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Recombinant Enzymes - From Basic Science to Commercialization

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Chapter 10
Case Study: Recombinant Bromelain Selection

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Abstract This chapter presents an investigation that we performed prior to the decision to proceed cloning and producing recombinant bromelain. The criteria that we examined were the following: (1) easy access to a DNA source; (2) broad application; (3) an enzyme size amenable to the cloning strategy; (4) available data in free online databases; (5) broad industrial application.

Keywords Animal feed industry · Bread industry · Brewing industry · Bromelain · Bromelain mechanism of action · Cysteine protease · Diarrhea treatment · Fish industry · Food industry · Formulation · Meat industry · Media formulation · Nutraceutical and pharmaceutical industries · OFAT design · Optimizing · Personal care industries · Soy source industry

10.1 Why Bromelain?

Bromelain is an enzyme that is extracted from pineapples. Two types of bromelain, fruit and stem, exist. As indicated by the names, fruit bromelain is extracted from pineapple juice and stem bromelain is extracted from pineapple stems. Bromelain is classified as a proteolytic enzyme or protease because it exhibits proteolytic activity in nature, which indicates that it acts on a protein substrate. It is used in many industrial applications including the food, pharmaceutical, nutraceutical, and cosmetic industries as well as in the preparation of animal feed and human food supplements.

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Bromelain is a group 3 (hydrolase) and subgroup 4 (peptide bond hydrolase) enzyme, based on the International Union of Biochemistry and Molecular Biology (IUBMB) enzyme classifications. Bromelains are further subdivided into endo-peptidases or exo-peptidases, depending on their ability to hydrolyze internal or terminally localized peptide bonds. Additionally, proteases can be classified based on their mechanism of action. There are six mechanistic classes based on their catalytic sites [1], and bromelain is identified as a cysteine endopeptidase (EC 3.4.22). Cysteine protease (CP) activity depends on a catalytic dyad consisting of cysteine and histidine and the preferred arrangement of Cys and His (Cys-His or His-Cys) residues differ among family members [2]. The protease structure exhibits an α-helix and β-barrel-like motif, separated by a groove containing the active site which is formed by the Cys-25 and His-159 residues that are located at each side of the groove and are evolutionarily conserved among all family members [3]. Therefore, this unique enzyme and its biochemical properties potentiate its various applications in multiple industries.

10.1.1 Biochemical Properties of Bromelain

Several diverse properties of bromelain have been well studied such as stability, pH, optimum temperature and molecular weight. The enzymatic activity of bromelain on various substrates including casein, gelatin and other synthetic substrates can be determined under optimal pH and temperature conditions. Numerous publications have reported the molecular weight, as well as the optimal pH and temperature for bromelain activity. For example, Suh and co-workers estimated the molecular weights of the stem and fruit bromelains at approximately 37 and 32.5 kDa, respectively [1]. These researchers observed the maximum activity for the stem and fruit bromelains at pH 7.0, 60 °C and pH 8.0, 70 °C, respectively. Both enzymes were completely inhibited by sulfhydryl reagents and the Ki values for the stem and fruit bromelains for p-chloromercuribenzoate were 0.10 and 0.18 mM, respectively. In addition, the optimal pH for stem bromelain activity was between 6 and 7 for the two substrates studied (Z-Arg-Arg-NH-Mec and Bz-Phe-Val-Arg-NH-Mec). Additionally, stem bromelain had a molecular weight of 28 kDa and was inhibited by E-64 [2]. Similarly, Ketnawa and co-workers revealed that stem bromelain had the highest relative activity at pH 7.0 and 55 °C [3], but they later reported that the molecular weight of bromelain was 29 kDa and had the highest relative activity at pH 8.0 and 60 °C [4]. In another study, crude bromelain extracts from pineapple cultivars displayed caseinolytic activity over a broad pH range of 3–9 [5]. Other researchers have also reported molecular weights of 25, 24.5, 26 and 30 kDa for bromelain [6–8]. Table 10.1 summarizes results from different studies that assessed bromelain's molecular weight and the optimal pH and temperature for its activity.

