

## Protease Inhibitors in Processed Plant Foods

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### ABSTRACT

Plants contain a wide variety of protein protease inhibitors. However, most is known about the serine protease (trypsin and chymotrypsin) inhibitors found in legumes, particularly soybeans. These inhibitors in unheated legume protein (a) impair the protein's nutritional quality, (b) induce pancreatic hypertrophy in some but not all experimental animals, (c) enhance the action of chemical pancreatic carcinogens in Wistar rats but not hamsters or mice, (d) are reported to be carcinogenic to the pancreas of Wistar rats and (e) inhibit certain experimental tumors in rats, mice and hamsters. The physiological significance of the low residual protease inhibitor levels in commercially processed plant proteins and human foods prepared from such proteins remains to be resolved. Plant proteins prepared for human consumption, however, contain low levels of protease inhibitor activity which are of no nutritional concern in animals or humans.

Protease inhibitors are widely distributed throughout nature and are found in plants, animals and microorganisms. The scope of this overview will be restricted to protein protease inhibitors occurring in plants, particularly plants contributing to the human diet. The overview will include a brief description of protein protease inhibitors, a discussion of their nutritional, physiological, toxicological, and therapeutic significance, and a review of the effects of food processing and food preparation procedures on protease inhibitor activity.

### OCCURRENCE AND PROPERTIES OF PROTEIN PROTEASE INHIBITORS IN PLANTS

Protease inhibitors have been found in a great variety of plants, including most legumes and cereals and certain fruits (apples, bananas, pineapples and raisins) and vegetables (cabbage, cucumbers, potatoes, spinach and tomatoes) (4,43). It has been estimated that between 5 to 10% of the soluble proteins in barley, wheat and rye grains are protease inhibitors (35). These levels of inhibitors approximate the lower estimates of the levels found in mammalian pancreas and plasma (4,49). The quantity of protease inhibitors depends on variety and physiological status of the plant and on levels of insect

infestations or damage. Hence, the protease inhibitor activity found in seeds or tubers will vary with variety, timing and conditions of harvest, and duration and conditions of storage (43). Plant inhibitors have been identified for enzymes from four major protease classes. The enzymes of importance regarding mammalian protein digestion and nutrition which are susceptible to plant protease inhibitors include: the carboxyl protease, pepsin; the serine proteases, trypsin, chymotrypsin and elastase; and the metallo-proteases, carboxypeptidases A and B. There are also plant protease inhibitors which inhibit the mammalian plasma serine proteases, kallikrein and plasmin, and the plant sulfhydryl proteases, bromelain, ficin and papain (43).

The protein protease inhibitors found in plants generally contain little or no carbohydrate and have a molecular weight ranging from 4,000 to 80,000. The widely studied Bowman-Birk protease inhibitor from soybeans contains 71 amino acids with a molecular weight of 8,000, and the Kunitz inhibitor from soybeans contains 198 amino acids with a molecular weight of 23,000. Many of the larger protease inhibitors are polymeric containing up to four subunits. The amino acid sequences of many protease inhibitors have been determined, and a characteristic feature is a large degree of sequence homology both within the same inhibitor and between inhibitors from different plants (43). All protease inhibitors have one or more peptide bond (reactive site) which interacts with the corresponding enzyme active site. The reactive sites are normally found closely associated with half cystine residues linked by a S-S bridge (24,25). Often two or more enzymes can be inhibited by the same protease inhibitor. In these instances, the different enzymes can compete for the same site on the inhibitor or can be inhibited by separate sites on the inhibitor molecule. The reactive sites of inhibitors react with active sites on the corresponding enzyme in a similar manner to the interaction between substrates and enzyme. Unlike substrates, however, the inhibitors form an extremely stable complex with the enzyme which dissociates slowly. It has still not been entirely resolved why these proteins are inhibitors, not just substrates for their respective enzymes (24).

