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***Review Article***

# Legume seed protease inhibitors: their functions, actions and characteristics

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**ABSTRACT**

Protease inhibitors are found in many legumes, especially their seeds. They are classified into families based on the amino acid sequence, the position and nature of reactive site, and the number of disulfide bridges. These inhibitors inhibit the pancreas proteases of higher animals and impair the nutritional quality of seeds by reducing protein digestibility and absorption by inducing pancreatic hypertrophy and by depressing growth. Several functions of these proteins have been proposed, including the regulation of endogenous proteinases and the protection of plants from insects and microorganisms. Clarification of the antinutritional status of legumes, especially of protease inhibitors, is important, not only because these proteins may be included at higher levels in diets than hitherto assumed, but also because, in general, protease inhibitors are desirable proteins from the view point that their content of sulphur containing amino acids as well as for their potential rules in protecting seeds and plants from insect attack. Protease inhibitors are very effective in their ability to suppress carcinogenesis in many different *in vivo* and *in vitro* assay systems. Although the mechanisms for the anticarcinogenic activity of protease inhibitors are unknown, many hypotheses have been proposed.

## INTRODUCTION

Legume seeds are important staple foods, particularly in developing countries, due to their relatively low cost, ease of preservation, and high nutritional value.

A lot of legume seeds are consumed by various segments of Egyptians and it is known that these seeds are an important source of energy and protein. However, their wider use is some how limited by the presence of undesirable components known as antinutritional factors (ANFs) in the seeds which may have adverse effects for human or animal nutrition. Some examples of these compounds include protease inhibitors (PIs), lectins (heamagglutinnis), α-amylase inhibitors, phenolics, phytates and α-galactosides (raffinose family sugars) (El-Morsi 1996, 1997, 1998; Zaki *et al*. 1999; Trugo *et al*. 2000; Ismail & Fawzi 2001).

This article deals with PIs in the seeds of some Egyptian legumes.

**PIs content in the seeds of some Egyptian legumes:** The nutritional properties of various PIs have been well documented. The results of determining PIs in the seeds of four legume crops studied by Aboul-Fotouh *et al.* (1998) are shown in table 1. TI activities were detected in both albumin and globulin fractions, but their levels were higher in albumin fractions than in globulin fractions in all samples. The highest level of total TIA was recorded in kidney bean Nomad variety (40.4 mg/g) and the lowest amount was found in pea variety Master B (2.5 mg/g). Of the two kidney bean varieties, Belfin contained about one third of the TIA recorded for the Nomad variety. The levels of TIA in cowpea and mungbean were lower than those recorded for kidney beans but higher than peas (Table 1). With the exception of kidney bean Nomad variety all other legumes demonstrated TIA (Table 1) lower than that recorded for soybean (26.5 mg/g) (EL-Morsi *et al.* 1990).

Cowpea variety 331 (Table 1), produced higher levels of TIA than that reported by Ismail, *et al.* (1995a) for black-eyed cultivar. The levels of TIA in Kawmy 1 mungbean seeds (Table I) were much lower than the values recorded for two mungbean genotypes studied by Zaki (1996). The value of TIA in pea seeds was within the range reported by other workers (Abdel-Galil 1998; Van der Poel *et al.* 1992).

Table 1: Trypsin inhibitory activity (TIA) in dehulled legume seeds (Mean ±SD)*

| Legume | TIA mg/g | | |
|---|---|---|---|
| | Albumin | Globulin | Total |
| Kidney bean | | | |
| Belfin variety | 10.6±0.1 | 2.5±0. | 13.1 |
| Nomad variety | 35±1.1 | 15.4±0.3 | 40.4 |
| Cowpea | | | |
| 331 variety | 6.8±0.2 | 3±0.1 | 7.1±0.2 |
| Pea | | | |
| Master B variety | 1.9±0.1 | 0.6±0.01 | 2.5±0.1 |
| Mungbean | | | |
| Kawmy I variety | 3.2±0.1 | 1.0±0.01 | 4.2±0.2 |

*Each value is the mean of three identical determinations ± standard deviation

i & Gad l-Hak 1985).