10.1.2 Molecular Structure of Bromelain

The amino acid composition of bromelain was compared with two other types of pineapple proteases (ananain and comosain) extracted from the pineapple stem. The

Table 10.1 Bromelain molecular weight and optimum pH and temperature for its activity

Type of bromelain	Optimum pH	Optimum temperature (°C)	Molecular weight (kDa)	Reference
Stem Fruit	7.0 8.0	60 70	37 32.5	[1]
Stem	6–7	–	28	[2]
Fruit	7.0	55	–	[3]
Fruit	8.0	60	29	[5]
Fruit	3–9	50–60	–	[4]
Fruit	2.9–7.7	37–59	–	[12]
Stem	–	–	29	[7]
Fruit			24.5	[13]
Stem	–	–	26	[8]
Fruit	–	40	–	[14]
Stem	–	–	30	[6]
Fruit	6.0	70	–	[15]
Stem	–	50–60	–	[16]

Table 10.2 Reported amino acid composition of ananain, comosain and bromelain [10]

	No. of residues		
Amino acid	Bromelain	Ananain	Comosain
Asx	18	19	18
Thr	9	8	7
Ser	17	18	17
Glx	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

stem bromelain differed from the other enzymes in the number of polar and charged amino acids, particularly lysine and arginine, and in the number of several aliphatic amino acids, such as alanine and isoleucine (Table 10.2). Bromelain has been further differentiated from other pineapple proteases as reported by Maurer [15], and the details are presented in Table 10.3.

Table 10.3 Cysteine proteases derived from pineapples

Name (EC number)	Molecular mass (Dalton)	Isoelectric point	Sequences	Glycosylation	References
Stem bromelain (EC 3.4.22.32)	23,800 (sequence + sugar)	> 10	212 amino acids	glycosylated	[17]
Ananain (EC 3.4.22.31)	23,800 [10]	> 10	216 amino acids	Not glycosylated	[9]
Comosain	24,400	> 10		Glycosylated	
Fruit bromelain	23,000	4.6		Not glycosylated	[2]

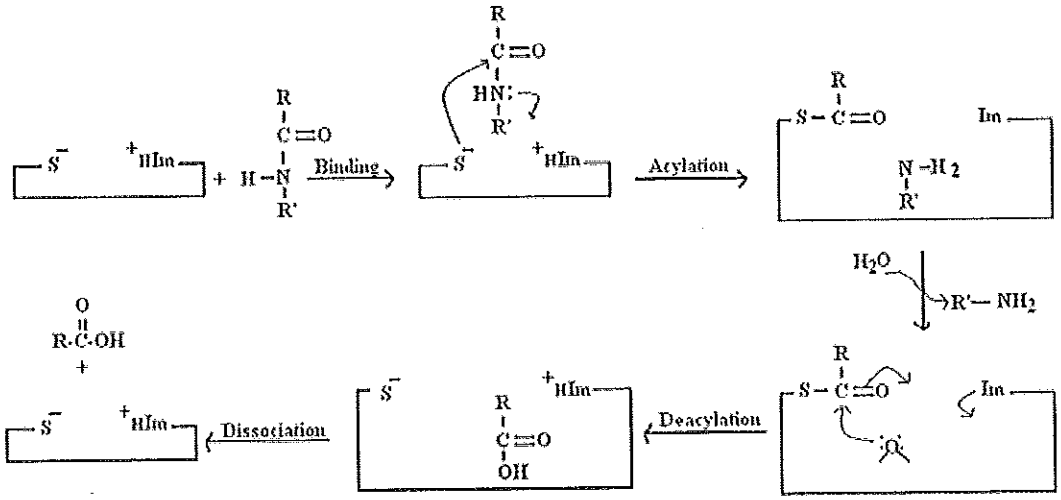

Fig. 10.1 Cysteine protease mechanism of action [18]

The amino acid sequences of these enzymes were initially discovered by Lee [9]. The amino acid sequence alignment indicates that the enzymes share the same active site Cys-25. Similarly, the position of the active site His-157 is identical among ananain, stem bromelain, papain, chymopapain and actinidin. Consequently, these proteases belong to the cysteine protease group [10]. The latest partial coding sequence for stem bromelain can be accessed at http://www.ncbi.nlm.nih.gov with the reference number JF332148 [11]. Our most recent search for a bromelain crystal structure on the protein database was not successful, only the papain crystal structure has been identified. Therefore, studies focused on obtaining the bromelain crystal structure are required.

