraction of bromelain from pineapple peels using different extractants. They obtained the highest bromelain activity when it was extracted with 100 mM phosphate buffer with 15 mM cysteine and 2 mM EDTA at pH 7.0. The addition of a small amount of reducing agent, such as cysteine, and chelating agents, such as EDTA, prevents protein oxidation and traps heavy metals that can boost the oxidation of thiols to inhibit protease action (Chaiwut et al. 2007; Gräslund et al. 2008; Janson 2011). The conventional extraction of bromelain from cooled pineapple juice involves grinding, filtering, and centrifugation. For instance, Shian et al. (2005) and Gautam et al. (2010) employed a homogenization method that disrupts the pineapple stem cells and releases bromelain using sodium acetate solution as an extraction buffer. Later, the crude extracts are filtered and centrifuged to remove cellular debris and insoluble. Once the desired bromelain is free from other particulate residue, the purification continues until a desirable level of purity is achieved, depending on its intended use.

Thorough investigations on the operational design are needed to reduce the cost and number of purification steps in downstream processing. Pre-purification steps such as precipitation, ultrafiltration, and extraction are most commonly employed in the early stage to concentrate and increase the recovery of the target protein. Once a soluble concentrate is obtained, successive purification steps are performed to remove remaining impurities and increase the level of purity. Occasionally, a single pre-purification is enough, but the degree of purification will be low compared to the high-resolution steps such as chromatography. Therefore, chromatography steps with ion exchange, affinity, and gel filtration are crucial to provide higher selectivity and purity of the end product. Some novel purification technologies have recently been applied to the purification of bromelain. These include reverse micellar extraction, aqueous two-phase systems, and adsorption. These are currently being employed at a laboratory scale and have yet to be explored in large-scale processing. Here, a brief description of ultrafiltration, precipitation, and chromatography are provided below.

Ultrafiltration

Ultrafiltration is the best method of concentrating proteins and has been used extensively in the lab and large-scale processes. The ultrafiltration membrane has a molecular weight cutoff ranging from 3 to 100 kDa, while microfiltration has a pore diameter ranging from 0.1 to 10 μm. The retentate containing the protein of interest is retained behind the membrane and is further recycled until the desired concentration is achieved. The permeate is usually discarded as waste (Walsh 2002;

Wheelwright 1991). Doko et al. (1991) have reported on the application of sequential batch filtration, which included microfiltration (8 μm) and ultrafiltration (100 kDa cutoff), followed by the additional steps of ammonium precipitation and ultracentrifugation prior to freeze-drying to remove contaminant residues, resulting in 50 % recovery. The recovered product contained 98 % protein. However, the proteolytic activity of bromelain was lost during the protein adsorption onto the ultrafiltration (UF) membranes relative to the level of concentration reached. A similar trend of enzymatic activity was observed when another study applied microfiltration (1.2 and 0.2 μm) and ultrafiltration (10 kDa) before bioethanol fermentation process (Gimeno et al. 2010). Apparently, the bromelain recovery obtained by Lopes et al. (2009) (90 %) and Hebbar et al. (2012) (92.4 %) was more than reported by Doko et al. (1991). The additional steps of microfiltration, RME, and ATPS before ultrafiltration caused a significant increase in activity recovery.

Precipitation

Protein precipitation involves the addition of salts, polar solvents, nonpolar solvents, and organic polymers into cell extracts or by varying the temperature or pH. When the solubility of proteins in solution is reduced by increasing the concentration of precipitating agents, salting out (precipitation) will occur. In a large-scale process (1–5 l of homogenate), ammonium sulfate is the agent of choice for salting out the proteins because it is effective and relatively cheap. Under saturation (4 M ammonium sulfate), most protein will precipitate from the solution. Low temperature is favorable to avoid denaturation during precipitation. To recover the protein of interest, additional steps such as centrifugation and dialysis are needed to remove contaminant proteins (Janson 2011). Shian et al. (2005), for example, successfully precipitated 1 l of homogenate pineapple juice at 55 % of ammonium sulfate. Partially purified fruit bromelain was also obtained at 30–80 % saturation by Ko et al. (1996). Similarly, Devakate et al. (2009) described the precipitation of fruit bromelain and achieved 2.81-fold purity at 40–60 % ammonium sulfate saturation. Organic solvents such as ethanol, ketones, methanol, propanol, and other alcohols in protein precipitation have been widely employed.