TIA was detected in the albumins of all ten cowpea varieties studied by El-Morsi *et al.* (1993). The amount of the inhibitor ranged from 2.52 to 7.36 mg TI/g protein. The albumin of *Pusa phatgoni* contained the highest amount of TIA followed by blackeye No. 5, whereas the lowest level was record in Cream 7.

Screening for TIA in ten genotypes of mungbean was reported by Ismail (1995) who observed that the albumins of all genotypes contained higher levels than the corresponding globulins. TIA ranged from 3.2 to 7.8 and 1.2 to 2.7 mg/g albumin and globulin, respectively.

Recently, Abdel-Galil (1998) studied the effect of maturity on TIA in pea seeds and reported the detection of the inhibitor at the very early stage of seed development at a level of 1.1 and 1.3 mg/g sample of Little Marvel (LM) and Progress respectively which was collected at 10 DAA. Trypsin inhibitor concentration was increased as maturation progressed to reach 2.5 and 3.2 mg/g of mature seed of LM and Progress respectively and the levels of TIA paralleled the crude protein content of two pea varieties.

Recently, Zaki *et al*. (1999) determined the level of TIA (3.28 mg/g defatted meal) in the seeds of an unspecified variety of guar. The guar seed TIA was found to be concentrated in its albumin fraction and the level was 5.2-fold of that recorded for its globulin fraction (El-Morsi *et al*. 2000).

**Chemical nature of PIs:** Hafez & Mohamed (1983a) determined the difference between two fractions having anti-tryptic activity in soybeans and Winged beans. The trypsin inhibitor in the seed extracts is referred to as total trypsin inhibitor activity (total TIA) (TTIA). The proteins in seed extracts were precipitated by the addition of either trichloroacetic acid (TCA) to a final concentration of 16% *(*wlv*)* or saturated ammonium sulphate, and the nonprotein trypsin inhibitor activities (NPTIAs) were determined in the protein-free supernatant. The NPTIA constituted *27-55%* of the TTIA in the soybeans and 5-14% of TTIA in Winged beans depending upon cultivars. Hafez and Mohamed (1983b) identified the NPTIA and nonprotein chymotrypsin inhibitor activity (NPCIA) in soybean as active peptides which form complexes with trypsin and chymotrypsin, as revealed by two-dimensional, thin layer chromatography.

Almost without exception the proteinaceous PIs found in legume seeds are relatively small proteins with little or no additional carbohydrate moieties. These inhibitors have been classified into two families on the basis of their molecular weights and cystine contents (Norioka *et al*. 1988). The inhibitors classified as the Kunitz family have molecular weights of about 20 kD and have two disulphide bridges. One member of this family is soybean trypsin inhibitor (Kunitz), which was isolated and crystallized from soybean seeds by Kunitz

in 1946. The other family, the Bowman-Birk family, has molecular weights of about 8.0 kD and is characterised by a high cystine content (seven disulphide bridges) One member of this family is soybean Bowman-Birk inhibitor, which was isolated from soybean seed by Bowman (1946) and later characterised by Birk *et al.* (1963).

**The reactive sites of PIs**: The plant inhibitors contain the reactive (or inhibitory) sites which interact with the proteases affected. Thus it has been reported (Richardson 1980) that several of the inhibitors are" double-headed" proteins capable of inhibiting both trypsin and chymotrypsin at separate peptide bond sites. However, some inhibitors have a single peptide bond which serves as the reactive site for a single inhibited proteinase or acts against several enzymes. Such examples are referred to as "single headed-inhibitors".

The reactive site of the inhibitor is defined as the part of the molecule that enters into direct molecular contact with the centre of the enzyme upon formation of the enzyme-inhibitor (El) complex. The amino acid side chain responsible for the substrate analogous accommodation in the specificity pocket of the enzyme is that of the "reactive amino acid residues. For trypsin inhibitors, reactive sites are almost always formed by arginine or lysine residues linked to another amino acid (Laskowski & Sealock 1971). By analogy with trypsin inhibitors, the amino acids tryptophan, tyrosine, phenylalanine and leucine were found in the reactive sites of the chymotrypsin inhibitors (Tschesche 1974).