## NUTRITIONAL AND PHYSIOLOGICAL SIGNIFICANCE OF PROTEASE INHIBITORS

It has been known for many years that the nutritional quality of the protein of legumes can be improved by heat treatment (17). The main heat labile factors in legumes are protease inhibitors and lectins and have been extensively reviewed by Liener (29,30). Although protease inhibitors are present in almost all legumes, the heat-labile trypsin inhibitors in soybeans have been most extensively examined due to the importance of soy protein as a component in animal and human nutrition. In the 1940's, it was first realized that the improved nutritional quality of soybeans for rats, chickens and mice due to heating could be attributed to destruction of these heat-labile trypsin inhibitors (29). Rackis et al. (41) have clearly demonstrated that as soy flakes are treated with moist heat for increasing periods of time, the trypsin inhibitor activity decreases and protein quality as measured by protein efficiency ratio (PER) or apparent nitrogen digestibility increases progressively.

Trypsin inhibitor activity, however, is generally not a good predictor of the nutritional quality of soy protein preparations. Kakade et al. (21) measured trypsin inhibitor activity and PER of 25 different varieties of soybean and found no correlation between PER and trypsin inhibitor levels. In a further experiment, Kakade et al. (18) demonstrated that, for soy flakes, removal of trypsin inhibitor by affinity chromatography increased the PER from 1.4 to 1.9, but heat treatment causing a similar decrease in trypsin inhibitor activity effected a greater increase in PER from 1.4 to 2.7. The lower PER of trypsin inhibitor-depleted soy protein compared to heated soy protein may be partially due to removal of the sulfur amino acid-rich component (trypsin inhibitor) of a protein limiting in sulfur amino acids. However, measurements of *in vitro* digestibility and pancreatic hypertrophy support the author's conclusion that not all growth-inhibiting activity can be attributed to trypsin inhibitors, but that native soy protein can be resistant to digestion by proteolytic enzymes (18). Therefore, trypsin inhibitor activity alone cannot be used to assess soy protein quality as other factors, including sulfur amino acid availability and protein digestibility, are also involved.

The addition of trypsin inhibitor preparations to diets containing free amino acids, and therefore not requiring intestinal digestion, results in decreased growth (29). Hence, the growth-impairing effects of trypsin inhibitors are not completely due to a decreased intestinal proteolysis and digestion of dietary protein but some other mechanisms are also involved. One such mechanism is attributed to pancreatic hypertrophy and hyperplasia, presumably due to the overstimulation of exocrine pancreatic secretion, which always appears to be associated with feeding inadequately heated soy protein preparations to rats (39). The degree of pancreatic hypertrophy shows a strong inverse correlation with protein quality or growth in rat studies investigating different varieties of soybean

(21), varying heat treatments (41) and various soy protein fractions (37). Pancreatic hypertrophy and the over-secretion of pancreatic enzymes can cause part of the growth depression in rats fed unheated soy protein because sulfur amino acids are first-limiting in soy protein and pancreatic enzymes are rich in sulfur amino acids (29).

The following mechanism has been proposed to explain the pancreatic hypertrophy caused by trypsin inhibitors in rats. The level of pancreatic secretions and pancreas size are normally regulated through cholecystokinin by the level of free trypsin in the intestine. After ingestion of trypsin inhibitors or protein, free trypsin levels fall and the pancreas is stimulated to secrete more enzymes, including trypsin (10,31). The persistent stimulation of pancreatic secretion elicited by dietary trypsin inhibitors in the rat results in pancreatic hypertrophy. Protease or trypsin inhibitors from other plants have not been as extensively examined as those from soybeans, but available data indicate they exert their effects on physiology and nutritional quality of the protein by similar mechanisms (29).