10.1.3 Bromelain Mechanism of Action

The mechanism of action of bromelain is referred to as general CP activity. The mechanism of action for CPs involves the hydrolysis of carboxylic acid derivatives through a double-displacement pathway composed of a general acid-base formation and hydrolysis of an acyl-thiol intermediate [18]. The initial catalysis step (as described in Fig. 10.1, where Im and +Him refer to the imidazole and protonated

imidazole, respectively) involves the noncovalent binding of the free enzyme to the substrate to form a complex. This is followed by the acylation of the enzyme and the formation and release of the first product, the amine R'-NH_2. Deacylation follows, where the acyl-enzyme reacts with a water molecule to release the second product while regenerating the free enzyme, as exhibited in Fig. 10.1.

10.2 Bromelain in Food Industries

10.2.1 Bread Industry

Bromelain is used in baking such as in bread making because it can strengthen gluten. Gluten is a protein that is present in foods processed from wheat. Bromelain proteolysis hydrolyzes both gliadins and glutenins in gluten, thus improving the dough structure. It encourages dough relaxation, preventing dough shrinkage, and promotes better bread volume. This enzyme is suitable for industrial applications due to its rapid reaction and broad optimal pH and temperature ranges [19]. Bromelain is active in the dough and during unbaked bread leavening, but is inactivated by high temperatures during baking. Some of the residues remain in the inactivated enzyme form, which can be metabolized similar to other proteins [20]. Furthermore, bromelain has been used to generate hypoallergenic wheat flour due to its ability to degrade the wheat glutenin IgE epitope Gln-Gln-Gln-Pro-Pro [21].

10.2.2 Brewing Industry

Crude bromelain and other proteases [22] are used in the brewing industry to obtain good colloidal properties at low temperatures, which eliminate cloud formation [23]. The bromelain protease activity prevents aggregation of insoluble complexes by hydrolyzing the proteinous substances that normally precipitate polyphenols and oligosaccharides during cold storage [24]. Additionally, bromelain can solubilize protein from barley adjuncts and release peptides and amino acids that can then be utilized during fermentation as a nitrogen source [25].

10.2.3 Meat Industry

Meat softness and tenderness are the most vital determinants of consumer satisfaction and taste perception [26]. Proteolytic enzymes are typically used to improve meat tenderness. Furthermore, myofibril integrity and the contribution of connective tissue affect meat tenderness. Bromelain is extremely powerful hydrolyzing fibrous proteins and connective tissue [27], thus, it is used to tenderize tough meat. Additionally, bromelain increased tenderness and degraded collagen more extensively

than contractile proteins, whereas ficin exhibits the most balanced degradation of both myofibrillar and collagen proteins [28].

10.2.4 Fish Industry

Countries with large fishing industries generate problematic waste materials at manufacturing plants processing marine species. Endogenous visceral enzymes are used to produce fish protein hydrolysate (FPH) to obtain higher added values for such wastes. These processes may either generate sources of bioactive peptides or used as nitrogenous substrates for microbiological media [29, 30]. Consequently, plant proteases such as bromelain, papain, ficin, and alcalase (contains ananain) have been widely used to generate FPHs [31].

10.2.5 Soy Source Industry

Soybeans are well-established, rich food sources due to their large content of qualitative protein. Proteases such as bromelain have been widely used to prepare soy sauce and other soy products. The proteolytic hydrolysis of soy proteins enhances their functional properties. Consequently, treating soy proteins with bromelain or alcalase generates soluble hydrolysates that exhibit good yield and low bitterness [24].