The involvement of arginine or lysine in the active sites of the inhibitors are readily examined though the use of cyclohexane-1 ,2-dione (CHD) or 2,4,6-trinitrobenzene sulphonic acid (TNBS) as chemical modifier for arginine and lysine, respectively. Protein inhibitors of trypsin are generally classified into two groups, arginine and lysine inhibitors (Laskowski & Kato 1980). Table 2 presents the different reactive sites which have been identified in some legumes by chemical and enzymic modifications.

Table 2: The reactive (inhibitory) sites of some PIs from legume seeds.

| Enzyme inhibited | Reactive site residues | Legume | Reference |
|---|---|---|---|
| Trypsin | Arg-lle | Soybean (Kunitz) | Richardson (1980) |
| | Lys-Ser | Soybean (Bowman-Birk) | Richardson (1980) |
| | | Chick pea | Richardson (1980) |
| | | Adzuki bean | Yoshikawa and Ogura (1978) |
| | Arg-Arg and Arg-Ser | Peanut (Bowman-Birk) | Norioka and lkenaka (1983) |
| | | Faba beans (Bowman-Birk) | Asao et al. (1991a,b) |
| | Arg-X | Peanut (Bowman-Birk) | Mahmoud (1993) |
| | Lys-Ser and Arg-Ser | Mungbean | Li *et al* (1994) |
| | Lys-X | Fiald bean | Ismail *e( al* (1995b) |
| | Arg –x | Pea | Mahmoud (1998) |
| | Arg-X | Mungbean | El-Morsi *et al*.,( 2001) |
| Chymo-trypsin | Leu-Ser | Soybean (Bowman-Birk) | Richardson (1980) |
| | Arg-Iie | Soybean (Kunitz) | Richardson (1980) |
| | Tyr-Ser | Adzuki bean | Yoshikawa and Ogura (1978) |
| | | Faba beans (Bowman-Birk) | Asao *et al* (199la,b) |
| | Tvr-X or Phe-X | Field bean | Ismail *et al* (1995b) |

Based on the knowledge of the active sites of a small number of trypsin inhibitors, Laskowski and Sealock (1971) attempted to make some generalisations concerning these sites. They can be summmarised as follows: (a) an enzyme-susceptible bond is required, (b) the amino acid terminal residue in the cleaved site is ILe, Leu or Ala, (c **)** the reactive site is in a disulphide loop, (d) there should be not be any negative charges in the vicinity of the susceptible bond, (e) there should be a proline in the vicinity of the active site.

Since the anti-trypsin active site of trypsin inhibitors resembles a trypsin substrate

(Arg-x, Lys-x), it is reasonable to assume, as a working hypothesis, that the active site of chymotrypsin inhibitors would resemble a chymotrypsin substrate. It was proposed, but not proven (Frattali & Steiner, 1968), that the antichymotrypsin active site could be in the form of Tyr-x or Phe-x peptide bond. The susceptibility of certain Leu-x peptide bonds to chymotrypsin hydrolysis has been reported (Bidlingmeyer *et al.* 1972).

**Some properties of field bean PIs:** In Egypt, field bean *(Vicia faba)* seeds are widely used by both humans and other animals. These seeds contain trypsin inhibitor as well as other anti-nutritional factors (Ismail *et al.* 1995b). Unlike the trypsin inhibitor in soybean and *Phaseous vutgaris* seeds, little is known about proteinase inhibitors of *Viciafaba* seed (El-Morsi 1982b). Knowledge about the properties and function of the proteinase inhibitors can enable one to devise better methods of processing. In addition, these inhibitors are excellent models for the research on protein-protein interactions.

El-Morsi (1982a) separated the water-soluble proteins of field bean into six components by gel filtration chromatography on a Sephadex G-200 column. Assay for trypsin inhibitor activity indicated the location of the inhibitor in fraction E eluted from the column.

