

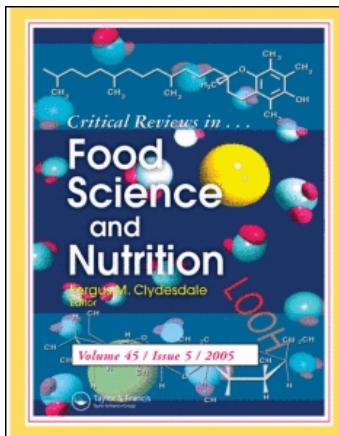
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The Biochemical and Functional Food Properties of the Bowman-Birk Inhibitor

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The Bowman-Birk inhibitor (BBI) is a small water-soluble protein present in soybean and almost all monocotyledonous and dicotyledonous seeds. The molecular size of BBI ranges from 1,513 Da to about 20,000 Da. BBI is to seeds what α_1 -antitrypsin is to humans. Soy-based food products rich in BBI include soybean grits, soymilk, oilcake, soybean isolate, and soybean protein concentrate. BBI is stable within the pH range encountered in most foods, can withstand boiling water temperature for 10 min, resistant to the pH range and proteolytic enzymes of the gastrointestinal tract, bioavailable, and not allergenic. BBI reduces the proteolytic activities of trypsin, chymotrypsin, elastase, cathepsin G, and chymase, serine protease-dependent matrix metalloproteinases, urokinase protein activator, mitogen activated protein kinase, and PI3 kinase, and upregulates connexin 43 (Cx43) expression. Several studies have demonstrated the efficacy of BBI against tumor cells in vitro, animal models, and human phase IIa clinical trials. FDA considers BBI as a drug. FDA also approves labels claiming that consumption of at least 3 to 4 oz of tofu or 8 oz of soymilk or soy protein may reduce the risk of coronary heart disease and breast cancer. This review highlights the biochemical and functional food properties of the Bowman-Birk inhibitor.

Keywords Bowman-Birk inhibitor, soybean (*Glycine max*), functional foods, cancer, angiogenesis, tryptase and/or chymase inhibitor

INTRODUCTION

Epidemiological observations during the last century have shown that cancer incidence and mortality vary significantly across regions of the world (Kelloff et al., 2000; Willett, 2000; Stewart and Coates, 2005). Populations whose major diets are mainly centered on plant foods tend to have a lower rate of cancer (Munoz de Chavez and Chavez, 1998). In the western world, the rate of colon, breast, and prostate cancer are high (Kelloff et al., 2000; Willett, 2001). The rates of cancer among populations migrating from low- to high-incidence countries can change markedly. In most cases, they approximate the rates in the new region within one to three generations (Willett, 2000). This indicates that the primary determinants of cancer rates are not genetic factors, but rather environmental and lifestyle factors and could, in principle, be modified to reduce cancer rates in high-risk areas (Kelloff et al., 2000). While in most cases, the genetic determinants appear to affect 5–10% of the population,

in the last two decades the primary factors of interest, apart from tobacco, has been diet.

Proteases catalyze many physiological as well as pathophysiological processes such as food digestion, tissue remodeling, host defense, blood coagulation, the activation of proenzymes and prohormones, to name a few. The role of proteases is crucial in normal cellular metabolism. Natural protease inhibitors constitute the final regulatory step in the control of protease activities. During homeostasis there is a biological balance between proteases and their natural inhibitors. However, under certain physiopathological conditions, the balance between endogenous proteinase inhibitors and proteases is lost. An imbalance between proteases and their endogenous inhibitors in favor of proteases in the body may trigger uncontrolled proteolysis which leads to irreversible tissue destruction such as inflammation processes, rheumatoid arthritis, periodontitis, hypertension, gastric ulcer, muscular dystrophy, pathological angiogenesis, or tumor growth and metastasis (Von der Helm et al., 2000; Noel et al., 1997). Factors that contribute to the physiological imbalance include (1) reduced natural inhibitor activity due to proteolytic degradation by lysosomal enzymes, (2) saturation of inhibitor with excessive enzymes released from the lysosomes, (3)

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inhibitor inactivation by reactive oxygen derived from H_2O_2 catalyzed by a myeloperoxidase released by neutrophils in granulocytes, and (4) increased stability of lysosomal cathepsins which are inactive in the extracellular space (Otto and Schirmeister, 1997). Prevention of excessive proteolysis represents a strategy for chemoprevention.

Natural proteinase inhibitors are small molecules such as fatty acids, phenolic compounds, amino acids, peptides, or large molecules such as polypeptides and proteins (α_2 -macroglobulin, α_1 -proteinase inhibitor, α_2 -antiplasmin, α_1 -antichymotrypsin, antithrombin III) which have the ability to specifically or non-specifically inhibit the catalytic activities of proteolytic enzymes in biological systems. Natural proteinase inhibitors are endogenous to the human body and can be found in viruses, bacteria, protozoa, plants, and mammals.

A comprehensive report by the National Cancer Institute (NCI) of the United States emphasized the importance of protease inhibitors is disease therapy and recommended that research for a cure for cancer should (i) identify sources of protease inhibitors, (ii) determine the concentration of protease inhibitors in human foods, (iii) determine the relationship between protease inhibitor concentration in diets and the rate of cancer in human population, (iv) determine the efficacy of protease inhibitors in animal models, and (v) investigate the mechanism of action of protease inhibitors (Troll, 1993).

This review focuses on the biochemical and functional food properties of the Bowman-Birk inhibitor (BBI). A brief introduction on proteinase classes is provided.

Proteinase Classification

Proteinases are classified, on the basis of their catalytic mechanisms and according to inhibitors, into five families: serine, cysteine, metallo, aspartic, and threonine proteinases.

Serine Proteinases (EC. 3.4.21)

All serine proteinases have similar catalytic sites characterized by a serine hydroxyl group at residue 195 in the active site. Serine proteinases also exhibit esterolytic activity towards ester bonds. Trypsin, chymotrypsin, elastase, chymase, cathepsin G, plasmin, thrombin, and subtilisin are classical examples of functional serine proteinases. Maximal enzymatic activity occurs at about pH 8.0. In the free enzyme, the gamma-hydroxyl group at active site residue 195 forms a tetrahedral intermediate with the carbonyl group of the scissile amide bond of the substrate (Stroud et al., 1975). Inhibitors of serine proteinases react with the active site Ser-195 to form inactivated enzyme derivatives. There are three major groups of serine proteinases: the trypsin-like (tryptases), the chymotrypsin-like (chymases), and elastases. Trypsin preferentially cleave substrate containing positive residues such as Arg or Lys at the P1 site due to interaction with Asp-189 in the S1 pocket of this group of enzymes. Chymases and elastases preferentially hydrolyze

substrate containing aromatic or large hydrophobic and small aliphatic residues at P1. Classical inhibitors of this class are the Kunitz type inhibitors (bovine pancreatic and soybean trypsin inhibitors, the Bowman-Birk type inhibitors, potato type I and II inhibitors, and the Kazal type inhibitors (pancreatic secretory trypsin inhibitors).

Cysteine Proteinases (EC. 3.4.22)

A catalytic histidine imidazole group, His-159 and cysteine sulfhydryl group, Cys-25 are located at the active site of cysteine proteases. Cysteine proteases catalyze the hydrolysis of peptide, amide, ester, thiol ester, and thiono ester bonds. The hydrolysis generates a thioester intermediate whose acyl group is transferred to a water molecule with concomitant release of the carboxylic acid portion of the substrate and regeneration of the active enzyme. These enzymes are found in yeast, fungi, and worms. The molecular basis of cysteine protease hydrolysis is not well known (Otto and Schirmeister, 1997). It is not well known if Cys25 and His159 are sufficient for full catalytic activity. Papain, ficin, bromelain, calpain, and Cathepsin A, B, and C are members of this class. Inhibitors of this class consist of bovine nasal cartilage, human α_1 -serum protein, IgG, egg white cystatin, and rice cystatin.

Metalloproteinases (EC. 3.4.34)

The catalytic residue of most metalloproteinases contain zinc atom and four ligands-two histidine residues, a glutamic residue and a water molecule arranged in a tetrahedral geometry. The interaction of the P1' side chain with the S1' subsite is recognized as the primary recognition between the enzyme and substrate. This is followed by the binding of the substrate to the active site with the scissile carbonyl group coordinating to the active site zinc. Then the zinc polarizes the scissile peptide carbonyl and an adjacent glutamic acid residue acts as a general base catalyzing the addition of water to the substrate (Powers et al., 1993). Classical examples of metalloproteinases include the angiotensin converting enzyme, carboxypeptidases, and all the more than twenty-five matrix metalloproteinase enzymes (collagenases, gelatinases, and stromelysins) discovered to date (Ala-Aho and Kahari, 2005; Handsley and Edwards, 2005; Woessner, 2002; Heath and Grochow, 2000; Laskowski and Kato, 1980). Naturally-occurring inhibitors include bovine aorta cartilage, bovine nasal cartilage, potato inhibitor, and β_1 serum protein (Powers et al., 1993; Heath and Grochow, 2000).

Aspartic Proteinases (EC. 3.4.23)

Carboxyl side-chains of two aspartic acid residues Asp-32 and Asp-215 have been suggested to be responsible for operating the catalytic activity among aspartic proteases (Kay et al., 1982). Most aspartic proteinases have a high content of dicarboxylic and hydroxy amino acid residues and a low content of basic amino acids. Pepsin, chymosin, cathepsin D, cathepsin E,

gastricsin, ornithine decarboxylase, and HIVprotease are classical examples of aspartic proteinases. Pepsin, chymosin, and gastricsin are gastric proteases. Some of these enzymes are also found in the prostate (gastricsin and cathepsin D), breast (cathepsin D), and colon (cathepsin D and ornithine decarboxylase). Rennin is found in the plasma, kidney, and submaxillary gland. Their molecular weights usually range between 30,000 and 42,000 daltons (Ryle, 1970), except for some cathepsins that have a molecular size higher than 90 kilodaltons. The pH of optimal activity for most aspartic proteinases is between pH 1.5–5.5. Aspartic proteinases have extensive sequence homology among them and there is particularly a high conservation of residues around the catalytic Asp-32 and Asp-215.

An enzyme is classified as aspartic proteinase when it is inhibited by pepstatin, diazoacetyl norleucine methyl esters, and EPNP-epoxy (*p*-nitrophenoxy) propane. Chymosin and pepsin are extensively used in the food industry as processing aids. Chymosin or rennet is used for cheese curd formation at pH 6.2–6.7. Pepsin from porcine and bovine stomach is used for the production of peptides for medical purposes.

While inhibitors of serine, cysteine, and metalloproteinases are ubiquitous in nature there exists a limited number of naturally-occurring food grade aspartic proteinase inhibitors known to date. Pepstatin, an acylated pentapeptide, the best known of aspartic proteinase inhibitors is obtained from various species of Actinomycetes. A 17,000 daltons protein from *Ascaris lumbricoides*, a 16–17 amino acid residues released during activation of pig/cow pepsinogen, and an extract from yeast are some of the best aspartic proteinase inhibitors known to date. Food sources of cathepsin D inhibitor include potato, squash, and cucumber. However, these food-derived inhibitors do not resist the acidic pH of the stomach. Their addition to foods would be meaningless unless they are protected to bypass the acidic environment of the stomach.

Threonine Proteinases (EC. 3.4.25.1)

The involvement of N-terminal Thr as nucleophile in the catalytic mechanism of proteasome classifies this enzyme as belonging to N-terminal nucleophilic hydrolases. The catalytic mechanism of threonine proteinase involves threonine as the N-terminal nucleophile and a water molecule acting as a virtual base (Duggleby et al., 1995; Lupas et al., 1995). Examples of threonine proteinases include the intracellular proteasome whose catalytic active site is a threonine residue. The proteasome is a multicatalytic protease that possesses a 20S core constituted by four stacked rings and each of the two inner rings is composed of seven different β subunits. Each β -ring contains three different proteolytically active sites of which the β 1 subunit contains a postacidic active site, the β 2 subunit is associated with a trypsin-like activity, and the β 5 subunit exerts a chymotrypsin-like proteolytic function. All proteolytic sites have a catalytic mechanism similar to those of serine proteases and utilize an N-terminal threonine residue as nucleophile. Usually, inhibitors of proteasome interact with the catalytic threonine. The 26S pro-

teasome hydrolyzes the cytoplasmic inhibitor inhibitory kappaB ($\text{I}\kappa\text{B}$), and as a result allows cytoplasm-to-nucleus translocation and subsequent activation of the transcription factor nuclear factor kappaB (NF- κB). Selective proteasome inhibitors block $\text{I}\kappa\text{B}$ degradation and consequently NF- κB activation in a variety of cellular systems. However, there are conditions where $\text{I}\kappa\text{B}$ degradation and NF- κB activation are not mediated by the proteasome system.