The addition of organic solvents will decrease the activity of water, as part of the water has been replaced by organic solvents, and cause the proteins to precipitate. Heinicke and Gortner (1957) and Soares et al. (2011) reported the use of acetone and ethanol as the precipitating agents, respectively. Surprisingly, ethanol precipitation yielded 2.07-fold purification, compared to 4.44-fold obtained by ammonium precipitation (Soares et al. 2011). Likewise, Soares et al. (2012) only managed to attain a purification factor of 2.28-fold at 30–70 % ethanol saturation. So far, organic solvent precipitation must

operate at subzero temperature until the desired concentration (10–100 %) is achieved to avoid denaturation (Wheelwright 1991). Nonetheless, recently, Silva et al. (2010) improved the quality of stem and rind bromelain recovery and activity in ethanol precipitation with the development of a fed-batch stirred precipitation tank equipped with conventional proportional-integral-derivative (PID) and adaptive PID controllers. They overcame the required temperature increment as a result of heat generated upon the addition of ethanol during precipitation. Organic polymers such as polyethylene glycol (PEG) can precipitate protein and obviate the need for temperature control during the process. Increasing the concentration of PEG increases the amount of precipitated bovine serum albumin (BSA) (Kumar et al. 2009). However, bromelain does not show the same effect as BSA (Soares et al. 2011).

Variations in the temperature, volume of homogenate, mixing conditions, and biochemical properties between fruit and stem bromelain caused the discrepant saturation levels between the above studies. The decrease of repulsive forces and the dominance of attractive forces at the isoelectric point cause protein aggregates to reduce their solubility. Thus, by manipulating the pH, contaminant proteins in the solution can be salted out. Low temperature is favorable in reducing the solubility and precipitating proteins, whereas higher temperature denatures proteins (Walsh 2002; Wheelwright 1991).

Aqueous two-phase system (ATPS)

ATPS involves the formation of two immiscible phases after the mixing of polymer and salts or the combination of two incompatible polymers (PEG and dextran) in an aqueous solution. The distribution of protein samples between these two phases is influenced by the ionic strength, pH, temperature, molecular weight of the polymer, and type of salt. For instance, under favorable conditions, the target protein tends to partition into the top phase, which is less dense, less polar, and hydrophobic (PEG phase), while contaminant proteins are found in the lower partition, which are more dense, more polar, and hydrophilic (dextran phase) and can be removed through centrifugation (Walsh 2002). A considerable amount of literature has been published on the purification of bromelain using aqueous two-phase systems. Bromelain has been extracted from pineapple fruit by ATPS containing PEG-potassium phosphate (Babu et al. 2008; Hebbar et al. 2012) or PEG-phosphate buffer (Ferreira et al. 2011). Bromelain has also been extracted from pineapple wastes by ATPS. For example, Ketnawa and Rawdkuen (2011) isolated bromelain from pineapple peels using a PEG/magnesium sulfate system, while Coelho et al. (2013) had purified bromelain from stems, barks, and leaves using a PEG/ammonium sulfate system. From all of these studies, it can be concluded that the highest

recovery of bromelain is obtained at the highest concentration of PEG and salts applied. This is due to the influence of volume exclusion and the salting out effect, whereby both the bromelain and total protein are in the top PEG-rich phases. However, the partition of bromelain on the top phase decreases as the molecular weight of PEG increases. This is probably due to the structural alteration of enzyme sites in the presence of high molecular weight PEG as well as the fact that bromelain is relatively partitioned in the bottom phase (Babu et al. 2008; Ketnawa et al. 2010). Analogously, thermoseparation of bromelain using an aqueous solution of polyethylene oxide (PEO)-polypropylene oxide (PPO) block copolymers done by Rabelo et al. (2004) had increased the activity recovery by approximately 79.5 %, with a purification factor of 1.25, but the values were relatively lower compared to those of Babu et al. (2008), Ketnawa et al. (2010), and Coelho et al. (2013).