A trypsin inhibitor was purified from the seeds of *Vicia faba* variety Giza 2 by EL-Morsi (1982b). The molecular weight was 12 kDa when determined by gel filtration on Sephadex G- 100 and 11,6 kDa from the amino acid composition, which revealed a high content of half-cystine, aspartic acid glutamic acid residues. The absence of free sulfhydryl groups and tryptophan was also observed.

A trypsin/chymotrypsin inhibitor from seeds of field bean *Vicia faba,* variety Giza 402) was purified and characterised by Ismail *et al.* (1995b*).* The molecular weight of the inhibitor was approximately 12 kDa, as estimated by gel filtration chromatography and SDS-PAGE. The inhibitor was characterised by high contents of half-cystine, aspartic, glutamic acid and serine, which accounted for about 44% of the total 104 amino acids. The inhibitor contained neither tryptophan nor free sulfhydryl groups and 8 disulphide bridges were present. One mole of field bean inhibitor formed a complex with 0.95 mole of trypsin (T-I complex) or 1.15 mole of a-chymotrypsin (C-I complex). The T-I complex retained only 50 % of its chymotrypsin inhibitory activity (CIA) but the trypsin inhibitory activity (TIA) in C-I complex was not affected by the presence of chymotrypsin. Thus, this inhibitor is a double-headed one.

**Effect of pH and heat on the stability of the inhibitor:** The effect of pH on the stability of the field bean inhibitor, when incubated at $37^0$C for 24hr is presented in Fig. 1.

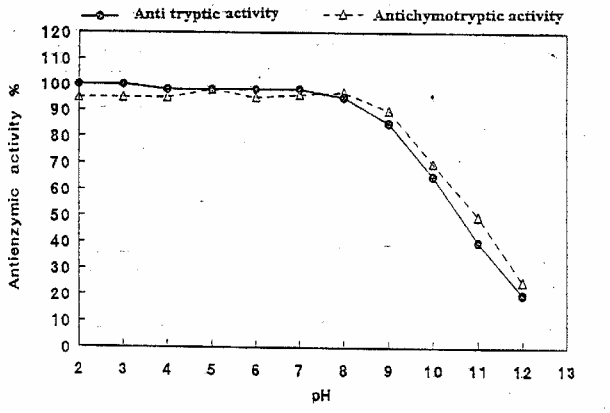


Fig.1: Effect of pH on the stability of field bean inhibitor

The inhibitor was found to be stable at pH ranging from 2 to 8. However, TIA rapidly dropped to 25 and 20% of the initial antichymotryptic and antitryptic activity at pH 12. The effect of heating the inhibitor at $100^0$C under three different pH values (2, 7 and 11) is shown in Fig. 2. The effect of pH on the inhibitory activities was apparent.

It is evident from Fig. 2, that, although considerable stability of the inhibitor was maintained at acidic and neutral pH, at alkaline pH (11) the inhibitor lost antitryptic and antictcymotryptic activities after 20 and 30 min. of heating, respectively (Fig.2.) After heating for 60 min., the inhibitor maintained 50 to 60% of its inhibitor activities at pH 2.and lower values (20-30%) at pH 7.

The unusual stability of protease inhibitors, in general, is their most remarkable property. This property has considerable bearing on the nutritional quality of many legume meals. Field bean inhibitor, studied here, has bean found to be quite stable, especially under acidic and neutral pH conditions and at high temperatures. The presence of as many as 8 disulphide bridges in the molecule and the extensive intrapeptide cross-linking cotes the stability of inhibitor.

**Effect of pepsin on the stability of the inhibitor:** The purified field bean inhibitor had no inhibitory activity against pepsin when it was treated at pH 2, for 1 hr.

The stability of the purified inhibitor towards pepsin treatment was studied at $37^0$C in a solution of HCl-KCl buffer pH 2, containing 1:1 weight ratio of inhibitor and pepsin. The inhibitory activities of the inhibitor against trypsin and chymotrypsin were not altered by this treatment.