## TOXICOLOGICAL ASPECTS OF PROTEASE INHIBITORS

Rapidly growing tissue has an increased susceptibility to chemical carcinogens. Therefore, it is not unreasonable that feeding raw soy flour to rats would cause pancreatic hypertrophy and increase the susceptibility of the organ to chemical carcinogens. This phenomenon was first reported by Morgan et al. (36) in 1977. They demonstrated that after male Wistar rats were fed raw soy flour and given weekly intraperitoneal injections of a pancreatic carcinogen, azaserine, for 19 wk, all pancreatic sections examined had nodules in the acinar tissue. Control rats fed heated soy flour and injected with saline had no nodules and other rats receiving either raw soy flour and saline injections or heated soy flour with azaserine injections had fewer pancreatic nodules (36). The same investigators have extended and verified these preliminary observations in Wistar rats and in all instances the carcinogenicity of azaserine was increased by feeding raw soy flour diets relative to heated soy flour diets (32,34). Enhanced carcinogenicity of the pancreatic carcinogen, N-nitrosobis(2-hydroxypropyl)amine, has also been observed in Wistar rats fed raw soy flour diets (28). The susceptibility of rapidly growing pancreatic tissue to chemical carcinogenesis was further demonstrated by the increased carcinogenicity of azaserine in Wistar rats recovering from DL-ethionine-induced pancreatitis (33).

In addition to the synergistic effects of pancreatic carcinogens and raw soy flour on pancreatic cancer, McGuinness et al. (34) observed that male Wistar rats fed raw soy flour for extended periods of time and receiving no known carcinogen developed a high incidence of preneoplastic pancreatic lesions, a few of which ultimately progressed to carcinomas. In the same study, feeding heated soy flour in the absence of any known

pancreatic carcinogens also led to the appearance of some preneoplastic nodules in the pancreas, indicating that these male Wistar rats may have a high susceptibility to "spontaneous" pancreatic cancer. In a U.S. Department of Agriculture study of trypsin inhibitor, male Wistar rats fed unheated soy flour or experimental unheated soy protein isolates (trypsin inhibitors) for 2 years developed preneoplastic pancreatic lesions in a dose-dependent manner (11). In contrast to the experiments of McGuinness et al. (34), however, carcinomas appeared with a similar low frequency (ca. 1%) among control rats and those fed unheated or heated soy protein in the USDA study (11). A further experiment with male Wistar rats failed to reveal either preneoplastic or neoplastic histological changes in pancreata from rats fed trypsin inhibitors as egg white or as commercially available soy protein isolate for 18 months (44). This variability in the response of pancreas of Wistar rats to soy trypsin inhibitors implies that factors other than trypsin inhibitors are also involved. Diets containing high levels of polyunsaturated fat, as used by McGuinness et al. (34), are known to enhance azaserine-induced pancreatic carcinogenesis (45). In addition, because there are at least ten distinct trypsin inhibitors in soybeans (47), different soy preparations are likely to have different trypsin inhibitor profiles. It is not known which of the trypsin inhibitors is associated with the abnormal pancreatic histology in Wistar rats. There are no reports to date of soy-induced neoplastic pancreatic lesions in any strain of rat, other than the Wistar.

Diets containing trypsin inhibitors as unheated soy have been fed to two other animal species (hamster and mouse) in long-term carcinogenesis studies. Hamsters fed either raw or heated soy flour had similar low (4%) incidences of pancreatic lesions (13). Furthermore, an anticarcinogenic effect of raw soy flour was evident, as hamsters injected with the pancreatic carcinogen, N-nitrosobis(2-oxopropyl)amine, and fed raw soy flour had a 9% incidence of pancreatic cancer compared to an 88% incidence in animals fed heated soy flour (13). The experiments with mice demonstrate that raw soy flour, either in the absence or in conjunction with a pancreatic carcinogen, azaserine, produced similar effects on pancreatic histology as heated soy flour (14). Hence, there are species and perhaps strain differences regarding the appearance of pancreatic lesions in response to dietary protease inhibitors (Table 1).

From available data, it appears that there is no correlation between the susceptibility of a particular animal to pancreatic hypertrophy or hyperplasia and the development of pancreatic lesions after prolonged feeding of unheated soy or trypsin inhibitors. Interspecies extrapolations regarding the potential development of preneoplastic and neoplastic pancreatic lesions in response to dietary protease inhibitors need to consider differences in (a) susceptibility of the inhibitors to gastric digestion (23), (b) susceptibility of pancreatic proteases to the inhibitors (see below) and (c) regulatory

TABLE 1. Effects of trypsin inhibitors as unheated soy protein on the pancreas of various animal species<sup>a</sup>.