Adsorption

The adsorption process can be defined as the binding of a dissolved solute to a solid adsorbent. Currently, the use of adsorption with a wide variety of adsorbents is becoming more common in bromelain preparation. For instance, Chen and Huang (2004) applied a magnetic nano-adsorbent using iron oxide nanoparticles as the core and polyacrylic acid (PAA) as the ionic exchange group. Notably, the process successfully produced 87.4 % bromelain activity after adsorption at pH 3–5 and desorption at pH 7. As bromelain has the property of having a single oligosaccharide chain attached to the polypeptide, Gupta and Saleemuddin (2006) used affinity binding to a Sepharose matrix pre-coupled with the lectin concanavalin A (Con A), which resulted in 60 % bromelain activity at pH 11. Meanwhile, Silveira et al. (2009) integrated the expanded bed adsorption (EBA) to investigate the effect of the expansion degree of Amberlite IRA 410 resin and the residence time distribution, which resulted in 13-fold bromelain purification. Recently, the use of dye ligand affinity membrane chromatography seems more promising because it offers high selectivity, a larger surface area, and excellent thermal and mechanical properties. The electrospun polyacrylonitrile (PAN) nanofibers developed by Zhang et al. (2010) were chemically modified by tethering them to chitosan as a composite bilayer membrane and Cibacron Blue F3GA (CB) as a dye ligand. This successfully captured 161.6 mg/g of bromelain. Sai-Nan et al. (2009) and Song et al. (2011) employed Reactive Red 120 (Red 120) as a dye ligand immobilized onto chitosan-coated magnetic nanoparticles (CS-MNPs) to achieve bromelain adsorption from aqueous solution. In these studies, 88.2 % activity of bromelain was achieved, and the bromelain adsorption isotherm fits well with the Freundlich model.

Reverse micelle extraction (RME)

The reverse micelle system is a liquid-liquid extraction technique for biomolecule separation in downstream processing. Reverse micelle systems consist of droplets of aqueous phase separated from an organic solvent by the presence of surfactants in the interface. The surfactants possess both hydrophobic and hydrophilic tails. The polar head (hydrophilic) is located inside the micelle, and the nonpolar tail is in contact with the solvent outside the micelle. The movement of protein inside and outside the micelle can be induced by manipulating the pH, the ionic strength of aqueous phase, and the surfactant concentration (Wheelwright 1991). Several reports on the application of RME for separation and purification of bromelain from pineapple fruit (Yin et al. 2011), juice (Fileti et al. 2009), and wastes (Chaurasiya and Umesh Hebbar 2013; Hebbar et al. 2012; Umesh Hebbar et al. 2008) are available. Yin et al. (2011) and Umesh Hebbar et al. (2008) have demonstrated that the forward extraction efficiency, purification fold, and specific activity of bromelain are optimal at pH 7.0–8.0. The proteins transfer from the organic solvent to the reverse micelle if the pH value of the aqueous phase is higher than the isoelectric point of bromelain (4.6). This is because of the electrostatic attraction that occurs when the protein has a positive charge and the internal surface of reverse micelle has a positive charge (cationic surfactant). Chaurasiya and Umesh Hebbar (2013) and Umesh Hebbar et al. (2008) concur that the concentrations of 150 mM cetrimonium bromide (CTAB) and 0.1 M sodium chloride (NaCl) for forward extraction and 0.5 M potassium bromide (KBr) in back extraction produce high-efficiency extraction of bromelain. The protein extraction rates increase when a low ionic strength of salt and a high concentration of surfactant are applied, which in turn favors the forward extraction process. The high concentration of the surfactant increases the stability and number of reverse micelles, while the high ionic strength of salt in the back extraction may reduce the enzymatic exclusion from the interior surface of reverse micelles (Yin et al. 2011). Hebbar et al. (2012) employed the optimized conditions for reverse micelles from Umesh Hebbar et al. (2008) and integrated the system with an ultrafiltration process, resulting in a bromelain activity recovery of 95.8 % and purification fold of 8.9. Fileti et al. (2009) improved the purification factor to 4.96 by continuous extraction of reverse micelles using a microcolumn with pulsed caps after applying the optimum conditions from the batch operation. Recently, affinity-based reverse micellar extraction and separation (ARMES) has been used to purify bromelain from pineapple core. The authors achieved 12.32-fold purification with 185.6 % activity recovery, which was higher than those reported by Umesh Hebbar et al. (2008), through the affinity interaction between the bromelain and concanavalin A ligand that been incorporated in the reverse micelle system (Kumar et al. 2011).