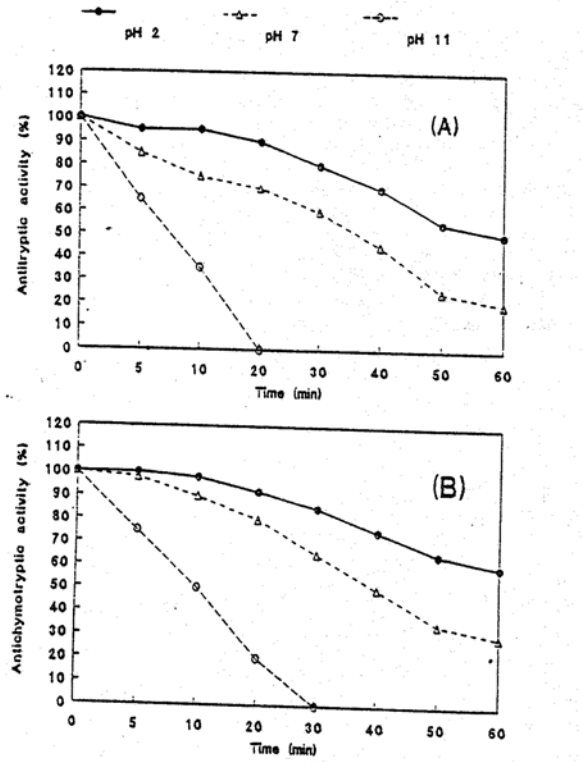


Fig. 2: Heat stability of field bean inhibitor. (A): TIA, (B): CIA.

When the inhibitor was treated with a large excess of pepsin (1:10 w/w), the CIA was more susceptible to pepsin treatment and about 50% of this activity was lost after 4 hr of incubation. Under the same conditions, only about 28% of the antitryptic activity was destroyed (Fig. 3). This may suggest that this inhibitor possesses a certain extent of resistance to attack by pepsin, especially around the region where the trypsin inhibitor reactive site is located.

In conclusion, the properties described here for the field bean inhibitor suggest that it belongs to the Bowman-Birk family of trypsin inhibitors.

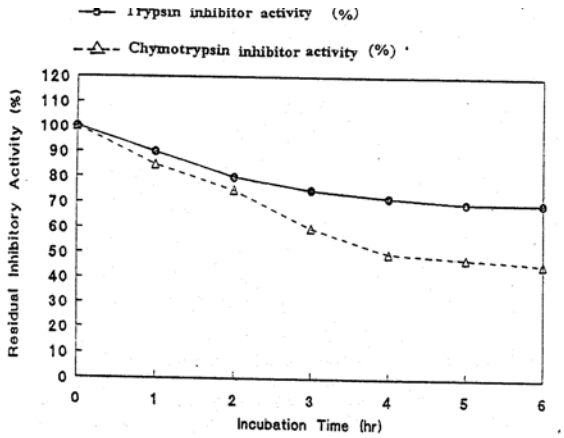


Fig. 3 : Effect of pepsin on field bean inhibitor.

**Physiological significance of PIs:** In plants, most postulated functions for PIs fit into three categories: (**a**) maintaining dormancy by preventing autolysis, (**b**) regulating protein synthesis and metabolism and ( **c** ) preventing attack by predatory insects.

Work by Gatehouse and collaborators (Gatehouse *et al*. 1985) led to the widely held assumption that trypsin inhibitors are totally, or at least partially, for the resistance of seeds of the TVu 2027 cowpea cultivar to the bruchid *Callosobruchus maculatus*. This view seemed to be strengthened by a report from the same group that leaves of tobacco plants transformed with a cowpea trypsin inhibitor gene do not support the growth of the tobacco budworm, a lepidopterous pest (Hilder *et al*. 1987), which is perhaps not surprising since it is known that lepidoptreous larvae rely on trypsin-like enzymes for protein digestion (Applebaum 1985).

