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Protein proteinase inhibitors in legume seeds -overview

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SUMMARY. Protein proteinase inhibitors are widely distributed in plant seeds, particularly in legumes. The specificity and potency of inhibition depend on defined inhibitory sites and on the animal species of the target proteinase. Feeding experiments on diets containing isolated soybean trypsin inhibitors (the Kunitz soybean trypsin inhibitor STI and the Bowman-Birk trypsinchymotrypsin inhibitor BBI) caused insignificant growth depression in ratas and chicks, but induced enlargement of the pancreas in ratas, chicks and mice but not in pigs, dogs, calves, monkeys and presumably humans. The trypsin-inhibitory site has been responsible for induction of the pancratic enlargement. The trypsin-chymotrypsin inhibitors from soybeans and from chickpeas inhibit insect midgut proteinases, supporting the hypothesis that proteinase inhibitors comprise a built-in defense mechanism of the seed against insects. Findings on the involvement of proteinase inhibitors, such as BBI, in prevention of tumorigenesis suggest a possible positive contribution of the inhibitors to the nutritional value of legume seeds. BBI is also an effective inhibitor of nephrotoxicity induced by the antibiotic gentamicin. BBI does not cause side effects and does affect the antimicrobial activity. The *in vitro* effects of proteinase inhibitors on animals should be interpreted with caution when related to humans.

RESUMEN. Avances en la investigación sobre los inhibidores proteicos de proteasas en semillas de leguminosas. Los inhibidores proteicos de proteasas se encuentran ampliamente distribuidos en las semillas de plantas, particularmente en las leguminosas. La especificidad y potencia de la inhibición depende de sitios definidos en el inhibidor y de la especie animal de donde se obtengan las proteinasas utilizadas. Experimentos nutricionales con dietas que contenían inhibidores purificados de soya (el inhibidor de tripsina de Kunitz, STI, y el inhibidor de tripsina y quimotripsina de Bowman-Birk, BBI) evidenciaron una insignificante reducción del crecimiento en ratas y pollos, pero indujeron hiperptrofia del páncreas en ratas, pollos y ratones, pero no en cerdos, perros, terneras, monos y, presumiblemente, tampoco en humanos. Se ha atribuido al sitio de inhibición para la tripsina la responsabilidad de promover el crecimento del páncreas. Los inhibidores de tripsina-quimotripsina de soya y de garbanzo inhiben las proteinasas del tracto intestinal medio de insectos apoyando la hipótesis de que los inhibidores representan un mecanismo de defensa de las semillas en contra de los insectos. Los hallazgos sobre la participación de los inhibidores de proteasas, tales como el BBI, en la prevención de la tumorogénesis, sugieren una posible contribución positiva de los inhibidores al valor nutricional de las semillas de leguminosas. BBI también es un potente inhibidor de la nefrotoxicidad inducida por el antibiótico gentamicina y, aunque no causa efectos secundarios, si afecta la actividad antimicrobiana del antibiótico. Los efectos *in vitro* e *in vivo* de los inhibidores de proteasas sobre los animales deben ser interpretados con cautela cuando se refieren a los seres humanos.

INTRODUCTION

The increasing interest in protein-rich legume seeds for use in human and animal nutrition is bringing also into perspective the possible, long -and short-term nutritional effects of the endogenous proteinase inhibitors. The inhibitors are proteins with molecular weights in the range of 7 to 25 kd and they amount to 0.1% of the seed protien content. Their physiological role in the plant is still being questioned. The fact that legume seeds contain inhibitors of growth and of gut proteinases of several stored-product insects suggested the hypothesis that the inhibitors have evolved as a defense mechanism against predatory insects. The first trypsin inhibitor from legume seeds was isolated and characterized by M. Kunitz in 1947 (1). By now the presence of proteinase inhibitors in all legume seeds is an established fact. The inhibitors differ in specificity and in capacity to inhibit one or two proteinases at the same time. Several kinds of inhibitors can be present in a single tissue, as in soybeans. The possible antinutritional effects of legume seed proteinase inhibitors have been studied