OCCURRENCE OF THE BOWMAN-BIRK INHIBITOR

Sources

There are several sources of BBI. While the soybean BBI is the classical representative of the BBI family and has been extensively studied, related BBI from other monocotyledonous and dicotyledonous seeds are being gradually identified and characterized (Luckett et al., 1999; Rakwal et al., 2001; Zablotna et al., 2002; Song et al., 1999; Rao et al., 1999; De Freitas et al., 1997; Prakash et al., 1994; Weder and Haussner 1991; Norioka et al., 1982 Tashiro and Maki, 1979; Tashiro et al., 1987). For simplicity, soybean BBI will be referred to as BBI and BBI from other sources will be identified using a symbol representing the seed followed by BBI. BBI was isolated for the first time in soybean by Bowman in 1946, characterized by Birk in 1947, and was therefore named after the two scientists. Since, the presence of similar BBI has been reported in almost all monocotyledonous and dicotyledonous seeds (Luckett et al., 1999; Rakwal et al., 2001). BBIs are found in the seeds of Fabaceae family of Leguminous plants. The Fabaceae family has 50 tribes of which the Phaseoleae, Glycineae, and Viciae tribes are morphologically close. To name a few, BBI have been isolated from rice bean (*Vigna umbellata* T), red kidney bean (*Phaseolus vulgaris*), brazilian pink bean (*Phaseolus vulgaris*), lima bean (*Phaseolus lunatus*), adzuki beans (*Phaseolus angularis*), chickpea (*Cicer arietinum*), pea (*Pisum sativum*), lentil (*Lens culinaris*), and pigeon pea (*Cajanus cajan*, syn. *Cajanus indicus*) (Godebole et al., 1994; Wu and Whitaker, 1991; Prakash et al., 1996; Kiyohara et al., 1981; Funk et al., 1993). Sunflower (*Helianthus annuus*) seeds contain a cyclic 14-amino acid BBI with an M_r , determined by electrospray mass spectroscopy (ESMS), of 1513 (Luckett et al., 1999). Wound-induced BBI has been reported in alfalfa and tobacco leaves (Brown et al., 1985; McMunnus et al., 1994). BBI-deficient soybeans, although in a small percentage, has also been reported (Stahlhut and Hymowitz, 1983; Domagalski et al., 1992).

Biosynthesis

Hammond et al. (1984) isolated and analyzed the gene encoding soybean BBI and determined its nucleotide sequence by constructing cDNA clones from mRNA of developing soybean seeds. The mRNA for BBI was shown to accumulate early during

embryogeny and to be about four hundred fifty nucleotide long. BBI genes have no introns.

BBI is biosynthesized as a preinhibitor containing sixteen residues extension at the N-terminus (Joudrier et al., 1987; Hilder et al., 1989; Domoney et al., 1995). The pro-BBI is converted to BBI following cleavage of a Ser-Asp bond. BBI is found in the intracellular spaces of cotyledons of ungerminated seeds and is rapidly released from the seed within the first eight hours of imbibition (Horisberger and Tacchini-Voulathan, 1983).

The sequences of cDNA clones encoding the Bowman-Birk proteinase inhibitor (BBPI) and its isoinhibitors (D-II and C-II) have been identified (Baek et al., 1994). Nucleotide sequence homologies among these clones and between the two reactive domains in each clone were very high. It was suggested that the high nucleotide sequence homologies among these clones and between the two reactive domains was indicative of an evolution from a common ancestral inhibitor. Legume seeds, such as *Erythrina variegata*, do not have the N- and C-terminal segments of their BBI, and may have lost these terminals during evolution (Kimura et al., 1994).

Screening/Identification

Traditionally, the characteristics properties of BBI, i.e. low molecular weight (about 8.0 kDa), electrophoresis, and trypsin and chymotrypsin inhibition were used to identify BBI. Brandon et al. (1989, 1991) developed and patented monoclonal antibodies and immunoassay methods to detect BBI in processed foods. Arentoft et al. (1993) used high-performance capillary electrophoresis (HPCE) to characterize the association complexes between soybean and pea BBI and mAb against BBI. Polyclonal antibodies of low specificities against BBI have also been reported (Sreerama and Gowda, 1997; Hwang et al., 1997).

Quantitative Assays

Immunoassays, specifically ELISA using polyclonal or monoclonal antibodies to BBI are ideal methods for BBI quantitative determination in a protein matrix (Brandon et al., 1989, 1991; Sreerama and Gowda, 1997; Gladysheva et al., 2000). Gladysheva et al. (2000) raised antibodies against BBI polymerized with glutaraldehyde to quantitate BBI in soybean preparations. However, the lack of commercially available antibodies against BBI makes it difficult for investigators to measure BBI quantitatively in soybean products that claim the health enhancing properties of BBI. Most investigators have used active site titration with trypsin or chymotrypsin to determine BBI activity (Larionova et al., 1993; Ware et al., 1997). Rapid and cost-effective methods for the quantitative determination of BBI in processed foods are needed to meet the demand of processors, researchers, and consumers.

MOLECULAR PROPERTIES OF BBI

Isolation/Purification

Historically, Read and Haas (1938) were the first to demonstrate that an aqueous fraction of soybean flour contain enzymes that inhibit trypsin (soybean trypsin inhibitor or STI) and trypsin and chymotrypsin (BBI) (Birk, 1961). However, Bowman (1944) pioneered the isolation procedure while Birk and associates purified the protein that now carries the name Bowman-Birk inhibitor (Birk, 1961; Birk et al., 1963). The method described by Bowman involved the precipitation of an acetone-insoluble powder. Defatted soybean is often the best starting material. Fratali and Steiner (1969) used ion exchange chromatography on DEAE cellulose to improve the isolation procedure. The method of Odani and Ikenaka (1977) remains the classical method of BBI isolation. The Odani method involves extraction of soybeans with 60% ethanol at room temperature followed by addition of two volumes of cold acetone, filtration, pH adjustment of the filtrate to pH 5.3, and acetone precipitation. The sticky acetone precipitate is dissolved in water, dialyzed against distilled water and ion exchanged through 5 mM sodium acetate equilibrated CM-cellulose at pH 4.0. Elution of BBI is carried with a salt gradient from 0 M NaCl to 0.5 M NaCl. Additional purification using DEAE in ammonium acetate at pH 6.5 was required to produce a purified BBI. BBI obtained was homogenous but the yield was very low. Tan-Wilson et al. (1982, 1985) used a combination of gel filtration and anion exchange chromatography on DEAE cellulose columns to separate ten isoinhibitors of BBI. Yeboah et al. (1996) combined ammonium sulfate precipitation, hydrophobic interaction chromatography, and gel filtration to purify 37 mg of BBI, with a minimum of 95% purity, from 200 g of starting material. Using titration with α -chymotrypsin, Gladysheva et al. (2000) measured 2.27 mg BBI per gram of soybean flour (variety GOST 17109-88) and 0.23 mg BBI in thermally treated soybean flour. Freshly milled soybean seeds are the best sources of BBI (Gladysheva et al., 2000).

Chemistry and Structure

The molecular weight of soybean BBI (Fig. 1) computed from the amino acid sequence of 71 residues is 7975 Da (Birk, 1985). Sedimentation equilibrium and gel filtration have reported values of 8,000 daltons (Millar et al., 1969; Kakade et al., 1970). Electrospray-MS of pea BBI isoinhibitors gave molecular values of 6916, 6807, 7676, 7844, 7848, and 7944 Da (Ferrasson et al., 1995). Horsegram (*Dolichos biflorus*) inhibitor was 8,321 daltons by amino acid sequence analysis and 8,625 Da by electrospray mass spectrometry (Prakash et al., 1996). Molecular weight obtained by MALDI-TOF-MS of soybean was 9857 Da (Losso et al., 2004). The precision of MALDI-TOF-MS is 0.05%, the precision of ESMS is also 0.05%, while the precision of SDS-PAGE is about 5%. In aqueous solutions and

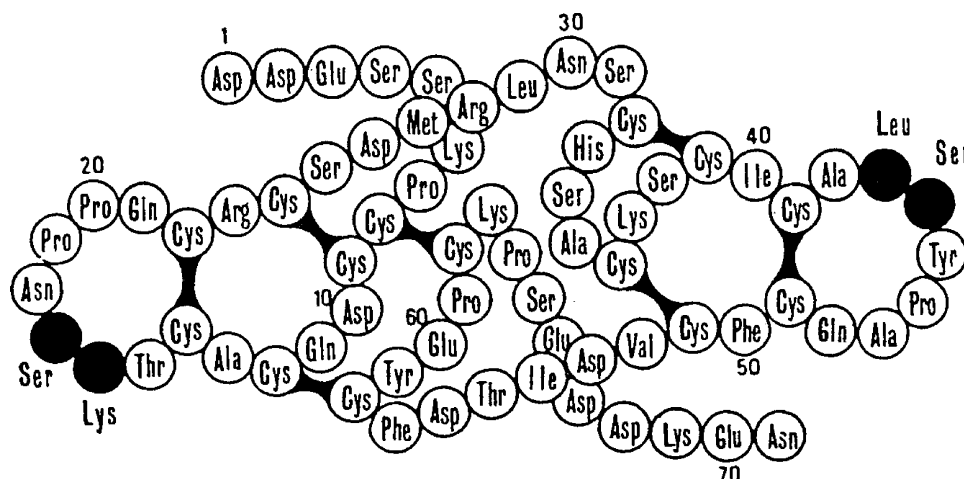


Figure 1 Structure of soybean BBI. Active site residues are shown in black and the disulfide bridges are also indicated in black. Reproduced with permission from Oxford University Press.

on SDS-PAGE under non-denaturing conditions, BBI exists as a mixture of monomer, dimer, trimer, and/or tetramers which is concentration-dependent as a result of self-association (Birk, 1985 and Fig. 2). Self-association of BBI monomers into dimers and trimers have also been reported with lima and kidney beans (Haynes and Feeney, 1967; Pusztai, 1968).

The isoelectric point of BBI is pH 4.0 to pH 4.2. For various legumes and fruits the pI values vary between pH 4.0 and pH 9.77 (Weder and Haussner, 1991). The variation is due to the difference in amino acid residues of various BBI. Soybean BBI has an extinction coefficient at 280 nm of 4.3 (Turner et al., 1975) and values of 4.4 to 4.8 have been reported (Birk et al., 1963). BBI is rich in cystine amino acid residues (about 20%). The covalent structure of BBI was elucidated by Odani and Ikenaka (1973).

Primary, Secondary, and Tertiary structure

BBI consists of 71 amino acid residues. BBI analogs containing between 54 and 133 amino acid residues have been reported in fenugreek, horsegram, barley, wheat, and rice bran (Song et al., 1999; Tashiro et al., 1987; Prakash et al., 1996; Nagasue et al., 1988; Odani et al., 1986). Overall the structure of BBI is stabilized by seven disulfide bridges (Cys9-Cys24, Cys14-Cys22, Cys8-Cys62, Cys12-Cys58, Cys36-Cys51, Cys32-Cys39, Cys41-Cys49). High homology of the amino acid sequence, mostly on the conserved cysteine positions has been reported. The large number of disulfide bridges in all BBI is mostly responsible for the stability of the protein molecule towards acid and enzyme hydrolysis, and heat. BBI and its analogs are highly structured proteins. The trypsin reactive site is held within Cys9-Cys24, Cys14-Cys22, Cys8-Cys62, Cys12-Cys58. The chymotrypsin/elastase reactive site is held within Cys32-Cys39, Cys36-Cys51, and Cys41-Cys49. As a result, the fragments of the reactive sites generated upon CNBr cleavage and pepsin hydrolysis are not totally separated

but kept together by the disulfide bridges. Fenugreek BBI has 5-6 disulfide bridges per half molecule, rice bran BBI, barley BBI and Job's tears BBI contain five disulfide bridges per half molecule, (Tashiro et al., 1987; Nagasue et al., 1988; Ary et al., 1988). Primary amino acid sequences of more than 100 BBI types are available from the SWISSPROT databank (Prakash et al., 1996).