A search was undertaken to identify legume sources of proteinaceous cysteine PIs (CPI) that can be used to control the proteolytic activity of endogenous digestive cysteine protease in the midgut of some larval bruchid insect larvae of insects destroyed stored legume seeds, such as dry beans (*Phaseolus vulgaris* L.) and cowpea (*Vigna unguiculata*) (Hines *et al*. 1990). There are a few published reports of CPI activity in legume seeds (Ryan, 1990). Coleopterans, or beetles which include bruchids, commonly utilise a cysteine digestive proteinase (Murdock *et al*. 1987).

Several lepidopteran insects show poor growth and an increased rate of larval mortality when fed on a Kuntiz inhibitor (KI)-or Bowman-Birk inhibitor (BBI)-supplemented diet (see Boulter, 1993 for a review; results were confirmed by *in vitro* tests where both KI and BBI inactivated the main digestive enzyme of these insects (Christeller *et al*. 1992). Marchetti *et al*. (1995) determined the activity of the soybean KI and BBI on 14 proteases of fungal and bacterial origin, as a preliminary step before analysing inhibition directly on microorganisms, with the aim of protection, not only from small mammals, but also from insects or pathogenic microorganisms. Their results indicated that microbial proteases are frequently inhibited by KI and BBI and that proteases with the same EC number (e.g. subtilisin Carlsberg and subtilisin BPN) may equally give different responses to the inhibitors.

**Nutritional significance:** The exact nutritional significance of PIs in the diets of animals and humans is difficult to evaluate due to the presence of other ANFs in legume seeds (Liener. 1979). The first implication of these proteins as the cause of certain nutritional disorders came when growth inhibition was observed. In animals fed with plant products known to contain high levels of PIs. Later feeding experiments, in which rats and chickens were supplied with raw soybeans or with supplements of the partially purified trypsin inhibitors, resulted in marked pancreatic hypertrophy and excessive enzyme secretion. It was also noticed that the inhibitors appeared to cause metabolic disturbances in the utilisation of the sulphur amino acids methionine and cysteine (Richardson 1980).

Poor nutritional performance of rats fed soya-bean containing diets appeared to be due to apparently reduced digestion and absorption of dietary protein, coupled to changes in systemic metabolism leading to a poor overall nitrogen balance (Grant *et al*. 1986). Aqueous heat treatment greatly reduced, but did not eliminate, the anti-nutrional effect. The whey protein (pH 4.8 soluble extract) fraction contained the bulk of TI and haemaglutination activity and gave the poorest net protein utilisation value. However, an eight-fold increase in TI content did not significantly affect apparent nitrogen digestibility. Therefore, inhibition of gut proteolytic enzyme activity *in vivo* by soybean Tls did not account fully for the poor nutritional performance.

The main ANFs in soybean are proteins which inhibit the action of intestinal proteinases, mainly trypsin, chymotrypsin and elastase (Huisman & Janman 1991). Thermal treatments denature ANFs and are needed to make soybeans suitable for feeding nonruminants. In ruminants, it is assumed (Susmel *et al.* 1995) that rumin microbial population can degrade these compounds in raw soybeans. Assuming that the average retention time of soybean in the rumin is about 12-14 hr, a considerable fraction of ingested TIA (about 50%) could pass through the rumin and to exert its effects on the host animal.

Several studies have suggested that animals fed diets containing peas. or pea extracts, having a high TI content, do not perform as well as animals fed control diets of peas with a low TI content (Huisman & Le Guen 1991; Jondreville *et al.* 1992). Other feeding trials, however, have been less conclusive, and a major flaw with this experimental approach is that the pea material utilised also varied in components other than TI, making any conclusion difficult.

**PIs as protectants:** Substantial research shows that PIs are one of the most powerful anti-carcinogens we have in our arsenal. They have proved to be particularly protective against

cancer of the colon, breast and prostate. The ability of PI to serve as cancer-chemopreventive agents *in vitro* (Kennedy 1993a) and *in vivo* (Kennedy 1993b) has been reviewed. Although the mechanisms for the anticarcinogenic activity of PIs are unknown, many hypotheses have been reported (Kennedy 1993a,b; Troll *et al*. 1984).