10.3 Bromelain in the Nutraceutical and Pharmaceutical Industries

Pineapples, which are a large source of bromelain, have been traditionally and widely used by South American, Chinese and Southeast Asian populations [32]. Bromelain systemically affects several cellular and molecular targets. Some bromelain's relevant therapeutic applications are summarized in this section.

10.3.1 Bromelain as an Anti-Inflammatory Agent

Bromelain has been demonstrated to be an effective anti-inflammatory agent. The major mechanisms of action appear to be proteolytic in nature and mediated via the following factors: increased serum fibrinolytic activity [33], reduce plasma fibrinogen levels [34] and decrease bradykinin levels, which result in reduced vascular permeability and thereby a reduction in edema and pain [35]. Bromelain can modify the leukocyte expression of cell surface molecules. Specifically, bromelain can remove cell surface molecules, including the CD128 chemokine receptors, by preventing the firm adhesion of leukocytes to blood vessels at the site of inflammation [36]. Studies

conducted by Bhui and co-workers, Huang and co-workers and Gaspani and co-workers [37–39] suggested that bromelain inhibit cyclooxygenase-2 (Cox-2) expression and thus decreases other inflammatory cascade proteins, including prostaglandin E2 (PGE2). Cox-2 is an enzyme (EC 1.14.99.1) that participates in the formation of important biological mediators called prostanoids, including prostaglandins, prostacyclin and thromboxane. Hale and co-workers [40] also observed that bromelain removed several types of cell surface molecules, thereby reducing leukocyte adhesion and activation and thus decreasing inflammation. Moreover, Gaspani and co-workers [38] revealed that bromelain-treated rats exhibited reduced concentrations of prostaglandin E2 (PGE2), which is a key mediator of the immune response. In addition, bromelain decreases the levels of PGE2 and thromboxane A2 and modulates certain immune cell surface adhesion molecules, which participate in the pathogenesis of arthritis [40–44]. Bromelain can reduce cell surface receptors, such as the hyaluronan receptor CD44 that is associated with leukocyte migration and an induction of proinflammatory mediators [40, 45]. Manhart and co-workers [46] have supported those findings when they demonstrated that bromelain significantly reduced CD4+ T lymphocytes, which are the primary effectors in animal models of inflammation. Several in-vivo results have revealed that bromelain acts as an anti-inflammatory agent. In fact, numerous clinical trials with bromelain have demonstrated its efficacy in treating various inflammation-based conditions, including sepsis in children [47], rhinosinusitis [48], breast engorgement during lactation [49], urogenital inflammation [50] and osteoarthritis of the knee and hip [51, 52]. In a mouse model of inflammatory bowel disease, bromelain decreased the clinical and histological rigorousness of spontaneous colitis and colonic inflammation [53]. Moreover, Secor and co-workers [54] established that systemic bromelain decreased the inflammatory process in a mouse model of allergic airway disease. Additionally, anecdotal evidence indicates that bromelain may be effective in treating mild ulcerative colitis [55].

10.3.2 Bromelain as an Anti-Tumor Agent

Studies have demonstrated that bromelain can reduce tumor growth. Bromelain was first reported to reduce malignant growth in 1972 by Gerard [56], followed by Nieper in 1974 [57]. The anti-cancer effects of bromelain are largely attributed to its protease activities [58]. Bromelain is believed to act on cancer cells via the proteolysis of extracellular proteins such as CD44. CD44 is a glycoprotein adhesion molecule that critically participates in tumor growth and metastasis [59, 60]. It is a cell receptor for hyaluronic acid and participates in many stages of cancer metastasis [61–63]. Multiple studies have implicated chronic inflammation, immune suppression and deregulation of the hemostatic system in carcinogenesis [32]. This suggests that bromelain may target pathways that directly participate in cancer initiation, growth and development. Current evidence reveals that bromelain may represent a potential target for developing oral enzyme therapies for cancer. In the past, adjuvant therapy with external proteases has produced positive results in cancer treatment; the therapy side effects were reduced and survival was prolonged [64]. *In*