Wu and Sessa (1994) reported native BBI to contain 61% β -sheet, 38% unordered structure, 1% β -turn, and no helices. Reduced BBI had 53% β -sheet, 5% β -turn, and 42% unordered. BBI contained no helical structure and no tryptophan (Fratali and Steiner, 1969). de Freitas et al. (1997) studied the secondary structure of blackeye pea BBI by circular dichroism and reported the protein to be non-helical with a deep negative band at 201 nm due to the disulfide bonds and small positive ellipticity values at wavelengths above 218 nm. The IR spectrum of blackeye pea BBI, in a solid film, in the amide I-II and V regions suggested the occurrence of the unordered, antiparallel β -strand. Several investigators have used CD to determine the structure of BBI (Millar et al., 1969; Wu and Sessa, 1994; Harry and Steiner, 1969; Jibson et al., 1981). Horse gram BBI contained 125 amino acid residues with a secondary structure of eleven β -strands and no α -helices (Song et al., 1999). The absence of α -helix conformation appears to be a characteristic of BBI and analogs. BBI obeys the standard mechanism proposed by Lakowski and Kato (1980) which stipulates that BBI is a specific limited proteolysis substrate for trypsin and chymotrypsin. Upon hydrolysis, P_1 and P_1' are split but held together by the numerous disulfide bonds in the molecule.

The tertiary structure of BBI has been studied in considerable details using X-ray crystallography and NMR (Zhu et al., 2001; Chen et al., 1992; Werner and Wemmer, 1991). The structure has two domains namely the antitryptic and the anti-chymotryptic domain, respectively (Werner and Wemmer, 1991). X-ray crystallography revealed that the inhibitory domains of BBI are very exposed and easily accessible to proteolytic enzymes. A high

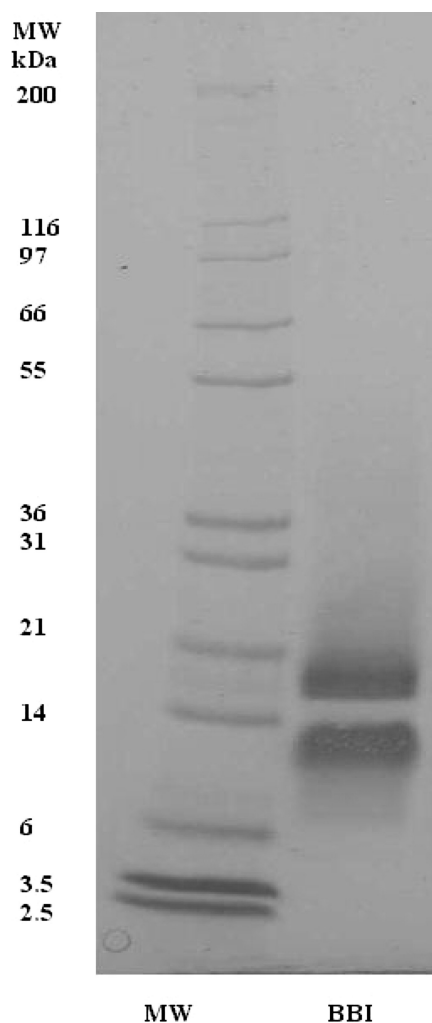


Figure 2 SDS-PAGE profile of BBI under non denaturing conditions. Reprinted from Bawadi; et al., 2004. With permission from The American Chemical Society.

level of sequence identity and a conserved cysteine bridging pattern was demonstrated among several representatives of BBI. The distribution of polar and non-polar residues throughout the BBI molecules and the distribution of chymotrypsin inhibitory sites are highly conserved. X-ray crystal structure of BBI revealed exposed hydrophobic patches and a polar domain interface solvated by internal water molecules (Philipp et al., 1998). As a result of exposed hydrophobic patches, BBI has a pronounced tendency to self-association (Voss et al., 1996). The crystal structure of BBI at 2.3 Å resolution revealed that the sub-domain Leu₄₄-X, which has been reported to be chymotrypsin specific, is actually also trypsin and elastase specific (Koepke et al., 2000).

Homology

BBI analogs exist all across the plant kingdom. Four double-headed BBI type inhibitors were isolated from peanuts by No-

rioka et al. (1983). All five isotypes showed two reactive sites for trypsin with different K_i and one reactive site for chymotrypsin. Unlike the soybean BBI, peanut BBI type showed weak inhibitory activity towards chymotrypsin and a preferential inhibitory activity against trypsin. Rice bran contains BBI analog which shows strong inhibitory activity against trypsin and weak against chymotrypsin (Tashiro and Maki, 1979). Wheat germ trypsin inhibitors I (MW = 14,500 daltons) and II (MW = 7,000 daltons) are highly homologous to BBI of leguminous plants (Odani et al., 1986). Lima bean BBI showed 70% sequence homology to BBI (Prakash et al., 1996). BBI and lima bean BBI share several similar chemical and physical properties (Seidl and Leiner, 1971).

Monocotyledonous seeds contain single-headed inhibitors of about 8 kilodaltons situated at the N-terminal which inhibit only trypsin and the second reactive is inactive (Prakash et al., 1996). The second reactive site of BBI in monocotyledonous seeds is strongly hydrophilic due to the flexibility and water accessibility of this region in the absence of disulfide bridge. The loss of chymotrypsin reactive site in monocotyledonous seeds appear to correlate with the crucial disulfide bond Cys₁₀ and Cys₁₁ which connects the amino carboxy termini of the active site loop in dicotyledons. In dicotyledonous seeds inhibitors, the amino- and carboxy-terminal ends are very hydrophilic. Rice bran has a duplicated structure of the soybean BBI composed of four domains, domain I and III, and domain II and IV which are homologous to the first and second domain of the soybean BBI (Tashiro et al., 1987).

Heterogeneity

BBIs are the products of multi-gene families and the derivation of multiple isoforms has been associated with protein processing at both the amino- and carboxylic ends (Domoney et al., 1995, 1993). The presence of multiple genes and the possibility of hydrolysis have been cited as evidence of the existence of a large number of BBI isoinhibitors (Kalume et al., 1995). A multitude of BBI isoforms has been reported to exist in a single soybean cultivar. A cultivar of soybean can easily have up to twelve isoinhibitors (Odani and Ikenaka, 1977; Hwang et al., 1977; Tan-Wilson et al., 1985; Larionova et al., 1993; 1994; Gladysheva et al., 2000). Trypsin inhibitors from winter pea seeds (c.v. Frilene) consist of six protease inhibitors (PSTI I, II, III, IVa, IVb, and V) which all belong to the Bowman-Birk family of inhibitors (Quillien et al., 1997; Ferrasson et al.,

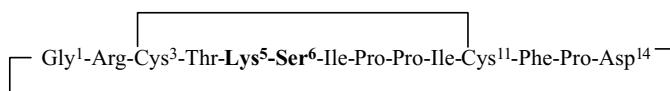


Figure 3 The structure of sunflower trypsin inhibitor-1 (SFTI-1). Reprinted from *Biochem. Biophys. Res. Commun.* 292, Zablotna, E., Kazmierczak, K., Jaskiewicz, A., Stawikowski, M., Kupryszewski, G., Rolka, K. Chemical synthesis and kinetic study of the smallest naturally occurring trypsin inhibitor SFTI-1 isolated from sunflower seeds and its analogues, 855-859, Copyright (2002), with permission from Elsevier".

1995). BBI analogs (TFI-A8, TFI-N2, and TFI-B2) were isolated from fenugreek seeds (*Trigonella foenum-graecum* L.) (Weder and Haussner, 1991). The mustard-type trypsin-chymotrypsin variant Chy8 has also been reported (Ceci et al., 2003). The existence of several BBI analogs across the plant kingdom brings about several differences in terms of amino acid sequence and amino acid residues at active sites of different BBI analogs although in most cases the reactive site loop residues are conserved. Table 1 shows the residues at the active sites of BBI and analogs.

Quaternary Structure-Molecular Interactions

BBI forms a 1:1 complex with trypsin and the interaction occurs at Lys₁₆-Ser₁₇ amino acid residues in the amino terminal half of BBI. BBI also forms a 1:1 complex with chymotrypsin and the interaction occurs at Leu₄₃-Ser₄₄ in the carboxyl terminal half of the BBI molecule. In the presence of a mixture of trypsin and chymotrypsin, BBI forms a ternary complex with both enzymes. BBI can also interact simultaneously with trypsin and elastase (Birk, 1987).

Table 1 Residues P₁ and P'₁ and the reactive peptide bond of BBI and analogs

	Source	Amino Acid Length	Molecular Weight (Da)	Number of disulfide bridges	Active Site	
					Trypsin	Chymotrypsin
1	Barley	124	13,823		R17-S18	R75-S76
2	Rice	195	21,570	9	K76-T77 K142-K143	
3	Peanut A-II	70	7633	7	R19-S20	R47-S48
4	Job's Tears	64	7270	5	R17-S18	
5	Dioclea glabra	67	7242	7	K13-S14	
6	Azuki bean	82	9130	7	K26-K27	Y53-S54
7	Soybean BBI precursor	110	12092	7	K55-S56	L82-S83
8	Wheat	56	6223		R17-S18	V43-G43
9	Azuki bean I-A	78	8675	7	K26-S27	R53-S54
					K26-S27	Y54-S54
10	Peanut B-II	63	6795	7	R13-R14	R41-S42
11	Kidney bean	79	8587	7	A25-S26	R52-S53
12	Foxtail millet FMTI-II	67	7679	5	K16-S17	
13	Soybean C II precursor	83	9201	7	A29-230	R56-S57
14	Wheat II-4	53	5952		K15-S16	T41-S42
15	Dolichos DE-3 axillaris (Macrotylona axillare)	76	8292	7	K24-S25	F51-S52
16	P.V.Kidney bean (French bean) PVI-3	85	9217	7	K29-S30	F56-S57
17	Foxtail millet FMTI-III	67	7680	5	K16-S17	
18	Glycine max-D II (precursor)	83	9468	7	S32-M33 R59-S60	
19	Wheat TI JPC 13: 187-194	71	7962	6	R19-M20	
20	Macrotylona axillare					
	DE-3	76	8369	7	K23-S24	L50-S51
	DE-4	76	8369	7	K23-S24	L50-S51
	EJB 97:85-91					
21	Dolichos axillaries				K23-S24	L50-S51
22	Apple leaf (DE #4)	80	8805	7	R25-S26	F52-S53
23	Kidney bean PVI-4	85	9231	6	K30-S31	F57-S58
24	Erythrina varigata	61	6717	7	K12-S13	L38-S39
25	Mung bean (Vigna radiata)	72	7959	7	K20-S21	R47-S48
26	Lima bean	83	9079	7	K26-S27	L53-S54
27	Vigna Angustifolia	72	8038	7	R16-S17	Y42-S43
28	Cow pea	83	9100	7	K26-S27	F53-S54
29	Broad bean	63	6989	7	K16-S17	Y42-S43
30	Maize	102	10976		F52-S53	
31	Dolichos biflorus				K24-S25	F51-S52 L37-K63
32	Swor bean	72	7807	7	K 21-S22	L48-S49
		73	7920	7		
		75	8163	7		
33	Rye				R33-A34	
34	Garden bean		8086 8371 8884		K53-S54 R53-S54	A26-S27
35	Sunflower	14	1513	1	K5-S6	

BBI shows interesting association characteristics. De Freitas et al. (1997) used light-scattering to demonstrate that in aqueous solution, BBI exists in equilibrium between a monomer and several forms of multimers (dimer-trimer-tetramer-hexamer). The anomalous distribution of hydrophobic and hydrophilic amino acids in blackeye pea BBI was cited as thermodynamically unfavorable for the protein to be monomeric in solution. The protein associates possibly because it contains more aspartic acid residues which are capable of forming additional hydrogen bonds in the pH region where it is undissociated. The presence of methionine residue at position 27 may provide a free thiol which may contribute to protein polymerization. Beta-mercaptoethanol, DTT, 4 M urea, or 5 M guanidine chloride may be used to keep BBI in the monomeric form.