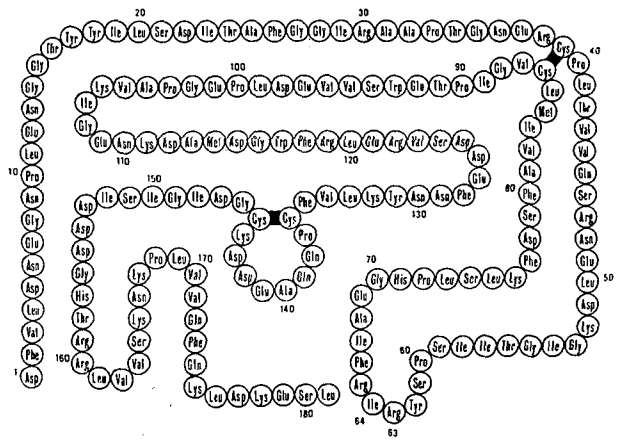
extensively (reviewed, 2). However, the quantification of the effects of proteinase inhibitors *per se* is hampered by several factors such as the form in which the inhibitors are present - raw seeds or purified inhibitors- the feeding strategy and the protein status of the animal (3). The evidence that proteinase inhibitors constitute a hazard to health is often only presumptive since the research relating to its harmfulness has been done with experimental animals consuming large quantities of a particular constituent over a lengthy period of time - conditions quite different from the level of the inhibitor in a normal varied diet.

The epidemiologic evidence for a cancer protective role of proteinase inhibitors in populations consuming legume seeds rich in inhibitors comes primarily from correlation studies (4). The epidemiologic observations led to extensive investigations of proteinase inhibitors as cancer chemopreventive agents of major human cancers (reviewed and summarized, 5). The possible therapeutic contribution of dietary proteinase inhibitors to the prevention of certain types of cancer open a new era in the research of legume seed proteinase inhibitors possible therapeutic contribution of dietary proteinase inhibitors.

EXPERIMENTAL RESULTS

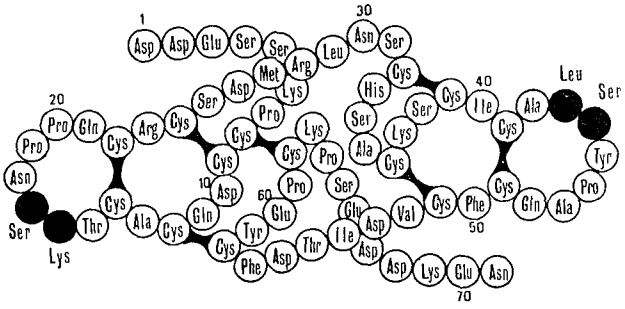
A summary representing the current knowledge on the distribution of legume seed proteinase inhibitors and their specificity of inhibition is given in Table 1 (6,7). The Kunitz soybean trypsin inhibitor (STI), molecular weight~22 kd (1,8), inhibits trypsin *via* the inhibitory site at Arg63- Ile64 (Fig. 1).

FIGURE 1
Amino acid sequence of the Kunitz soybean trypsin inhibitor (8)



It has been also studied thoroughly in numerous laboratories and served also for the establishment of the Standard Mechanism of Inhibition (9). STI is inactivated by heat and by gastric juice (10). However, only a few inhibitors homologous to STI have been found in common legume seeds. The predominant type of inhibitor in legume is the Browman-Birk trypsin-chymotrypsin inhibitor (BBI) from soybeans (Fig. 2) (11,12).

FIGURE 2
Amino acids equence of the Bowman-Birk soybean trypsin and chymotrypsin inhibitor (12)



It has a molecular weight of~8 kd and it forms a 1:1 complex with either trypsin or chymotrypsin and a ternary complex with both enzymes *via* the inhibitory sites at Lys16-Ser17 and Leu43-Ser44 against trypsin and chymotrypsin, respectively. BBI inhibits bovine, porcine, avian, fish and human trypsins and chymotrypsins and is also a potent inhibitor of trypsin - and chymotrypsin-like enzymes from the digestive tracts of insects. The inhibitor is relatively stable to heat and to gastric juice and has unusual resistance to proteases, such as pepsin and pronase. Inhibitors homologous to BBI have been found in lima beans, garden beans, adzuki beans, mung beans, ground nuts and chickpeas (summarized 2, 13, 14). Six proteinase inhibitors have been recently isolated from winter pea seeds. Their partial sequence suggests that they belong to the Bowman-Birk family of trypsin inhibitors (15). Extensive studies have been carried out on lentil seeds, from which twenty-three proteinase inhibitors, belonging to the Bowman-Birk inhibitor family, have been isolated and partly characterized (16). Recently, the expression of BBI and of several active mutants has been achived in *E. coli* (17).