vivo studies have reliably demonstrated the tumor-inhibitory effects of bromelain. In chemically induced mouse skin papillomas, bromelain decreased tumor formation and tumor volume and promoted apoptotic cell death [65]. These findings are consistent with other studies where bromelain decreased metastasis [66] and local tumor growth [67], thereby increasing survival rates. Moreover, *in vitro* bromelain treatment in mouse tumor cell lines inhibited cell growth and Matrigel invasion capabilities [68]. Another study revealed that bromelain inhibited cell adhesion and migration in glioblastoma cell lines [69]. The same study also discovered that bromelain reduced the invasive capacity of glioblastoma cells and de novo protein synthesis [69], suggesting that bromelain is a good cancer therapy candidate.

10.3.3 Burn Debridement and Wound Healing

Bromelain has been extensively used in burn debridement [70–72]. Therefore, the enzyme could be a tenable option for surgical escharotomy in deep burn patients. An *in vitro* study demonstrated that bromelain preparations can effectively debride full-thickness burns in pig skin within 1 day. The enzyme affected only burned skin and resulted in minimal blood loss [73]. In a porcine model of burn-induced compartment syndrome, circumferential limb burns treated with bromelain exhibited a significant reduction in intra-compartmental pressures [71]. In addition, Rosenberg an co-workers [70] reported complete scar debridement (burned and traumatized tissue) after one to two brief bromelain applications with minimal side effects and no blood loss. Moreover, topical bromelain cream has been reported to achieve complete debridement of experimental burns in rats within approximately 2 days [74].

Similarly, the enzyme may exert beneficial effects on soft tissue wound healing. More rapid reductions in edema and bruising have been reported in patients with episiotomy wounds treated with bromelain [73]. In a study of wound healing, bromelain treatment at the early phase decreased the soft tissue wound healing period [75]. In another study conducted by Hu [76], bromelain greatly simplified the management of high-velocity gunshot wounds in a pig model. Moreover, bromelain was also demonstrated hydrolyze devitalized tissue in wound tracks without apparent injuries to the surrounding normal tissue and enhanced firearm wound healing [77].

10.3.4 Diarrhea Treatment

The anti-diarrheal activity of bromelain has been previously established [78, 79]. These studies have suggested that the proteolytic activity of bromelain inactivates a specific glycoprotein receptor located on the intestinal mucosa and blocks attachment of enterotoxigenic bacteria. Studies conducted by Mynott [80] have indicated that stem bromelain exhibited anti-secretory properties by preventing fluid secretion mediated by secretagogues that act through cAMP (cyclic-3, 5- adenosine monophosphate), cGMP (cyclic-3, 5- guanosine monophosphate)

and calcium-dependent signaling pathways. Because toxins that cause diarrhea activate one of these pathways, bromelain likely exerts anti-diarrheal effects. The enzyme can also block secretory changes caused by prostaglandin E, theophylline, calcium-ionophore A23187, 8-Br-cAMP 2 (8-bromocyclic-3, 5-adenosine monophosphate) and 8-Br-cGMP (8- bromocyclic-3, 5-guanosine monophosphate), which are well-characterized intracellular mediators of ion secretion. Experiments performed by Roselli [81], which assessed the effects of different plant extracts and natural substances (PENS) on membrane damage in pig intestinal cells, demonstrated the protective effects of bromelain.