Chemical Modification

Guanidination of BBI does not affect its inhibitory activity. Esterification does not affect the trypsin inhibitory activity. Maleylation, succinylation, and reaction of BBI with nitrous acid selectively abolish BBI trypsin inhibitory activity without affecting its chymotrypsin inhibitory activity (Seidl and Liener, 1971). Mercaptoethanol reduces BBI and performic acid oxidizes BBI and both reagents abolish BBI inhibitory activity against both trypsin and chymotrypsin (Birk, 1976). CNBr treatment cleaved BBI into two active fragments (Birk et al., 1986). Sodium borohydride partially reduces the inhibitory activity of BBI. However, BBI inhibitory activity may decrease to zero if four or more disulfide bonds are split. Regeneration of BBI activity against trypsin and chymotrypsin may occur in a disulfide-thiol redox buffer. N-acetylcysteine- and sodium sulfite-treated BBI (sulfitolysis) lost its inhibitory activity towards trypsin and chymotrypsin (Sessa et al., 1988). In the presence of acetone, BBI may undergo deamidation. In order to inhibit trypsin, all the cysteine residues in BBI must be present in the form of intact disulfide bonds in order to maintain its three-dimensional structure. In the presence of reducing agents such as 2-mercaptoethanol, BBI loses its three-dimensional structure and becomes incapable of inhibiting trypsin and chymotrypsin. The data on synthesis, characterization, and pharmacological activities of BBI-conjugates with naturally-occurring and synthetic polymers as well as with receptor-binding ligands have been reviewed by Larionova et al. (1997). Modification of BBI with plasma expanders: hydroxyethylstarch (Larionova et al., 1997) and clinical dextran (Gladysheva et al., 2001a) only slightly decreased effective inhibition of trypsin, chymotrypsin, and human leukocyte elastase (HLE). Larionova et al. (1996) modified BBI with a monoaldehyde derivative of a block copolymer of ethylene oxide and propylene oxide, MW 2 kDa, exhibiting membranotropic properties. This BBI-polyester conjugate effectively inhibited α -chymotrypsin (K_i of 40 nM) and HLE (K_i of 1.2×10^{-7} M), and retained the full ability to suppress radiation transformation in vitro (Larionova et al., 1996). The total clearance of BBI-polyester conjugate decreased by a factor of 8, body

mean residence time increased by a factor of 5 in comparison with BBI (Larionova et al., 1996). The conjugate of BBI with monoclonal antibody against the mucin-like human epithelial membrane antigen via clinical dextran was prepared for tumor targeting (Gladysheva et al., 2001a). BBI-dextran-mAb conjugate retained the high antiprotease activity of BBI and immunohistochemical activity of mAb (Gladysheva et al., 2001a). Another approach to the development of targeted delivery systems capable of bringing proteinase inhibitors to defined tumor cells consists of conjugating them with lectins. Chimeric protein, containing BBI and Con A (Mw 200 kDa), maintained 43% of antitrypsin activity (Lin and Hu, 1986). The conjugation of BBI and Con A not only increased (6-fold) the binding of the inhibitor to the sarcoma-180 cells but also resulted in internalization as measured by the intracellular trypsin inhibitory activity (Lin and Hu, 1986). The mechanism involved in the cellular uptake of the conjugate seems to be receptor-mediated endocytosis due to specific binding of Con A to the D-glucose-cell surface receptors. Furthermore, Bowman-Birk inhibitor/Con A showed a higher inhibitory effect on the growth of LKT- and NIH3T3, as well as Hela cells, than the mixture of an equivalent dose of free lectin and the inhibitor.

Persiani et al. (1991) conjugated BBI to poly(D-lysine) and poly(L-glutamate) as an anti-carcinogenic agent for lung cancer. The results obtained showed that BBI-poly(D-lysine) retained full anti-carcinogenic activity and more BBI-poly(D-lysine) was retained in lung tissues, after 24 h, than BBI-poly(L-glutamate) or native BBI. BBI was cationized by conjugation with dicationic, tetracationic, or polycationic carriers such as poly-D-lysine (Ekrami et al., 1995). Polycationic-BBI conjugates accumulated in the lung better than the other carriers. It was concluded that size alone was not important to increase BBI accumulation in the lung, BBI-conjugate positive charge density was more important in increasing BBI accumulation to the lung. The size of the cationic conjugate became an important factor only when the charge density of the conjugate was maintained. Polylysine and spermine were used as carriers for BBI delivery to the lung and liver (Ekrami et al., 1993). BBI-spermine conjugate retained 50% of the original BBI activity against chymotrypsin. Spermine-BBI conjugate was less toxic than polyD-lysine-BBI conjugate, more BBI-spermine conjugate was found in the lung compared to the unmodified BBI (Ekrami et al., 1993).

Recently, a number of mucoadhesive polymer/proteinase inhibitors conjugates was developed as an ingredient of peptide delivery systems providing an intimate contact to the mucosa, thereby reducing the peptide drug degradation between the delivery system and the absorbing membrane in the intestine. Approximately half of the incorporated insulin was shown to remain undegraded by the mixture of trypsin, chymotrypsin and elastase in physiological concentrations for 4.5 h at 37°C (Bernkop-Schnürch et al., 1998). BBI along with aprotinin was used for the development of microparticulate delivery systems with improved oral bioavailability of polypeptide drugs. Larionova et al. (1999b) prepared starch microcapsules capable for sustained release of the total encapsulated antichymotrypsin activity of BBI

during degradation by α -amylase. Undegraded model protein (BSA) was detected after enzymatic digestion (by the mixture of α -amylase and trypsin) of microcapsules prepared with the proteinase inhibitor (Larionova et al., 1999b).

Lipophilic BBI preparations were synthesized by conjugating BBI to derivatives of palmitic (Honeycutt et al., 1996) as well as oleic, linoleic, and α -linoleic acids (Malykh et al., 2001b). BBI-unsaturated fatty acid conjugates retained 85% of the antitrypsin activity (Malykh et al., 2001b). All unsaturated fatty acids BBI derivatives inhibit α -chymotrypsin more efficiently than the native BBI does, diacylated preparations being more efficient ($K_i(\text{BBI})/K_i((\text{oleoyl})_2\text{BBI}) = 575$) than monoacylated derivatives ($K_i(\text{BBI})/K_i((\text{oleoyl})_1\text{BBI}) = 10$) and triacylated BBI conjugates ($K_i(\text{BBI})/K_i((\text{oleoyl})_3\text{BBI}) = 3$) (Malykh and Larionova, 2002). Mono- and triacylated conjugates of soybean inhibitor retain high affinity to HLE compared with the native BBI. The anti-elastase activity of BBI containing two fatty acid residues is significantly higher than that of the native BBI. Thus, k_{ass} for $(\text{oleoyl})_2\text{BBI}$ is 200 times larger than that for BBI and affinity increases approximately two orders of magnitude (Malykh and Larionova, 2002). The transepithelial transport of BBI-derivatives with modified NH_2 -groups across Caco-2 cells was 6–9-fold in comparison with native BBI (Malykh et al., 2001a). The cellular uptake of $(\text{oleoyl})_1\text{-BBI}$, $(\text{oleoyl})_2\text{-BBI}$, $(\text{lin})_2\text{-BBI}$ and $\alpha\text{-(lin)}_2\text{-BBI}$ by Caco-2 cells was 200-, 100-, 30-, 4-fold compared to the native inhibitor, respectively (Malykh et al., 2001c). A great improvement on stability and plasma half-life was found for $(\text{palmitoyl})_3\text{-BBI}$ (Honeycutt et al., 1996). Besides, kidney clearance was significantly reduced and more BBI-palmitic acid conjugate was detected in the liver than unconjugated BBI.

A nanopeptide containing amino acid residues 41–49 has a significantly reduced antichymotryptic activity compared to the entire BBI molecule (inhibitor constant of $3450 \mu\text{M}$ for the peptide vs $0.013 \mu\text{M}$ for BBI (Dittorman et al., 2001). Ser44 $\rightarrow$ Gly44 using limited enzymatic hydrolysis abolished the reactivity of BBI at the chymotrypsin inhibitory site suggesting that an α -methyl or methylene group was required at the carboxylic terminal direction of the reactive site for inhibitory activity against chymotrypsin (Odami and Ikenaka, 1978).

Enzymatic Modification/Hydrolysis

Enzymatic modification of P'_1 arginine residues in the trypsin subdomain active site of peanut BBI ($\text{Arg}_{16}\text{-Arg}_{17}$) to citrulline was associated with the disappearance of BBI trypsin-inhibitory activity (Kurokawa et al., 1978). The deiminated BBI preserved its chymotrypsin inhibitory activity. Deletion or blockade of the trypsin inhibitory site on the BBI molecule would provide an inhibitor whose activity is directed specifically against chymases. Among the seeds investigated, only peanut BBI contains a trypsin inhibitory active site that is amenable to imidation. The anti-chymotryptic activity of lima beans was lost when lima bean BBI was incubated with trypsin at pH 3.5 as a result of the cleav-

age of a single leucyl-serine bond (Krahn and Stevens, 1970). Pancreatic elastase hydrolyzes the Leu43-Ser44 bond and abolishes most of the chymotrypsin inhibitory activity (Getler and Birk, 1970).

During soybean germination, protease K1 initiates the degradation of BBI followed by extensive proteolysis by protease B2 (Papastoitosis and Wilson, 1991; McGain et al., 1989). K1 is a cysteine proteinase of 29,000 daltons which requires the presence of reduced thiol for activity. K1 is active against BBI at pH 4.0. K1 is stored in the protein bodies and is inactive before germination. McGain et al. (1989) indicated that protease B2 appears by day 9 after the initiation of seed imbibition. B2 also catalyzes BBI degradation at pH 4.0.

pH, Thermal, Chemical, and Storage Stability

BBI is soluble between pH 1.5 and 12. BBI is active at pH as low as pH 1.5 in the presence of pepsin at 37°C for 2 h with no loss of activity (Weder, 1986). BBI in dry state or in 0.02% aqueous solution is stable at 100°C or at 105°C for 10 min, respectively. BBI is stable at 100°C for up to 10 min over the pH range of 1.6 to 8. The stability to pH and heat is associated with the rigid structure provided by the seven disulfide bridges in the protein molecule.

BBI from rice bean is stable between pH 3 and pH 10, without any decrease in antitryptic activity (Maggo et al., 1999) whereas BBI from Jack fruit seeds could maintain its tryptic inhibitory activity between pH 3 and pH 12 (Annpurna et al., 1991). However, pigeonpea seed lost 20% of its tryptic inhibitory activity between pH 3 and pH 5 but retained full activity between pH 7 and 10 (Godebole et al., 1994). BBI is completely inactivated following autoclaving or reduction with 2-mercaptoethanol in the presence of urea.

Cyanogen bromide treated BBI was digested by pepsin into two active fragments, one with trypsin inhibitory activity and the other with chymotrypsin inhibitory activity (Odani and Ikenaka, 1973). BBI is stable to pronase. BBI binds trypsin and chymotrypsin at pH 8.2. Jensen et al. (1996) used high performance capillary electrophoresis to demonstrate that the sequence S-N-P-P-E-C at the trypsin inhibitory subdomain could be detected 10 min after trypsin hydrolysis. The same authors also reported that the sequence S-Y-P-A-E-C-F in the chymotrypsin reactive subdomain was detected after 60 min of incubation with trypsin.

Van den Hout et al. (1998) studied the inactivation kinetics of BBI and indicated that the inactivation of BBI in water and starch matrix followed a first-order reaction. When mixed with starch in the presence of cysteine, two-phase inactivation kinetics was observed, the first phase was associated with the sulfhydryl-disulfide interchange and the second phase was related to heat. Soybean seeds stored at 4°C under constant humidity retained maximal BBI activity for at least one year than seeds stored at room temperature under variable humidity conditions (Gladysheva et al., 2000).

ENZYME INHIBITORY ACTIVITY

Mechanism of Action of BBI against Serine Proteases

For any protein, structural characteristic and spatial orientation are important factors in the protein functionality. The occurrence of BBI in various plant species strongly emphasizes the significance of the structure-function relationship for BBI. The interaction of BBI with tryptases or chymases occurs via a well-defined disulfide-linked short beta-sheet region which generates a non-covalent complex that renders the serine protease inactive (McBride and Leatherbarrow, 2001). The conformation of the reactive site loop is complementary to the active site of the protease inhibited and allows BBI to bind tightly to the protease (Chen et al., 1992). Upon complex formation between BBI and protease, BBI is cleaved very slowly (Jensen et al., 1996).

Protease inhibitors make up about 5–6% of soybean/legumes/monocotyledon proteins. BBI is the second major soybean protease inhibitor. The enzyme inhibitory activity of BBI is associated with two subdomains of the BBI molecule, Ser₁₇-Lys₁₆ and Leu₄₃-Ser₄₄. Lysine residue is involved in the mechanism of trypsin inhibition but not in the chymotrypsin inhibition (Sreerama and Gowda, 1997). During trypsin inhibition, the Lys₁₆ of the BBI molecule extends into the primary pocket of trypsin forming a direct network of two hydrogen bonds to Asp₁₈₉ therefore blocking the active site of trypsin (Flecker, 1987). The complex BBI-trypsin inhibits chymotrypsin but is inactive toward trypsin, the complex BBI-chymotrypsin is inactive towards chymotrypsin but inhibits trypsin. Removal of the NH₂ or COOH terminal amino acid does not affect BBI inhibitory activity toward trypsin or chymotrypsin. Chymotrypsin inhibition involves the interaction of Leu₄₃ or Ser₄₄ with chymotrypsin active site. Using casein as a substrate, the K_i for bovine trypsin is 5.6×10^{-8} , and K_i for chymotrypsin is 5.0×10^{-7} (Kassell, 1970). Larionova et al. (1993) used synthetic substrates and reported K_i values of 0.14 nM for bovine trypsin and 6.4 nM for α -chymotrypsin, respectively. The ternary complex trypsin-BBI-chymotrypsin complex has an altered conformation which is associated with changes in absorption and fluorescence emission spectra (Werner and Wemmer, 1991).