The discovery of thermo-labile trypsin inhibitors in soybeans led to the assumption that the beneficial effect achieved by heat processing of raw soybean meal is due to the destruction of the inhibitors. Feeding experiments of rats and chicks carried out on properly heated soybean meal diets supplemented with STI, BBI, or both, resulted in an insignificant depression of animal growth rate, but the inhibitors were responsible for pancratic enlargement (18). In addition, feeding of rats with raw soybean protein isolates that had a very low trypsin inhibitor content resulted in remarkable growth depression (19). The fachure of soybean trypsin inhibitors to cause growth depression was also demostrated *in calves (20).*

TABLE 1
DISTRIBUTION OF PROTEINASE INHIBITORS PRESENT IN LEGUMES

Botamical name	Common name	Proteinases inhibited[a]
Arachis hypogaea	Peanut, groundnut	T,C,Pl, K
Cajanus cajan	Pigeon pea, red gram	T
Canavalia ensiformis	Jack bean, sword bean	T,C,S
Chamacrista fasiculata	Partridge pea	T
Cicer arietinum	Chick pea, Bengal gram, Garbanzo	T,C
Clittoria ternatea	Butterfly pea	T,C,S
Cyamopsis tetragonoloba	Cluster bean	T,C,S
Dolichos biflorus	Horse gram	T
Dolichos lablab	Hyacinth bean, Hakubenzu bean	T,C, Th
Faba vulgaris	Double bean	T
Glycine max	Soybean	T, C
Lathyrus odoratus	Sweet pea	T
Lathyrus sativus	Chickling vetch	T, C
Lens esculenta (culinaris)	Lentil	T, C
Lupinus albus	Lupine	T
Mucana deeringianum	Florida velvet bean	T
Plaseolus aconitifolius	Moth bean	T
Phaseolus angularis	Adzuki bean	T, C
Phaseolus aureus	Mung bean, green gram	T, endopeptidase
Phaseolus coccineus	Scarlet renner bean	T, C
Phaseolus lunatus	Lima bean, butter bean	T, C
Phaseolus mungo (radiatus)	Black gram	T, C, S
Phaseolus vulgaris	Navy bean, kidney bean, pinto bean, French bean, white bean, wax bean, haricot bean, garden bean	T, C, E, S
Pisum sativum	Field bean, garden pea	T
Psophocarpus tetragonolobus	Winged bean, Gao bean	T
Stizobolium deeringanum	Velvet bean	T
Vicia faba	Broad bean, field bean, faba bean	T, C, Th, Pr, Pa
Vigna unguiculata (sinensis)	Cowpea, black-eyed pea, Southern pea, serido pea	T, C
Voandzeia subterranca	Bambara bean	T

[a]C= chymotrypsin; E= elastase; K = Kallikrein; Pa= papain; Pl= plasmin; Pr= pronase; S= subtilisin; T= trupsin; Th= thrombin.
Source: Liener (6), compiled from Liener and Kakade (7).

The physiological effects of proteinase inhibitors differ between animal species (21). Among the sources of variation one may count the extent of the digestion of a diet, the specificities and morphology of the gastro-intestinal tract and the extent of inactivation in the stomach (3). Another important factor that should be taken into consideation is the stoichiometry of inhibitions which may depend on differences in number and potency of secondary binding sites between the inhibitors and the trypsins from different animal species. The inhibitory capacities of BBI, measured in terms of trypsin or chymotrypsin inhibited, are usually evaluated on bovine pancreatic proteinases. This has raised the question of validity and relevance of inhibition data with respect to trypsin and chymotrypsin from other animal species. Indeed, it has been shown that many inhibitors that strongly inhibit bovine trypsin do not inhibit human trypsin (22). STI inhibits more trout trypsin than bovine trypsin (23) and BBI inhibits more carp trypsin and chymotrypsin than the bovine enzymes (24). As for pea proteinase inhibitors, they inhibit more bovine and rat trypsin than porcine trypsin (25). Trypsin and chymotrypsin from rats, mink and pigs showed appreciable differences in their sensitivity toward pea proteinase inhibitors (26). The recently reported differences in the mode of action of lentil proteinase inhibitors against human and bovine chymotrypsin and trypsin have been attributed to a combination of the unsual, additional binding of human chymotrypsin at the trypsin inhibitory site and the weak binding of bovine chymotrypsin (27).

The most striking finding on the effect of proteinase inhibitors is the remarkable enlargement of the pancreas and the increase in pancreatic secretory activity. Ingestion of raw soybean meal or trypsin inhibitors caused pancreatic enlargement in rats, chicks, mice and young guinea pigs but not in adult guinea pigs, dogs, growing swine, claves and presumably, humans (reviewed, 28), and it has not been noted in primates even after 5 years of feeding on soybean-based protein diets containing trypsin inhibitors (29). Pancreata of rats and chicks adapted to raw soybean meal synthesized more trypsinogen and chymotrypsinogen than pancreata of rats adapted to heated soybean meal (30). The trypsin inhibitory site, rather than chymotrypsin inhibitory site of BBI, is involved in the enlargement of the pancreas and in the increase of pancreatic proteolytic activity (31).