Chromatography

Chromatography is a separation method in which protein samples migrate through the column at different rates and conditions of solid stationary and mobile phases. The packed column in chromatography comprises two types of mechanisms: adsorption (e.g., ion exchange, hydrophobic interaction, and affinity) and nonadsorption (e.g., gel filtration) (Wheelwright 1991). Among these methods, ion-exchange chromatography is commonly used to separate bromelain from pineapple fruit, juice and wastes. For example, Murachi and Neurath (1960b) obtained two major components, fractions 2 and 5, which were similar in their activities toward various substrates after ion-exchange fractionation of pineapple juice using Duolite CS101 at pH 6.05. The purification procedure was improved by Murachi et al. (1964) over the aforementioned method (Murachi and Neurath 1960) through the removal of contaminant carbohydrates and colored material. The latter study successfully produced 0.87 g of purified bromelain from 10 g crude commercial bromelain by integrating gel filtration and ammonium sulfate and acetone precipitation steps after ion-exchange purification. Similarly, Yamada et al. (1976) applied ammonium sulfate precipitation after anion-exchange chromatography, which yielded a homogeneous fruit bromelain with a high specific activity of 10 U/mg. Ota et al. (1964) supplemented the observations of Murachi et al. (1964) by further characterizing the extraneous end-group of stem and fruit bromelain after purification by gel filtration and ion-exchange chromatography. Further fractionation by successive use of gel filtration using Sephadex G-75 and ion exchange using carboxymethyl (CM)-Sephadex and diethylaminoethyl (DEAE)-Sephacel successfully separated stem bromelain into six proteolytic components (SBA and SBB 1–5) and two active components of fruit bromelain fraction A (FBA) and B (FBB) (Ota et al. 1985). Likewise, several studies on stem bromelain purification using cation-exchange and affinity chromatography yielded nonglycosylated components of stem bromelain, referred to as ananain (Napper et al. 1994; Rowan et al. 1990) and F9 (Harrach et al. 1995), as well as the glycosylated components comosain (Napper et al. 1994), F4 and F5 (Harrach et al. 1995), and SBA/a and SBA/b (Harrach et al. 1998). Meanwhile, Ko et al. (1996), Devakate et al. (2009), and Gautam et al. (2010) had employed additional steps of ammonium sulfate precipitation and dialysis prior to ion-exchange chromatography purification to remove most of the impurities in the stem and fruit bromelain samples. A summary of the purification condition of ion-exchange chromatography is presented in Table 4.