Soybean BBI has weak inhibitory activity against human trypsin and human plasmin but has no inhibitory activity against human thrombin (Kassell, 1970). Soybean BBI inhibited human trypsin to about the same extent as bovine trypsin (Weder, 1986). Fenugreek seeds contain several BBI isoinhibitors of which TFI-A8, TFI-B2, and TFI-N2 are well-characterized (Weder and Haussner, 1991). One mg of TFI-A8 inhibited 8.2 mg and 8.1 mg of human and bovine trypsin, respectively; one mg of TFI-A8 inhibited 0.8 mg and 1.0 mg of human and bovine chymotrypsin, respectively; one mg of TFI-B2 inhibited 7.5 mg and 5.1 mg of human and bovine trypsin, respectively; and TFI-B2 inhibited 1.8 mg and 1.9 mg of human and bovine chymotrypsin per mg BBI, respectively. Fenugreek isoinhibitor TFI-N2 equally inhibited human and bovine trypsin and chymotrypsin (5.0 mg and 4.1 mg of human and bovine trypsin per mg BBI, respectively;

and 4.9 mg and 3.7 mg of human and bovine chymotrypsin, respectively).

Cathepsin G may be involved in the pathogenesis of several human diseases (Stockley, 2001). BBI inhibited cathepsin G with a rate constant of $6.4 \times 10^6 \text{ M}^{-1} \cdot \text{sec}^{-1}$ (Gladysheva et al., 1994) and K_i of 1.2 nM (Larionova et al., 1993). The inhibition kinetics of human leukocyte elastase (HLE) by BBI occurred in two steps: the first step involved a rapid interaction between BBI and HLE to form BBI-HLE complex with a K_i of 28 nM, and the second step in which the BBI-HLE complex underwent a slow conversion/rearrangement to a tighter-binding BBI-HLE complex with a K_i of 2.3 nM (Larionova et al., 1997).

Duodenase combines trypsin- and chymotrypsin-like activities, is similar to cathepsin G and mast cell chymases, and belongs to the group of Janus-faced proteinases (Zamolodchikova et al., 1995). Soybean BBI (Gladysheva et al., 1999) and lima bean BBI (Gladysheva et al., 2002) interacted with duodenase through the chymotryptic inhibitory site, Leu-Ser, while the tryptic inhibitory site, Lys-Ser, remained available for interaction with trypsin. In a mixture containing BBI, trypsin, and duodenase, BBI preferentially binds to trypsin first ($k_{\text{ass}} = 1.1 \times 10^6 \text{ M}^{-1} \cdot \text{sec}^{-1}$) then the complex BBI-trypsin binds to duodenase (Gladysheva et al., 1999). The K_i of duodenase by soybean BBI and lima bean BBI were 4 nM and 23 nM, respectively (Gladysheva et al., 2002). The inhibition of cathepsin G of human leukocytes and chymase of mast cells of human skin by BBI occurs at the chymotrypsin binding site (Ware et al., 1997). Competitive and non-competitive type inhibitions have been reported for different BBI depending on the structure of the inhibitor reactive site (Godebole et al., 1994; Maggo et al., 1999; Annappurna et al., 1991).

Mechanism(s) of Action of BBI Against Metalloproteinases Activation

BBI does not directly inactivate metalloproteinases. Serine proteinases such as trypsin, chymotrypsin, and plasmin activate pro-metalloproteinase such as pro-MMP-9, pro-MMP-2, and pro-MMP-1 (Laskowski and Kalo, 1980; Duncan et al., 1998). BBI at 100 nM completely inactivated bovine trypsin activation of pro-MMP-1 to MMP-1 (Losso et al., 2004). Rice BBI at concentrations of 0.08–0.352 mg/mL dose-dependently inhibited the in vitro activation of 45 microg/mL pro-MMP-1 by trypsin (Bawadi et al., 2004).

Interaction of BBI and Proteasome

Chen et al. (2005) demonstrated that BBI specifically and potently inhibits the proteasomal chymotrypsin-like activity in vitro and in vivo in MCF-7 breast cancer cells through accumulation of ubiquitinated proteins and the proteasome substrates, p21(Cip1/WAF1) and p27(Kip1), accompanied with down-regulation of cyclin D1 and cyclin E. These authors suggested that BBI abates proteasome function and dose- and time

dependently up-regulates MKP-1, which in turn suppressed ERK1/2 activity. This new findings suggest that the chemopreventive effect of BBI is also associated with proteasome inhibition and further provide the evidence that soybean products containing active BBI have the potential to advance as chemopreventive agents.

BIOLOGICAL ADVANTAGE AND SIGNIFICANCE

Physiological Role in Seeds

BBI is to seeds what α_1 -antitrypsin is to humans. Seeds use protease inhibitors as (1) storage of sulfur, (2) regulators of endogenous proteases, and (3) defense mechanism against seed-eating insects, predators, and bacterial and viral infections (Flecker, 1987; Ryan, 1990; Azzouz et al., 2005).

During seed germination, the concentration of protease inhibitors, in general and BBI in particular, decreases as a result of BBI digestion by protease K1 and B2 (Papastoitis and Wilson, 1991; McGain et al., 1989). During soybean germination, protease K1 initiates the degradation of BBI followed by extensive proteolysis by protease B2 (McGain et al., 1989).

A 7.5 kilodaltons BBI isolated from broad bean (*Vicia faba*) inhibited the activity of *Mycosphaerella arachidicola*, *Fusarium oxysporum*, and *Botrytis cinerea* and exerted an inhibitory activity against HIV-1 reverse transcriptase activity (Ye et al., 2001). Wound-induced leaf expression of BBI was reported as an example of anti-pest function for BBI (Brown et al., 1985). Protease inhibitors (PIs) are natural defenses for plants and seeds, and have been recognized as a good strategy for insect pest control (Guerrero and Rosell, 2005; Carlin and Grossi-de-sa, 2002). PIs specifically target leaf-feeding insects which use digestive proteases, i.e. serine and cysteine proteases, to degrade ingested proteins. Although these approaches refer to seed protection, many of them can be in principle suitable for human and animal health, since the mechanism of action of these inhibitors on related enzymes is quite similar.

Cell Culture, Oncogene, and Animal Studies

Du et al. (2001) used a whole organ culture system of mouse mammary glands to investigate the effect of BBI or BBI-palmitic acid conjugate on 7,12-dimethylbenz[a]anthracene (DMBA)-induced nodule-like alveolar lesions. When added to cell culture during the development period after exposure to DMBA, BBI, and BBI-palmitic acid prevented DMBA-induced transformation at 35.9 and 53.4% respectively. However, BBI was less effective than BBI-palmitic acid (10.3% effective vs 32.2%) when the addition of the inhibitor occurred before the promotion period. The authors suggested that conjugation of BBI to palmitic acid enhanced the conjugate lipophilicity and its retention in mammary gland cultures.

Native BBI and (oleoyl)₁-BBI were tested on their influence on bovine herpes virus-1 (BHV-1) reproduction in embryonic

bovine kidney cell culture Taurus-1. The lipophilic derivative was shown to exhibit effective inhibition (>100-fold) of BHV-1 reproduction unlike native BBI (Larionova et al., 2003). The combined effects of the 200-fold increase of cellular uptake, even in the presence of serum-containing culture medium and the 100-fold increase in affinity to the elastase-like proteinase of (oleoyl)₁-BBI compared to the native inhibitor are most likely responsible for the observed antiviral action of (oleoyl)₁-BBI.

Prostate specific antigen, a neutral serine protease that lyses seminal proteins, is one of the clinical biomarkers for prostate cancer. Prostate specific antigen stimulates the generation of reactive oxygen species (Sun et al., 2001). The authors showed that BBI effectively blocked the generation of reactive oxygen species in prostate cancer cells. Bowman-Birk inhibitor concentrate (BBIC) inhibited the growth and clonogenic survival of BRF-55T, 267B1/Ki-ras, LNCaP, and PC-3 cells (Kennedy and Wan, 2002). BBI decreased the ability of LNCaP cells to invade across the reconstituted basement membrane (Matrigel) in the presence of PC-3 cell-conditioned medium as chemoattractant (Kennedy and Wan, 2002). BBI or BBIC was not toxic to normal human prostate epithelial cells.

In a study of human inflammatory bowel disease such as ulceratis colitis, Ware et al. (1999) demonstrated that BBIC at 0.5% level in diet of dextran-sulfate treated mice suppressed three of four scored histopathological inflammatory criteria ($P < 0.01$), total histopathological score ($P < 0.01$), reduced the mortality rate by 15%, and delayed the onset of mortality. Again, given the composition of BBIC and the presence of saponins known to be anti-inflammatory, the contribution of BBI per se in the study is not known.

Dittmann et al. (1998) reported that pre-incubation of normal human skin fibroblasts with BBI time-dependently blocked radiation-induced activation of tyrosine kinase and inhibited the activation of epidermal growth factor by enhancing specific tyrosine phosphatases.

BBI at 100 $\mu\text{g/ml}$ significantly protected Balb c/3T3 cells (clones A 31) exposed to 2.70 J/m² of UVC irradiation and reduced transformation of foci to 0.33 foci per dish (Baturay and Roque, 1991). BBI's ability to modulate superoxide anion radical production, DNA repair, immune effects, and arachidonic acid metabolism was associated with antioxidative properties (Lippman and Matrisian, 2000).

Chang et al. (1990) examined the effect of protease inhibitors on c-myc expression in normal and transformed C3H 10T1/2 cells. Incorporation of BBI into the growth medium was associated with reduced level (expression) of c-myc RNA in normal proliferating C3H 10T1/2 cells. St. Clair et al. (1991) used standard dot and Northern blot analysis to determine the level of c-myc, c-fos, c-Ha-ras, c-EGFR, and c-actin mRNA from colonic mucosa of rats irradiated in the presence of i.p. administered BBI. BBI selectively inhibited the overexpression of c-myc and c-fos, and did not interfere with radiation-induced overexpression of c-Ha-ras and c-EGFR. BBI also did not affect crypt cell proliferation and the constitutive expression of c-Ha-ras,

c-EGFR, or c-actin. Caggana and Kennedy (1989) showed that BBI was effective in reducing *c-fos* expression. BBI may also exert its anticarcinogenic activity by inhibiting a 125 kDa serine protease involved in the induction and/or expression of phenotype changes in intestinal epithelial cells (Habres and Billings, 1992).

Polycationic peptides such as polylysine and spermine were used as carrier for BBI delivery to lung and liver (Ekrami et al., 1993). BBI-spermine retained almost 50% of chymotrypsin inhibitory activity and had a pI between 9 and 10. BBI-spermine conjugate, injected i.v. into mice, was taken up into lung, liver, and kidney, remained in these organs, and reduced the number of tumors while being thirty-fold less toxic on C3H10T1/2 cells.

Blood syndrome patients have a high incidence of cancer rate as a result of high levels of chromosome abnormalities and sister chromatid exchange (Kennedy and Szuhaj, 1984). Cells from patients with blood syndrome and normal human diploid fibroblast cells were grown in the presence of 50 $\mu\text{g}/\text{ml}$ of BBI for 3 weeks (Kennedy et al., 1984). The authors reported a 50% reduction in the frequency of chromosomal abnormalities and sister chromatid exchange by BBI and suggested that BBI may have prevented the *novo* formation of aberrations and sister chromatid exchange (Kennedy et al., 1984).

Konijn et al. (1970) added BBI at 0.5% level in diets fed to rats, chicken, and quails. The birds and rats experienced pancreatic enlargement and growth depression. This study along with many others in which raw soybeans were fed to small animals concluded that BBI was an antinutrient that humans should avoid consuming (Roebuck et al., 1985; Liener and Hasdai, 1985). However, other animal studies showed that at micromolar concentrations, BBI could inhibit malignant transformations and help prolong life (Kennedy and Szuhaj, 1993). In mice treated with BBI and exposed to 12 Gy of irradiation in the abdomen, the expression of *c-myc* mRNA was lower than in control (St. Clair and St. Clair, 1991).