Kennedy (1994) suggested that PIs suppress both the initiation and promotion stages of carcinogenesis by stopping an ongoing process involved in carcinogenesis. It is known that many different PIs are capable of suppressing radiation and chemical carcinogen-induced malignant transformation in a variety of *in vivo* transformation systems (Kennedy 1993a).

Not all PIs are capable of suppressing transformation *in vivo*, and the inhibitory profiles of those that affect transformation have been discussed (Kennedy 1993b). For the suppression of transformation, the most effective of the PIs is chymostatin, a quite specific and potent inhibitor of chymotrypsin. With only picomolar concentrations in the medium, chymostatin has the ability to suppress radiation–induced transformation *in vitro* (Kennedy 1985). Inhibitors of the BBI family are effective at suppressing transformation at nanomolar concentrations (Yavelow *et al*. 1985). The structure of the soybean –drived BBI is shown in Fig.4. The two well charaterised protease inhibitory sites in BBI are shown in Fig.4 (Odani & Ikenaka 1973); only the chymotrypsin inhibitory site is involved in the suppression of transformation of BBI (Yavelow *et al*. 1985).

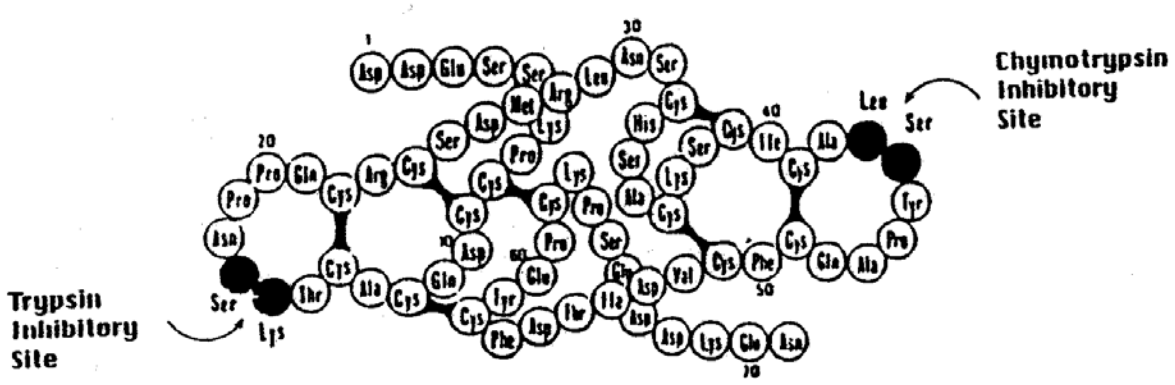


Fig. 4 : Covalent structure BBPI. Two PIs reactivesites are shown as *black circles* (Kennedy 1994).

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## الملخص العربى

# مثبطات الانزيمات المحللة للبروتين فى بذور البقوليات : وظائفها ؛ تأثيرها وصفاتها

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تحتوى بذور كثير من المحاصيل البقولية على مثبطات للانزيمات المحللة للبروتين وتقسم هذه المثبطات الى عائلات مختلفة بناء على تسلسل الاحماض الامينية المكونة لها وطبيعة مراكز التفاعل وعدد الروابط ثنائية الكبريتيد. تقوم هذه المثبطات بتثبيط الانزيمات المحللة للبروتينات فى الحيوانات المختلفة مما يؤدى الى تقليل معامل هضم البروتينات كما يسبب تضخم البنكرياس وتثبيط الحيونات. اما بالنسبة للنبات فقد اقترحت عدة وظائف لهذه المثبطات منها حماية البذرة اثناء فترة الكمون عن طريق تثبيط الانزيمات التى تحلل بروتينات البذور كما انها تقوم بحماية النبات من الحشرات والميكروبات. يتناول هذا المسح المرجعى دور مثبطات الانزيمات المحللة للبروتين فى النبات وفى التغذية وكذلك كيفية التخلص أو التقليل من اثر تلك المثبطات عند استخدام بذور البقوليات فى الغذاء. كما تطرق هذا المسح المرجعى الى دور هذه المثبطات فى علاج بعض الامراض .السرطانية.