Table 4 Purification conditions for ion-exchange chromatography

Type of bromelain	Type of ion exchange	Lysis buffer	Binding and washing	Elution	References
Stem bromelain F4 and F5	Cation (CM-Sephadex)	8.3 mg in 2 ml (50 mM bicine buffer, pH 8.9)	50 mM bicine buffer, pH 8.9	Gradient, 0–150 mM NaCl, F4 and F5 obtained at 50 and 60 mM of NaCl	Harrach et al. (1995)
Fruit bromelain	Anion (DEAE cellulose)	0.02 M potassium phosphate buffer + 100 mg sodium tetrathionate (pH 7.2)	0.02 M potassium phosphate buffer (pH 7.2)	0.4 M potassium phosphate buffer (pH 7.2)	Yamada et al. (1976)
Stem bromelain	Anion (DEAE cellulose)	1 g in 0.2 M potassium phosphate, pH 6.05	0.2 M potassium phosphate, pH 6.05	0.2 M potassium phosphate + 2 M KCl, pH 6.05	Murachi and Neurath (1960b)
Stem bromelain	Cation (CM-Sephadex)	3 g in 0.02 M sodium citrate buffer saturated with phenylmercuric acetate, pH 6.10	0.02 M sodium citrate buffer saturated with phenylmercuric acetate, pH 6.10	0.01 M sodium citrate buffer saturated with phenylmercuric acetate, pH 6.10	Ota et al. (1985)
Fruit bromelain	Anion (Q-Sepharose)	50 g in 0.1 M sodium phosphate buffer + 5 mM EDTA + 5 mM cysteine	50 mM Tris buffer, pH 8.5	50 mM Tris buffer + 0.3 M NaCl, pH 8.5	Bruno et al. (2006)
Stem and fruit bromelain	Anion (DEAE cellulose)	10 mM Tris-HCl buffer	0.5 M sodium phosphate buffer, pH 8	25 mM Tris-HCl + 50 mM NaCl, pH 8	Gautam et al. (2010)
Fruit bromelain	Cation	25 mM acetate buffer, pH 4	25 mM acetate buffer, pH 4	25 mM acetate buffer + 1 M NaCl, pH 4	Devakate et al. (2009)
Stem bromelain	Anion (Duolite A-2)	10 g in 100 ml (0.05 M potassium phosphate buffer, pH 6.1)	0.05 M potassium phosphate buffer, pH 6.1	0.05 M potassium phosphate buffer + 1 M KCl, pH 6.1	Murachi et al. (1964)
Stem bromelain	Cation (Mono Q HR)	8 mg in 3 ml (20 mM Tris-HCl, pH 8)	20 mM Tris-HCl, pH 8	20 mM Tris-HCl + 250 mM NaCl, pH 8	Harrach et al. (1998)
Fruit bromelain	Cation (SP-Sepharose)	20 mM acetate buffer + 0.1 mM EDTA, pH 5	20 mM acetate buffer, pH 5	20 mM acetate buffer + 1 M NaCl	Hale et al. (2005)

Future directions

Up to the present, bromelain is a plant protease of choice, owing to its multitude of applications in industries such as tenderization, food, beverage, and pharmaceuticals. However, the utilization of bromelain is still in its infancy due to the rising dispute whether pineapple crops should be designated for growth as food or for bromelain extraction. Additionally, as the main source of bromelain, pineapples carry issues of space required for cultivation, seasonal growth, differences in pharmacological activities, and accessibility of mass production. Therefore, variation in the availability of bromelain sources, such as waste generated from pineapple processing in the form of the crown, peel, stem, and core, is likely to be crucial to fulfill the demand for this enzyme. Separation and isolation of bromelain from pineapple crops are only in the developmental stage, where challenges in technology, purity, and the economic aspects of large-scale production still need to be explored. A full discussion of the pros and cons of all listed purifications is shown in Table 5.