Dittmann et al. (2001) treated confluent layers of normal human skin fibroblasts (HSF1 and HSF6) with BBI or a nonapeptide containing the amino acids 41–49 (sequence C-A-L-S-Y-P-A-G-C) of the original BBI molecule in the concentration range of 0–80 μM for 16 h. The cells were irradiated either with 100 J/m^2 of UVB or 4 Gy. The photo- and radioprotective activity toward BBI persisted in the presence of the nonapeptide although 20 μM of the peptide was required to mimic the photo- and radioprotective effect of 10 μM BBI. Alteration of the amino acids Ser 42 with a charged or neutral amino acid completely abolished the photo- and radioprotective effect of the nonapeptide. The substitution Ser $\rightarrow$ Val resulted in a significant reduction of photo- and radioprotection. While BBI provides in vivo photo- and radioprotection to normal human and animal cells with functional p53, BBI has not been shown to provide radioprotection to transformed fibroblasts with mutated p53 or to xeroderma pigmentosum fibroblasts defective for nucleotide excision repair (Dittmann et al., 2005, 2000, 1998a). Nondenatured soymilk or BBI when applied topically, once a day for

5 days, to SKH-1 hairless mice with a high risk of developing UV-induced skin tumors, inhibited the formation and growth of skin tumors (Huang et al., 2004). These authors also reported that topical application of nondenatured soymilk to miniswine skin once a day for five days prior to UVB irradiation reduced or completely eliminated UVB-induced formation of thymine dimers and apoptotic cells.

Physiological Advantage to Humans

Many phytochemicals used by plants for self-protection are also used to regulate the adaptive response system in mammals. Several serine proteinases have been associated with severe disorders in humans (Seemuller et al., 1980). Diet can prevent chronic degenerative diseases such as arthritis and cancer, for which standard cures are limited. Prostate cancer, a disease whose etiology remains complex, has become a worldwide contributor to mortality in men. Prostate cancer is histologically present in 30–50% of 30- to 50-year-old men. Prevention of clinically detectable prostate cancer requires earlier chemoprevention interventions that inhibit the growth and progression of the disease. BBI, 9-cis-retinoic acid, dehydroepiandrosterone, flasterone, and an isoflavone mixture were identified as prostate cancer chemopreventive agents in animal models (Bosland et al., 2002).

For more than two decades, nutritionists considered BBI as an antinutrient (Liener et al., 1988). In a short-term experiment using high levels of BBI, Liener et al. (1982) reported that BBI infused into the human duodenum was associated with increased secretion of trypsin, chymotrypsin, and elastase. These studies led to the hypothesis that BBI could be harmful to human health. As a result, suggestions were made to engineer seed depleted of antinutrients such as BBI or to inactivate the proteinase inhibitor before consumption (Domoney et al., 1995; Domoney and Welham, 1998). Consumption of BBI at 0.5% level in the diet, as per the animal study mentioned above, is too high to be considered for human consumption. A 100 g meal would contain 500 mg of BBI. A concentration of 500 mg BBI per day in a diet is (1) equivalent to drinking about 180 oz (22 cups) of soymilk per day and (2) is economically prohibitive. After encountering the massive evidence of numerous studies worldwide demonstrating the effectiveness of BBI at nanomolar concentration on a variety of in vitro and in vivo human ailments (Trou, 1993; Larionova et al., 1993; Dittmann et al., 2001; Kennedy, 1998; Sawey, 2001; Armstrong et al., 2000a, 2000b, 2003) some nutritional biochemists have begun to acknowledge that BBI could be helpful for human health.

The rationale of ingesting bioactive compounds, such as BBI, as food or food ingredient is that (1) foods are a relatively inexpensive way to deliver disease suppressing and health promoting agent such as BBI over the lifespan of an individual, (2) medical intervention is always costly once a degenerative disease has erupted, (3) most people do not have the discipline and perseverance to take pills over a sustained period of time, and (4)

epidemiological studies have suggested that people who consume soybean or pulses regularly have a reduced rate of some degenerative diseases that strike later in life.

Ip et al. (1994) viewed chronic diseases as a multiyear process that can be prevented by adopting the strategy of general chemoprevention which involves increasing consumption of food containing health enhancing compounds rather than toxic drugs. In light of the FDA approval of health claims associated with soy proteins, of which BBI is a part, and in light of the absence of toxicity reported in human clinical studies, BBI may contribute to healthier foods and lead the way to reduce incidence of some types of degenerative diseases, such as cancer, mostly those of the gastrointestinal tract occurrence.

Reluctance to use non-specific proteinase inhibitors, for disease prevention, lies in their indiscriminate actions toward both intended target (disease tissues) and normal substrate (normal tissue). Under normal physiological conditions, natural inhibitors such as α_2 -macroglobulin (α_2 -M) inhibits many kinds of endoproteinases of all four catalytic classes under a unique mechanism referred to as trap hypothesis (Barrett and Starkey, 1973). Under pathophysiological conditions, the concentrations of natural proteinase inhibitors such as the α_2 -M are often reduced. The concentration of other endogenous inhibitors such as the TIMPs was shown to be directly correlated to the activity of metalloproteinases (Baker et al., 2000). Tumor suppressor genes which inhibit cell proliferation were reported to be lost during tumorigenesis (Sawey, 2001). Ingestion of safe and bioavailable inhibitors, such as BBI, that can fulfill the same function as α_2 -M becomes warranted.

Trypsin inhibition by BBI triggered cholecystokinin release (Reseland et al., 1996). Cholecystokinin (CCK) is a gut hormone whose release has been associated with the feeling of satiety or fullness and reduced hunger in humans and animal models (Campfield, 1997). Pathophysiological changes such as pancreatic enlargement, increased CCK secretion, and growth depression do not occur in all species fed a soybean diet (Rackis, 1974). For instance, pig exhibits pancreatic secretion and growth inhibition but no pancreatic enlargement (Yen et al., 1977). Yen et al. (1977) suggested that trypsin inhibition may bring about some deleterious changes but in general some of the changes stem from differences in animal physiology. Pig is the animal closer to human as far as the gastrointestinal physiology is concerned. Increased CCK reduces appetite. However, if CCK production has to be increased in human as a result of BBI consumption, without the enlargement of the pancreas, BBI may be also be good for food intake and appetite control. BBI may provide the feeling of fullness and satiety and be good for obesity control.

Frenkel et al. (1987) studied the effectiveness of chymotrypsin-trypsin (BBI) and trypsin inhibitors (STI), from various commodities, in inhibiting H_2O_2 formation by TPA-activated human PMN's. Protease inhibitors from potato were more effective, at 10 μ M, than inhibitors from lima bean, and chicken egg white. About 50 μ M of BBI was required to provide about 40% inhibition of H_2O_2 formation.

Bioavailability and Metabolism of BBI

BBI is resistant to acid and alkaline digestion in the gastrointestinal tract and reaches the colon in intact form (Yavelow et al., 1983). However, slow in vitro hydrolysis of BBI, after prolonged hours, has been reported (Jensen et al., 1996). Experiments with laboratory animals have consistently shown that BBI is bioavailable (Kennedy, 1998). BBI enters the circulation via the intestinal epithelial cells or the paracellular mechanism (Billings et al., 1991; Billings et al., 1992). Billings et al. (1992) provided evidence that three hours following ingestion, up to 40% of ingested BBI was found in the blood stream, and about 17% of BBI ingested was found in the bladder (Billings et al., 1992). The excreted BBI maintained its protease inhibitory activity (Yavelow et al., 1983). One to two percent of ingested radiolabeled BBI was found present in all organs except the brain (Kennedy, 1998). In animal models in which 0.01% dietary BBI completely inhibited the induction of liver tumors, up to 5 μ g of absorbed BBI was detected in the liver, the breast, and the prostate (Yavelow et al., 1983; Yavelow et al., 1985). This minute concentration of BBI was effective in preventing or suppressing in vitro malignant cells transformation (Yavelow et al., 1985) or liver angiosarcoma induced by dimethylhydrazine in animals (St. Clair et al., 1990). But, antibodies developed against BBI could not detect BBI extracted from the bloodstream and excreted BBI (Brandon et al., 1989). It was suggested that BBI in the bloodstream and excreted BBI may have existed in a reduced form as a result of endogenous reducing agents such as glutathione. In studies involving humans, Kennedy (1998) reported that BBI was detected in the urine within 1–9 h following oral administration and the baseline was re-established within 24 h.

The concentration of BBI metabolites recovered from urine of human subjects fed 36 oz or 60 oz of soymilk (containing 105 or 175 mg BBI) was estimated to be less than 0.02% of the BBI ingested (Wan et al., 2000). It was suggested that the ingested BBI may have undergone conformational change in the presence of natural reducing agents such as glutathione, a chemical change which made it unlikely to be detected by the antibodies generated against native BBI. Ingested BBI may be bioavailable (Wan et al., 2000). Bioavailable BBI, if still active, may therefore provide protection in organs other than those of the gastrointestinal tract.

Neoplastic diseases are associated with enzymatic destruction of the extracellular basement membrane (ECM). The disruption of the basement membrane integrity facilitates the formation of new blood vessels, from pre-existing ones, and the spread of diseases through the newly formed vessels. If ingested BBI is still active and bioavailable, BBI in organs may inhibit serine- and metalloproteinases which contribute to the destruction of the basement membrane (Losso et al., 2004). Larionova et al. (1998) demonstrated inhibition by BBI of fibronectin hydrolysis by human leukocyte elastase. Besides BBI effectively inhibited the hydrolysis of elastin by human leukocyte elastase or cathepsin G (Tikhonova et al., 1994). BBI showed the same

efficacy in suppressing the stimulated hydrolysis of elastin by joint action of the two enzymes. The inhibitory effect was practically unaffected by both the adsorption of the enzymes on elastin and the prolongation of the enzymatic reaction (Tikhonova et al., 1995).

Synergism-The Hurdle Effect

BBI is not effective against all the proteinases associated with the onset and progression of diseases. But, BBI in combination with other proteinase inhibitors may achieve homeostasis. Metalloproteases and cysteine proteases contribute to the degradation of the basement membrane (Creemers et al., 1998). The hurdle effect is a combination of several agents or techniques in order to establish a series of inhibitory effects (hurdle) that any proteolytic enzyme that would catalyze a pathological change should not be able to overcome. Hurdle technology as applied to BBI would require the combination of cysteine, threonine, metallo, and aspartic proteases in order to achieve what BBI alone cannot accomplish. Each proteinase inhibitor individually affects the activity of an enzyme or a series of enzymes and concertedly controls the proteolytic activity of enzymes and makes it unlikely for any overexpressed enzyme to go uninhibited. BBIC, for instance, is a soybean extract which contain BBI, cystatin, saponins, isoflavones, and phytic acid. The contribution of each of these bioactive compounds to health is well-documented. However, excessive levels of proteinase inhibition may have significant side effects on physiology.

APPLICATIONS

Suitability/Adaptability

Although BBI languished for many years as an antinutrient, clinical trials have shown that BBI has now become an important protein for disease prevention (Kennedy, 1998; Armstrong et al., 2002a; Armstrong et al., 2002b; Meyskens, 2001; Tolcher et al., 2001). The fact that BBI is present in soybean, dry beans, pulses, and many mono- and dicotyledonous seeds, implies that it has been a component of the human diet for centuries. Extensive in vitro toxicity and clinical studies of BBI at level normally found in food and at higher levels have confirmed that BBI is safe (Kennedy, 1998; Malkowicz et al., 2001).

FDA considers BBI as a drug (Kennedy, 1998; Tolcher et al., 2001). At the same time FDA has approved labels claiming that the consumption of at least 3 to 4 ounces of tofu or 8 ounces of soymilk soy protein consumption may reduce the risk of coronary heart disease and breast cancer. Because BBI is present in most soy-based formulations and is present in soymilk that FDA has recommended as a healthy food that may reduce the risk of coronary heart disease and breast cancer, one should just consume soy products or more legumes and not wait for a prescription to buy BBI. BBI has the characteristics of a functional food.