Animal species	Hypertrophy/hyperplasia <sup>b</sup>	Increased incidence of adenomas/nodules
Mouse	+	- <sup>c</sup>
Rat, Wistar	+	+ <sup>d</sup> , - <sup>e</sup>
Rat, other strains	+	?
Hamster	+	- <sup>f</sup>
Guinea pig	+	?
Dog	-	?
Pig	-	?
Human	(-)	?

<sup>a</sup>Positive responses are indicated by "+", no response by "-", unknown responses by "?", and predicted responses are in parentheses.

<sup>b</sup>Data from Liener (29).

<sup>c</sup>Data from Hasdai and Liener (14).

<sup>d</sup>Data from McGuinness et al. (34) and Gumbmann et al. (11).

<sup>e</sup>Data from Richter and Schneeman (44).

<sup>f</sup>Data from Hasdai and Liener (13).

mechanisms for pancreatic enzyme secretion and function.

### THERAPEUTIC POTENTIAL OF PROTEASE INHIBITORS

Troll et al. (50,51) have proposed that protease inhibitors can be anticarcinogenic. A review of epidemiologic data led them to investigate the effect of feeding protease inhibitors in the form of soybean diets on carcinogenesis in rats. The incidence of radiation-induced mammary cancer in rats fed raw soybeans was 44% compared to 74% in rats fed a casein diet (51). The diets were fed before, during, and after radiation treatment so it is difficult to assess whether the effects of protease inhibitors are exerted during the initiation or promotion stages of carcinogenesis.

In mice, Becker (1) found that feeding diets containing an unheated soy protein isolate, Edi-Pro A, inhibited the induction of "spontaneous" hepatocellular tumors. As previously discussed, chemically-induced pancreatic carcinogenesis was unaffected by feeding raw soy flour to mice (14) but was inhibited by feeding raw soy flour to hamsters (13).

In addition to the anticarcinogenic effects of protease inhibitors when included in the diet of animals, it has also been observed, in a tissue culture system using mouse C3H/10T-1/2 cells, that oncogenic transformation and promotion can be inhibited by protease inhibitors. Kennedy and Little (22) demonstrated that Kunitz soybean trypsin inhibitor, while having no effect on the X-ray-induced transformation of these cells, did suppress the promotional effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) on transformed cells. A subsequent study using the same in vitro system, indicated that Bowman-Birk soybean trypsin inhibitor inhibited X-ray-induced transformation but had no effects on the promotion of transformed cells by either TPA or croton oil (52).

The efficacy of soy flour diets in the treatment of human chronic pancreatitis has been examined by Pap et al. (38). These authors concluded that feeding soy flour diets for 1 month represented an effective treatment for chronic pancreatitis with moderate pancreatic insufficiency.

#### MEASUREMENT OF PROTEASE INHIBITOR ACTIVITY

To follow the effects of various food processing techniques on protease inhibitor levels, it is necessary to have appropriate assay methods. The most commonly used assay procedure involves measuring the decrease in protease activity due to the presence of an extract containing the inhibitor. Protease activity can be readily measured by monitoring the conversion of a substrate to a product which is colored or has some other characteristic which facilitates quantitation. Many procedures based on this principle have been proposed for the measurement of trypsin inhibitor activity in materials containing soy protein (2,5,12,19,20,46). A common feature of all these procedures is that bovine trypsin is used as the protease source. The relevancy of applying protease inhibitor data obtained using bovine trypsin to other species, including man, has been challenged by Holm and Krogdahl (15). These investigators found that proteases from human pancreatic juice and commercially available purified bovine and porcine sources ranked the protease inhibitor activity of different soybean varieties differently. They also found that human gastric juice inactivates the Kunitz but not the Bowman-Birk soy trypsin inhibitor (23). (The inhibitor assays [2,5,12,19,20,46] do not differentiate between different types of inhibitor.)