Thanks to the advent of recombinant technology which exploited *E. coli* as the workhorse for gene expression systems, the cloned bromelain gene has finally opened up the possibility of tailor-made protein with desirable properties (Amid et al. 2011). Despite the extensive application of protein engineering, there are still research bottlenecks that should be alleviated in the downstream process of recombinant bromelain production. The insertion of a poly-histidine sequence at the N- or C-terminus of the protein-coding region seems to allow the binding of recombinant bromelain to affinity column that has metal ions attached to it, but such a protein might be susceptible to co-elute with the native *E. coli* proteins, which are referred to as contaminants during the purification process. Therefore, the optimization of purification conditions or re-engineering an *E. coli* strain by mutation or alternative tag addition can be a realistic solution to improve the purity of recombinant bromelain and reduce the number of purification steps. However, in order to avoid unnecessary protein binding to the column, sometimes additional steps such as centrifugation, ultrafiltration, or precipitation are required to remove remaining host cell proteins, polishing, and intensification of the desired protein. The multiple steps in the chromatographic method associated with the huge amount of manufacturing cost needed ultimately will cause a significant drawback in the large-scale downstream process.

Among nonchromatographic methods presented in the aforementioned literature, the aqueous two-phase systems (ATPS) could be promising with the burgeoning interest in bromelain research. This method was adapted successfully in various researches that involve recombinant proteins with a high degree of purity (Bhambure et al. 2013; Lan et al. 2013; Omidinia et al. 2010). This purification method is easy to scale

Table 5 Comparison of different purification strategies

Type of purification	Advantages	Disadvantages
Ultrafiltration	– Concentrate the protein – Efficient to remove trace contaminants	– Long separation process – Increase protein loading will cause the ultrafiltration membrane clogging
Precipitation	– Easy to scale up with simple equipment requirements – Low energy needed – Efficient to remove trace contaminants	– Need a dialysis step to remove excess salt during precipitation
Aqueous two phase	– Low cost and shorter separation time – Easy to scale up and produce higher yield – Efficient to remove trace contaminants	– Difficult recovery of target protein from phase-forming polymer
Reverse micelle System	– Easy to scale up and can be operated in a continuous mode – No loss of native protein and activity – Low interfacial tension	– Difficult recovery of target protein from surfactant-containing organic solvent
Chromatography	– Highly specific and scalable	– Low separation efficiency and higher cost – Small sample loading capacity – Multiple chromatography steps

up as it only comprises water, salt, and polymer which is a biocompatible environment. With the optimized condition of pH and composition of salt and polymer, phase separation using centrifugation results in just one-step separation. Nevertheless, few challenges still hampered large-scale ATPS especially in the mechanism of phase separation (interaction between the protein, salt and polymer), reusability and toxicity of the polymer, and mode of operation (batch, semicontinuous, or continuous) (Azevedo et al. 2009). Current research in the manipulation of protein affinity to a biospecific ligand in the phase-forming polymer will favor the selective partitioning of the target protein. Besides, in view of the resolution to overcome drawback in the conventional ATPS system which requires back extraction to recover salt or polymer, the use of thermoseparating polymer could be one promising candidate. The temperature-induced phase partitioning method will offer cost-effectiveness and recyclability to overcome the bulk industrial waste disposal of polymer. Finally, pre-evaluation and detailed cost comparison with other separation methods must be taken into account to determine the viability of the ATPS process in an industrial scale (Espitia-Saloma et al. 2013).

Conclusion

Bromelain is among the protease enzymes that have been studied extensively. With the development of recombinant bromelain, new applications are expected in the field of pharmaceutical industry. Since the demand for large quantities of pharmaceutical products increases tremendously, the aqueous two-phase system offers high load capacity and easy to scale up for separation process with no compromise in the purity aspects. Modern methods of protein engineering combined with high-throughput approaches such as automatic

instrumentation during data collection and analysis are expected to enhance experimental efforts and create systematic methods. Thus, extensive effort involving the cooperation of many experts from various scientific and technological fields should be devoted to identify previously unexplored strategies to characterize recombinant bromelain for novel applications.

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