Existing Applications

Clinical Applications for Chemoprevention

BBI is with no doubt a food ingredient. However, BBI has had more successful stories in the medical or paramedical scientific community than in the nutrition scientific community. Commercially available BBI is cost-prohibitive for clinical studies. A crude BBI concentrate (BBIC) was developed by Kennedy (1993) to allow clinical studies based on the properties of BBI. US Patent 5,338,547 5,217,717 describes a method for producing BBI from hexane defatted soybean (Kennedy et al., 1994). BBIC has undergone preclinical, phase I, and II of clinical trials. BBIC administered in a single oral dose between 25 and 800 chymotrypsin inhibitory units to 24 subjects with oral leukoplakia was well tolerated and showed no sign of toxicity (Armstrong et al., 2000a). BBIC contains BBI, soybean cystatin, STI, isoflavones, saponins (Lipman and Matrisian, 2000; Zimacheva and Mosolov, 1995). In vitro studies using BBI have demonstrated that BBI alone is an anticarcinogen that is effective at nanomolar concentrations and has irreversible effects on cancer cells (Ekrami et al., 1995; Kennedy, 1998; Koroma and de Juan, 1994). However, it is difficult to associate all the chemopreventive properties of BBIC to BBI alone because saponins and isoflavones have been reported as anticarcinogens and antiangiogenic (Koroma and de Juan, 1994; Kerwin, 2004; Kim et al., 2004). BBI has a broad and irreversible chemopreventive activity but the specific target protease that is inhibited by BBI or BBIC has not been specifically characterized although a range of proteases with a wide range of size has been suggested (Habres and Billings, 1992; Moy and Billings, 1994). Kennedy (1998) believes that the uniqueness of BBI lies in "its ability to reverse the "initiated" state of cells after initiation has occurred, a property ascribed to few anticarcinogens.

Otto and Schirmeister (1997) suggested that a pharmacologically effective protease inhibitor should interact irreversibly with the protease or the enzyme-inhibitor complex should possess a dissociation constant of less than 0.1 nM for lasting activity. Demuth et al. (1996) reported that most serine proteinase inhibitors might also react with cysteine proteinase. The similarity between BBI and the bromelain inhibitor has been reported (Hatano et al., 1996). However, the reactivity of BBI toward cysteine proteinases has never been documented.

Oral cancer has of the lowest survival rates of all cancers. In the US, 30,000 new cases are diagnosed annually, more than 8,000 deaths occur, and the survival rate is about 50% after 5 years (NIDCR, 2006). In other countries, the survival rate is as low as 25% (Jane-Salas et al., 2005). BBI was found to shrink precancerous lesions in the mouth that lead to oral cancer called leukoplakia. BBI, in the form of lozenges, was administered to 32 participants with leukoplakia. BBI helped shrink the precancerous lesions in the mouth in about one-third of the participants (Kennedy, 1993). BBI administered to 24 subjects with oral leukoplakia, as single oral doses for 1 month in a phase I clinical trial, at levels approximately four times those ingested

in Japanese diets was very well-tolerated and showed no toxic or allergic reactions (Armstrong et al., 2000a). In a phase II clinical trial, BBI at concentration between 200 and 1066 chymotrypsin units was administered to 32 patients with leukoplakia for 1 month (Armstrong et al., 2000b). BBI treatment produced a partial or complete response in 31% of the patients. This encouraging clinical trial was the first in human studies that demonstrated the chemopreventive properties of BBI against oral cancer. The best way to cure cancer is to prevent cancer (Meyskens, 2001; Armstrong et al., 2003). Regular and moderate ingestion of BBI with food may be the best way to prevent leukoplakia. BBI is a suppressing agent and may act early during the process, thereby preventing the disease.

Nineteen male subjects with benign prostatic hyperplasia and lower urinary tract symptoms participated in a phase I trial of BBIC (Malkowicz et al., 2001). A statistically significant reduction in the level of serum PSA was obtained with some patients showing a 43% reduction in serum PSA. Significant decrease in serum triglycerides and prostate volume was also recorded. BBI showed no dose-limiting toxicity. The clinical data were encouraging to allow a phase II clinical trial. At the National Cancer Institute Workshop on "New Clinical Strategies in Prostate Cancer Prevention," BBI was selected as a chemopreventive compound that reduces superoxide-induced DNA damage (Tolcher et al., 2001).

Other known properties of BBI include the prevention of hair and weight loss in animal models (Evans et al., 1992), increased lifespan (Kennedy and Szuhaj, 1993), ability to potentiate the cytotoxicity of cis-platinum in lung cancer treatment (Kennedy, 1998), ability to prevent and not cause some birth defects (Von Hofe et al., 1990). Kennedy (1998) pointed out that people from Asia who are regularly on a high soybean diet reproduce themselves without any sign of BBI toxicity.

Serine, metallo, aspartic, and cysteine proteinases involvement in malignancies is well-documented. Serine, cysteine, and metalloproteinases are responsible for the propensity of malignant cells to evade the connective tissue boundaries that limit their growth, invade, and metastasize into healthy tissues. Most cancers take years to develop and bring a pathological state. Rather than providing excessive and intensive medical intervention when symptoms appear, which in most cases is late, the presence of proteinase inhibitors in normal diet that maintain a normal level of anti-proteinase activity may be a better strategy for chemoprevention. Good nutrition is a key to prevention.

Other Degenerative Diseases

Excessive protein intake has been reported to contribute to the progressive deterioration of renal function in diabetes and other disease states (Anderson et al., 1998). As early as 1836, Bright suggested that excessive protein intake contributes to the progression of chronic renal disease (Anderson et al., 1998). Excessive protein intake may be associated with increased hyperfiltration, glomerular hypertension, and progressive deterior-

ation of kidney function whereas restriction or use of soy protein may slow the progression of nephropathy (Brenner et al., 1992; Anderson et al., 1998). BBI at a dose of 20 mg/kg body weight administered i.p. to 3-methylchloranthrene (MCA)-treated A/J mice caused a 15% reduction in total cholesterol (Kennedy et al., 2003). Because the liver is the major site for the production of cholesterol and since cholesterol contributes to heart disease, BBI may also contribute to the beneficial effects of soy protein in the prevention of heart disease.

Inflammatory Diseases

Chymase, a chymotrypsin-like serine protease found in mast cells of many mammalian species, is a chemoattractant that may catalyze the accumulation of inflammatory cells in the development of chronic inflammatory response of allergic and nonallergic diseases (Tani et al., 2000). The chemotactic activity of chymase was inhibited by BBI, suggesting that the hydrolytic activity of chymase may be responsible for its chemotactic activity (Tani et al., 2000; Wara et al., 1997). Inflammation associated with tumor promotion was inhibited by BBI (Kennedy, 1998). US Patent 6,767,564 (Morris et al., 2005) covers the use of BBI for the treatment of multiple sclerosis and other autoimmune diseases.

Potential Applications

BBI as a Functional Food Ingredient

Throughout history diet has shown the ability to affect the onset or progression of chronic diseases, but only recently that the molecular basis of diseases prevention by dietary intervention has been under scientific consideration (Dirsch and Vollmar, 2001). Proteinases levels in the body are biomarkers of health and performance. The identification of biomarkers of health and the development of food ingredients and technologies that address the issues of biomarkers will be keys to the success of functional foods. Diet has a strong influence on health. Functional foods are foods consumed as part of a normal diet rather than pills. BBI fits the definition of a functional food. BBI is a component of soymilk. Sound evidence from human studies using acceptable biomarkers is providing the basis for the promotion of BBI as a functional food ingredient.

Proteinase inhibitors, in general, are not considered as bioactive ingredients for food fortification as a result of previous nutritional investigations and the inability of proteinase inhibitors to be very specific. However, by essence functional food or nutraceuticals are bioactive compounds which have the ability to delay or prevent the onset of diseases. Most compounds that prevent diseases act by inhibiting enzyme(s) that catalyze the pathological process. Tylenol is a proteinase inhibitor, so is AZT. Resveratrol, a phenolic compound, is a proteinase inhibitor (Chang et al., 2001). Black tea is reported to inhibit dental plaque formation by inhibiting glucosyl transferase, thus limiting the biosynthesis of sticky glucan by *Streptococcus mutans*

and *S. sobrinus* (Hamilton-Miller, 2001). If all the good data about BBI could be substantiated in large scale population studies, BBI as a functional food could offer a very economical public health intervention.

The role of nutrition in the prevention and possibly the treatment of breast, colon, and prostate cancer is receiving considerable attention in the medical scientific community. In particular, the role of protease inhibitors of food origin such as BBI is being recognized by biomedical researchers as potential chemopreventive agents (Kennedy and Wan, 2002; Lippman and Matrisian, 2000; Kennedy, 1998; Sawey, 2001; Meyskens, 2001). Loewenstein (1979) hypothesized that gap junctions mediate cell-cell interactions important in growth suppression and regulation. Wilgenbus et al. (1992) indicated that mammary tumors were deficient in gap junction and Lee et al. (1992) reported that cells derived from mammary tumor do not exhibit gap junction-mediated cell-cell communication. Connexins are proteins found in gap junctions. Connexin 26, 43, and 32 (Cx26, Cx43, or Cx32) are connexin genes and are often coexpressed in mammary cells (Sawey, 2001). Sawey (2001) reported that radiation-transformed C3H/10T1/2 cells contained a low level of Cx43 while BBI-treated C3H/10T1/2 cells had a higher level of Cx43 (Sawey, 2001). Cx43 transcripts were detected in MCF-10 cells treated with 100 mg/ml of BBI. But Cx 43 transcripts were deficient in MCF-7 or BT-20 cells in the presence or absence of BBI (Sawey, 2001). MCF-7 and BT-20 are immortalized human epithelial cells from breast carcinoma (Soule et al., 1973). MCF-10 is derived from human fibroblastic mammary tissue and is nontumorigenic. Sawey (2001) suggested that the gap junction may function as tumor suppressor genes and may be regulated by BBI.

The contribution of protease inhibitors in the treatment of human chronic diseases has long been recognized. The rationale of providing moderate concentration of inhibitors in the diet to restore normal anti-protease level lies on the premises that when most disease erupts or symptoms appear, medical intervention becomes costly. Proteinase inhibitors have the advantage of blocking the carcinogenesis process at an early stage (Wattenberg, 1992).

BBI does not have a GRAS status. However, BBI is a natural component of water-based soy products and is present in many food products from mono- and dicotyledonous seeds. But, BBI has the characteristics of a nutraceutical. Soy infant formula contained at least 6.3 μg of BBI per ml (Friedman and Brandon, 2001).

The stability of BBI towards heat, pH, and gastrointestinal enzymes has been demonstrated (see previous paragraphs). For instance, individuals with genetic α -1-antiprotease deficiency have a high rate of lung function loss caused by uncontrolled activity of human neutrophil elastase. Therapeutically, the restoration of normal protease activity is carried by infusing purified anti-elastase in these individuals. BBI has shown inhibitory activity against elastase (Larionova et al., 1993; Bueno et al., 1999). BBI has also shown to be able to reach organs beyond the gastrointestinal tract.

Good nutrition is recognized as key to prevention. Food fortification has long been recognized as an effective strategy to overcome nutrient deficiency in affected populations. BBI is available in soymilk and the consumption of soymilk could serve as a means of ingesting BBI. However, many people do not drink soymilk because of the beany flavor that many consumers find unpalatable. Under these circumstances, food fortification with BBI extract may provide daily requirements of BBI for those in need. Strategies to consume BBI within its natural matrix include soymilk, tofu, or aqueous extract of soybean. Soymilk contains isoflavones, oligosaccharides, and saponins which are all health enhancing bioactive compounds. After observing that US born descents of Asian immigrants are equally susceptible to develop the common cancers ascribed to Americans, it was suggested that genetic difference may not account for the variation in cancer rate; rather eating habits may have a significant role to play (Kennedy, 1998).

German et al. (1999) noted that future food science ventures into functional foods should learn from examples of cholesterol reducing diets, probiotic bacteria, and prebiotic carbohydrate. The success of BBI as a functional food ingredient will depend on three critical factors: (1) the mechanism(s) by which BBI prevents disease and promotes health, (2) the biomarkers of health towards which BBI molecules should be targeted, and (3) published evidence that BBI is effective in disease prevention and health promotion.

BBI and Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing ones. Angiogenesis is important in normal and pathological processes. The formation of new blood vessels is critical for a tumor to grow beyond a few millimeters in size (Folkman, 1971; Carmeliet and Jain, 2000; Folkman, 2006; Carmeliet, 2005). Matrix metalloproteinases (MMPs) are a class of more than twenty-three zinc- and calcium-dependent enzymes recognized to participate in the destruction of the extracellular matrix (ECM) under pathological conditions such as arthritis, cardiovascular disease, inflammation, cancer, and Alzheimer's (Creemers et al., 1998; Deguchi et al., 2006; Stetler-Stevenson, 1999; Jia et al., 2000; Adair et al., 2004). In tumor such as breast, gastric, colon, neck, pancreatic, prostate, and lung cancer MMP-2 and MMP-9 are overexpressed compared to normal tissues (Laskowski and Kato, 1980; Hrabec et al., 2001; Haq et al., 2000; Baker et al., 2000). There are three classes of MMP: collagenases, stromelysins, and gelatinases (Laskowski and Kato, 1980). In the basement membrane, MMPs are secreted as inactive zymogens into the extracellular matrix and require activation by other enzymes. Trypsin has been recognized as one enzyme activator of MMP (Duncan et al., 1998). Matrix metalloproteinase -2 (MMP-2 or gelatinase A) and MMP-9 (or gelatinase B) hydrolyze gelatin, type IV and type I collagens. Together MMP-1, MMP-2, and MMP-3 can destroy all structural proteins of joints. BBI showed inhibitory activity against mast-cell chymases (Ware et al., 1997). BBI from soybean and rice

bran inhibited the activation of pro-MMP-9 by trypsin (Bawadi et al., 2004).