10.3.5 Bromelain as Anti-Thrombotic Agent

Bromelain can prevent the aggregation of human blood platelets and prevent or minimize the severity of angina pectoris and transient ischemic attacks (TIA). The enzyme is also essential for preventing and treating thrombosis and thrombophlebitis as well as for degrading cholesterol plaques and exerting fibrinolytic activity [82]. Hale and co-workers [40] revealed that *in vitro* bromelain treatment of leukocytes in whole blood modified 25% of the leukocyte markers studied. The bromelain induced loss of CD41 and CD42a can be expected to reduce platelet function and, hence, inhibit thrombus formation [74]. Similarly, Felton [83] suggested that a bromelain plasminogen activator may produce plasmin in rat experiments. Plasmin cleaves the Hageman factor and leads to a strong release of kallikrein but a weak release of thrombin; moreover, a combination of fibrinolytic and antithrombic properties appear to be effective, as two large-scale tests in heart patients demonstrated an almost complete elimination of thrombosis. In addition, bromelain was observed to increase the permeability of vessel walls to oxygen and nutrients while increasingly concentrating blood, both of which assist bromelain as antithrombotic agent [84] Furthermore, Metzig and co-workers [84] revealed that pre-incubating human platelets with bromelain completely prevented thrombin-induced platelet aggregation *in vitro*. Correspondingly, the authors reported that bromelain inhibited *in vivo* thrombus formation in a model of laser-induced thrombosis in rats.

10.3.6 Enhancing the Immune System

Bromelain bolsters the immune system by increasing cytokine production, which are hormones produced by white blood cells to improve immunity. Several studies have established the ability of bromelain to remove T-cell CD44 molecules from lymphocytes [40, 85, 86]. In a study conducted by Munzig and co-workers [87], highly purified bromelain protease F9 decreased the expression of CD44 ten times more than crude bromelain, resulting in an approximately 97% inhibition of CD44 expression. Similarly, Roep and co-workers [86] discovered that protease treatment reduced the expression of cell surface receptors on T-cells and antigen-presenting cells. Moreover, protease therapy has been reported to reduce CD44 expression

Table 10.4 Bromelain-based personal care products

Brand	Country	Remarks	Price (USD)	Function
NOW Food	USA	2500 GDU	29.99	Digestive aid
SOLARAY	UK	Mix with quercetin and vitamin C	28.09	Dietary supplement
Doctor's BEST	USA	Mix with quercetin	35.99	Food supplement
Natrol	USA	Maximum strength	21.99	Digestive aid
Natural Radiance	USA	Mix with glucosamine	58.98	Pain relieving cream
PERFECT IMAGE LLC	USA	Mix with papaya extract and alpha hydroxy acids	34.95	Facial peel s
Kate Somerville	USA	–	75.00	Dark circle eye cream

on lymphocytes from patients with multiple sclerosis [86, 87]). Furthermore, Hale [44] discovered that bromelain exhibits a strong immunogenicity subsequent to oral dosing. Additionally, Hale and co-workers [88] revealed that repeated exposure was necessary for the maturity of anti-bromelain antibodies with a dose-dependent exposure period of 3–6 weeks.

10.4 Bromelain in the Personal Care Industry

Bromelain has relatively recently become available for commercial purchase at numerous personal care outlets. It helps to degrade dead, dry surface skin cells, leading to softer and smoother skin. This effect is helpful for dry and/or blemished skin. In fact, bromelain degrades the connecting structure that holds surface skin cells together, which is exfoliating but can be irritating. However, more studies are needed to demonstrate how bromelain acts on the skin. Bromelain is also consumed as a food supplement and formulated as a topical cream to relieve pain. Table 10.4 displays several examples of bromelain-based personal care products including the brand, activity or strength offered and price.

10.5 Bromelain in the Animal Feed Industry

Feed enzymes are typically added to animal feed to increase nutrient bioavailability by acting on feed components prior to or after consumption. Theoretically, bromelain would digest proteins in animal feeds into smaller units and promote their absorption by the digestive track. Bromelain is used in animal feeds, especially for ruminant animals, as a digestive aid in the lumen and as a mastitis preventative. Additionally, bromelain supplements have been demonstrated to increase milk protein and milk fat in dairy goats [89]. An independent study by

Table 10.5 Bromelain sold as a food additive in animal feeds

Product	Company	Country
Bromelain powder	Rolling Dies Manufacturer	Thailand
Bromelain powder	Wisapple	China
Bromelain powder	K&G Global Business Corp	Taiwan
Bromelain powder	BIO-CAT Inc	Spain

Tománková and Kopečný [90] indicated that bromelain had the highest protein degradation efficiency compared with pronase E and papain [90]. Table 10.5 provides examples of companies that sell bromelain suitable for use in animal feeds.

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