The mechanism by which the inhibitor induces the pancreatic enlargement is not yet fully understood. It is explained by a feedback inhibition which depends on the level trypsin present at any given time in the small intestine. When the level of this enzyme falls below a certain critical threshold value, the pancreas is induced to produce more enzyme. The suppression of this negative feddback mechanism can thus occur if the trypsin is complexed with the inhibitor. It is believed that the mediating agent between trypsin and the pancreas is the hormone cholecytokinin (CCK), which is released from the intestinal mucosa when the level of trypsin in the intestine falls below its theshold level (reviewed, 6). The negative feedback mechanism of pancreatic enzyme secretion found in the rat exists also in the pig and calf, which do not develop pancreatic enlargement (28). A recent study has confirmed the existence of feedback control in human (32).

In view of the growing interest in the use of legume seed products in the human diet, it becomes important to assess the effect on human health associated with the consumption of trypsin inhibitors present in a wide variety of foods. In a study carried out in 1977 (33), it has been found that prolonged feeding of male Wistar rats on raw soybean meal enhanced the action of the pancreatic carcinogen azaserine. In the «USDA trypsin inhibitor study», male Wistar rats, which had been fed

raw soybean meal or experimental unheated soy protein isolates for 2 years, developed pancreatic nodular hyperplasia and acinar adenoma in a dose-dependent manner (34). However, similar long-term feeding of mice and hamsters on raw soybean meal, in the presence or absence of chemical carcinogens, failed to induce carcinogenic changes in the pancreata. Moreover, the raw soybean meal seems to have excerted a protective effect on the chemical induction of tumors in the hamster (35). In view of the difference in species response to the presence of inhibitors in the diet, the relevance of the above pancreatic effects in human remains to be elucidated.

Tribolium castaneum, the red flour beetle that causes serious damage to wheat, rice and corn but fails to develop on raw soybeans, served as a model for these studies. The *Tribolium* midgut trypsin-like proteolytic activity was fully inhibited by BBI when co-submitted to polyacrylamide gel electrophoresis in gels embedded with gelatin, but not at all - when assayed on a protein substrate in solution. This points towards the necessity for mutual, «solid state», exposure of enzyme to inhibitor, in a similar manner to the interaction of the insect with solid food (36).

In a series of studies in rats, BBI significantly counteracted the nephrotoxicity induced by the antibiotic amino glycoside gentamicin commonly used in clinical practice. BBI did not show any side effects and did not affect the antimocrobial activity gentamicin (37, 38).

Epidemiological studies have identified legumes as possible protective agents in the decreased occurrence of breast, colon and prostatic cancer in vegetarian population. The association of legume seeds rich in proteinase inhibitors with prevention of human cancers, stimulated the insight into the possible action of proteinase inhibitors as cancer chemopreventive agents. The epidemiologic evidence for a cancer protective role of the inhibitors comes primarily from correlation studies (4, 39) and from evidence based on *in vitro* models rather than on studies in humans. The *in vitro* studies of the anticarcionogenic proteinase inhibitors have recently been compiled by Kennedy (40), showing that BBI proved to be a potent anticarcinogenic agent *in vitro* (41). In experiments on transformation of C3H/10T1/2 cells, it has been shown that nanomolar concentrations of BBI suppress the X-ray-induced transformation *in vitro* and that the chymotrypsin-inhibitory domain of BBI is responsible for this effect (42). The recently isolated, thermostable, trypsin- and chymotrypsin-inhibitor from amaranth seeds effectively blocked estrogen-induced tumorigenesis of MCF7 breast cancer cells *in vitro*. Similar results were obtained with BBI (43,44). Recent studies on the effect of BBI *in vivo* have demostrated the ability of dietary BBI to prevent or suppress carcinogenesis in animal model systems *in vivo* (45, 46).

DISCUSSION

Proteinase inhibitors are a significant component of human food. The evidence that they constitute a hazard to health is frequently only presumptive and must be placed in perspective in relation to the level of total proteinase inhibitors in the overall diet. Most of the *in vivo* research has been done with small animals that consumed large quantities of a particular food component over a long period of time- a situation quite removed from the eating patterns and varied diets of humans. The reported findings point also at remarkable differences between animal species in response to ingested proteinase inhibitors. The *in vivo* differences are supported by the *in vitro* experiments demostrating the variability in inhibitory capacity of a certain trypsin inhibitor towards target trypsins from different animal species. The relevance of *in vitro* assays to conditions prevailing *in vivo*, and different potencies of inhibition achieved under different assay conditions, as found for *Tribolium* trypsin, should also be taken into account. These imply standardization of analytical methods and consequent quantitative evaluation of foods in *in vivo* experiments. Finally, the alleged anti-nutritional properties of legume seed proteinase inhibitors should be weighed against their potential in protecting valuable crops and foods in storage and also as possible anticarcinogens (47).

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