Pancreatic cancer has no real cure and patients have a very short, i.e. 3-6 months, survival rate (Haq et al., 2000). MMP-2 and MMP-9 have been reported to determine the aggressiveness of pancreatic cancer and inhibition of MMP-2 was the most important factor associated with survival rate (Haq et al., 2000; Kennedy, 1996) pointed out that almost all human pancreatic cancer is of ductal origin and soybean products containing high level of protease inhibitors significantly inhibited animal pancreatic cancer of ductal origin. The major protease inhibitors of soybean are trypsin and the trypsin and chymotrypsin inhibitors. Soybean also contains a small amount of cystatin. Recent work in our laboratory has demonstrated the effectiveness of BBI as inhibitor of pro-MMP-1, pro-MMP-2, and pro-MMP-9 activation (Losso et al., 2004; Bawadi, 2004). BBI at a concentration of 62.5 nM inhibited the activation of pro-MMP-2 by chymase. Most in vitro biochemical studies use APMA to activate pro-MMPs. However, in the human body, APMA does not exist. Trypsin or any other serine protease is the most likely enzyme to activate pro-MMPs. MMP-2 and MMP-9 were reported to potentially be critical in the early steps of angiogenesis (Jia et al., 2000). Collagenases and gelatinases are overly expressed in diabetic rats (Ryan et al., 2001). Effectiveness of nanomolar concentrations of BBI against pro-MMP-1 and pro-MMP-2 may delay the onset of MMPs-dependent degenerative diseases. Inhibitors that perturb matrix metalloproteinases in a particular cell type and interrupt tumor-specific proteinase activation cascades are heavily considered as a powerful approach that may lead to the development of better tools for disease prevention, diagnosis, and therapy (Gruss and Herlyn, 2001).

Delivery of bioactive food compounds larger than 11 Å, to the body, represents a challenge to proteinaceous drug delivery through the gastrointestinal tract. Although the uptake of BBI into circulation has been demonstrated, it remains a challenge for food scientists and nutrition technologists to deliver a large amount of BBI to circulation via the oral route. Conjugation of BBI to carriers may provide some answers.

BBI as Plant and Seed Protection Agent

Proteinase inhibitors with insecticidal activities have emerged as an interesting strategy for insect pest control (Azouz et al., 2005). Pea and soybean BBI induced significant mortality and growth depression on pea aphid *Acyrtosiphon pisum* (Rahbe et al., 2003; Ceci et al., 2003). Trypsin inhibitors are wound inducible. The level of trypsin inhibitory activity increased up to 8 h after leaves of rice bean (*Vigna umbellata* T.) were wounded on the periphery with pin holes (Maggo et al., 1999). Wound-induced expression of BBI-type genes in plants suggests a natural protective function of BBI against insect proteinases (Sakal et al., 1989). Wound-induced leaf expression of trypsin inhibitors in tobacco leaves (McMunnus, 1994) and alfalfa (Brown et al., 1994) indicate that BBI may have an anti-pest function.

Designer/Medical/Medicinal Foods

Medical foods are by essence designer foods because they are processed and formulated to meet the nutritional needs of hospitalized or compromised people such as cancer and AIDS patients, people with malfunctioning food/nutrient organs, people with disordered immune systems, and infants/people with inborn errors of metabolism such as PKU. Medical foods are FDA regulated and must be administered under medical supervision because of potential side effects. No side effects have been demonstrated for BBI in humans. BBI may qualify for incorporation into medical foods in pre- and post-surgery situations to enhance the healing process. Because of its anti-proteinase activity, BBI may provide additional healing and prevent a relapse of increased proteinase activity in the affected area.

BBI and the Macrobiotic Diet

The macrobiotic diet concept depicts the fundamentals of vegetable-whole foods diet as cancer chemopreventive. A macrobiotic diet consists of cereals, pulses, vegetables, occasional fish consumption, and no meat and dairy products (Kushi et al., 2001). BBI is a component of the macrobiotic diet.

LIMITATIONS

Safety, Tolerance, Efficacy

BBI does not have a GRAS status as food. However, BBI is a component of soy protein or soymilk approved by FDA for a healthy lifestyle. Brandon et al. (1991) reported that infants on soy formula may consume an average of 10 mg of trypsin and trypsin-chymotrypsin inhibitor per day. Yen et al. (1977) demonstrated that feeding BBI to pigs, animals that are physiologically closer to humans than rats, did not enlarge the pancreas or cause deleterious side effects in the pigs. BBI is safe in clinical trials (Kennedy, 1998; Armstrong et al., 2000a). Should BBI be marketed or produced as dietary supplement to avoid the food additive petition or GRAS determination process? O'Donnell (2001) mentioned that as of 1997 many food ingredients such as bovine lactoferrin, fructooligosaccharides, trehalose, tagatose, licorice, jellywort, chrysanthemum, and plant sterols have used the GRAS self-affirmation route to position themselves on the market. Through the GRAS self-affirmation process, a company submits a Food Additive petition for formal FDA approval. Often the FDA does not question the basis of the request and the product finds its way to the market. BBI may have to follow this route.

BBI and Allergenicity

Maki and Kennedy (1992) investigated the effect of orally and intraperitoneally administered BBI on antibody response to keyhole limpet hemocyanin and delayed-type hypersensitivity response to dinitrochlorobenzene and concluded that BBI does

not compromise humoral and cellular immune function. Individuals fed up to 1,000 mg/kg/day of BBIC showed no sign of antibodies against BBI in blood serum (Wan et al., 2002). Allergenicity associated with soymilk consumption derives from a soybean P34 protein, a 34 kilodalton protein, rather than BBI (Babiker et al., 2000). At concentration found in soymilk BBI may not cause allergenicity.

BBI and Acute Toxicity

Toxicological data in animal models showed conflicting tolerance levels of BBI depending on the diet, route of administration, and animal physiology (Yen et al., 1977; Liener et al., 1988). However, numerous recent studies have demonstrated that BBI is not responsible for previous abnormal growth of animals fed raw soybean (Kennedy, 1998). Raw soybeans contain lectins which are known to cause growth depression. Human diet containing soybean proteinase inhibitors do not contain active lectins and are therefore unlikely to cause pancreatic atrophy as observed in animal models. Raw soybean is not mainstream human diet. The upper limit of BBI that could cause adverse side effects in humans is not known. The challenge for nutritionists and the FDA is to help determine the upper limit of BBI in food products or the maximum daily BBI concentrations allowed in food products rather than prematurely classifying BBI as a drug.

Occurrence of diarrhea was reported in one clinical trial of BBI (Lippman and Matrisian, 2000). A potential explanation is that if BBIC is acetone extracted, then oligosaccharides such as raffinose which are easily extracted by acetone might be present in BBIC extract and may be responsible for the flatulence reported in those clinical studies.

Blood syndrome cells (VS strains) and normal human diploid fibroblast cells (AG 1522) were cultured in the presence or absence of BBI (50 μ g/ml) and antipain (25 μ g/ml) for 2 weeks (Kennedy, 1998). Results suggested that BBI and antipain were not toxic and did not interfere with the cell cycle of either cell. The same was demonstrated for the cultivation of embryonic bovine kidney cells Taurus-1 (fibroblast-like) in the presence of BBI and (oleoyl)1BBI (up to 3000 TIU/ml) for 15 days (Larionova et al., 2003).

BIOTECHNOLOGY

Large Scale Production

More than 2 billion bushels of soybeans were produced in the United States alone in 1999 (Friedman and Brandon, 2001). The cost of raw soybean is low. Soybean is highly available to enable production of large quantities of BBI by direct isolation from soybean. However, difficulty in obtaining purified BBI using conventional separation techniques may explain the high cost of commercially available BBI (\$90 per 50 mg protein). For many years, nutritionists labeled BBI as an antinutrient. For that reason, food technologists and nutritionists have

not shown interest in formulating BBI in food products. US patents 5,338,547 and 6,887,498 (Kennedy et al., 1994; Konwinski et al., 2005) cover BBI preparation from soybean for use as anticarcinogenic compound. In an effort to reduce the cost associated with BBI purification Kennedy et al. (2003) used a crude extract of BBI for clinical studies. The extract was as effective against chymotrypsin activity as purified BBI has shown significant health enhancing effects in clinical trials (Lippman and Matrisian, 2000; Armstrong et al., 2000a; Meyskens, 2001).

Soy phytochemical concentrate (SPC) is commercially produced by Archer Daniels Midland Company or ADM (Decatur, IL) and soy protein isolate (SPI) is commercially produced by Protein Technology International (St. Louis, MO). ADM uses hexane defatted and dehulled soybeans and 60% ethanol to prepare an extract from which SPC is produced (Kerwin, 2004). The certificate of analysis of SPC indicates that one gram of SPC contains 0.14 g of protein, 0.055 g of fat, 0.027 g of ash, 0.065 g of moisture, and undefined fraction rich in saponins (Kerwin, 2004). However, the composition of BBI was not defined. There is no information on the concentration of BBI in SPI. Gladysheva et al. (2000) measured 2.04 mg in soybean grits, 1.57 mg BBI in soymilk, 0.82 mg BBI in oilcake, 0.49 mg in soybean isolate, and 0.29 mg BBI in soybean protein concentrate.

Genetic Engineering

Recombinant BBI obtained by Lys16 $\rightarrow$ Arg16 and Ile27 $\rightarrow$ Met27 was fully active but had a reduced trypsin inhibitory activity (Flecker, 1987). Rice BBI was expressed in E.coli as a fusion protein bearing an N-terminal (His)₆ purification tag (Li et al., 1999). The recombinant rBBI, which accumulated in inclusion bodies, required 8M urea for solubilization and showed strong inhibitory activity against trypsin and a weak inhibitory activity against chymotrypsin. Lys16 $\rightarrow$ Trp16, Lys16 $\rightarrow$ Tyr16, Arg 16 $\rightarrow$ Trp16, or Arg16 $\rightarrow$ Tyr16 converted the trypsin inhibitory site to a chymotrypsin inhibitory site.

In 1996, Monsanto (St. Louis, MO) introduced glyphosate-tolerant soybeans (GTS) as the first biotechnologically improved and marketed soybean (Rogers, 1998). GTS has a built-in insect protection and tolerance to herbicides. Protein profile and composition of GTS were shown to be similar to protein profile and composition of the parent soybean line.

Inactive BBI and Kunitz inhibitor genes containing elevated sulfur amino acid content and having a high expression in soybean were designed in an effort to produce soybean varieties with no or low levels of BBI. Targeted and specific genetic manipulation using antisense technology have been suggested as way to interfere with the processing that affects BBI inhibitory activity (Domoney et al., 1995; Domoney and Welham, 1998). Jung et al. (Jung et al., 1997) reported that the expression of an albumin protein from Brazil nut (*Bertholletia excelsa*), that apparently was rich in sulfur amino- acid but less than the amount found in BBI, reduced the amount of BBI synthesized in the seed. Ser42 $\rightarrow$ Arg42, Ser42 $\rightarrow$ Glu42, or Ser42 $\rightarrow$ Gly42

completely eliminated the radio- and photoprotective activity of BBI, but Ser → Val42 reduced the radio- and photoprotective activity (Dittman et al., 2001). Leu43 → Ile43, Leu43 → Arg43, or Leu43 → Gly43 was associated with complete loss of radio- and protective activity while Leu43 → Glu43 or Leu43 → Val43 reduced the activity of BBI. Tyr45 → Gly45 showed reduced chymotryptic inhibitory activity and total loss of photo- and radioprotective effect. Opportunities may exist for protein engineering to enhance BBI yield in soybean variety.

FUTURE PROSPECTS

BBI is a component of a soy protein-based diet. Research is needed to study the contribution of BBI to health in physiologically healthy individuals and its potentials in chronic disease prevention using human subjects. Research is needed to evaluate BBI as functional and medical food. This could pave the way for food products fortified/enriched with BBI for the restoration or the maintenance of normal anti-proteinase level in the body, especially in the digestive tract. The development of analytical methodology for the determination of BBI in food products needs to be pursued. The development of low cost isolation techniques for BBI preparation and use in food products is another area worth pursuing. Consumer education on soybean components, other than isoflavones, and the benefits that can be provided for general chemoprevention, should be envisioned.

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