In addition, studies with purified inhibitors revealed that a strong inhibitor of trypsin from one species may be a very good substrate for trypsin from another species (24). The multiple forms of human trypsin is a further confounding factor concerning the relevance to human health and nutrition of data obtained using bovine or other sources of purified trypsin *in vitro*. The major or anionic trypsin component in human pancreatic juice is only poorly inhibited by the Kunitz soybean trypsin inhibitor, whereas the minor cationic form is inhibited at a stoichiometric inhibitor:enzyme ratio of 1:1 (6).

Despite these objections, the inhibition of bovine trypsin by soy preparations provides a feasible measurement of trypsin inhibitor activity. However, results from such assays need to be interpreted carefully and appropriate controls need to be used because artifacts can occur. When soy protein samples are being assayed, the presence of adequate calcium ions is imperative because most soy preparations contain phytate which can complex with calcium, an essential cofactor for trypsin, and decrease enzyme activity (26). For samples in which trypsin inhibitor in a small proportion of the total sample, large quantities must be added in the assay to obtain measurable responses. However, high protein levels may compete with the synthetic substrate for hydrolysis by trypsin

and result in the measurement of falsely high trypsin inhibitor activity. All protein preparations appear to have some trypsin-inhibiting activity in this assay system.

Immunochemical techniques have potential to quantify trypsin inhibitors in food preparations. They are unlikely to become widely used because the immunological identity of specific inhibitors may vary between soybean varieties and because there are at least ten different protein fractions in soybean with trypsin-inhibiting activity (47). Furthermore, it will be necessary to demonstrate that heat treatment and processing decrease the biologic activity and immunological identity of inhibitors by the same degree.

#### FOOD PROCESSING EFFECTS ON PROTEASE INHIBITORS

Some technologies with potential for eliminating protease inhibitors from processed plant foods are indicated in Table 2. Plant breeding to eliminate protease inhibitors are long-term programs, but, using food processing methods, immediate decreases in protease inhibitor activity can and are being achieved. Moist heat treatment in the form of home cooking or industrial food processing is extensively used to prepare plant materials for human consumption. Moist heat is an effective method for decreasing protease inhibitor activity and improving the nutritional quality of the plant protein (17,41). Normal cooking procedures significantly decrease trypsin inhibitor activity of eggs, broad beans, cabbage and potatoes (2,4). The trypsin inhibitor activity, on a protein basis, of tofu and cooked tofu is 19 and 11%, respectively, of that in raw soybeans (4).

In addition, industrial processing and heat treatment can result in toasted soy flours, soy protein concentrates and soy protein isolates which have less than 10% of the trypsin inhibitor activity of raw soy flour (2,19,46; Burns, unpublished data). Further heat treatments, including spray drying or canning and sterilization which are used to manufacture soy-based infant formulas from soy protein isolates, result in additional decreases in trypsin inhibitor activity so that the final level is approximately 3% of that in raw soy flour and is equivalent to the inhibition of approximately 2 to 5  $\mu\text{g}$  trypsin/mg protein (3; Burns, unpublished data). These levels of trypsin inhibitor in processed soy products are of no nutritional or physiological concern in rats (3,41) and

TABLE 2. *Potential technologies for eliminating protease inhibitors from processed plant proteins.*

1. Genetic manipulation of plants
2. Moist heat treatment, especially in the presence of thiols (cysteine, N-acetyl-cysteine)
3. Protein fractionation techniques
  - a. Precipitation of protein isolates at different ionic strengths or pH values.
  - b. Ultrafiltration
  - c. Affinity chromatography
4. Enzyme (protease) treatment

are unlikely to be of significance in human nutrition. Soy-based infant formulas supplemented with methionine promote growth and nitrogen retention of infants equivalent to milk formulas (7,8). Similarly, studies with children (48) and adults (16,42,53) demonstrate that commercially available soy protein isolates and concentrates are highly digestible and are nutritionally equivalent to high quality animal proteins.

In the presence of thiols, such as cysteine or N-acetyl-L-cysteine, moist heat treatment has a greater effect on the denaturation of trypsin inhibitors in soy preparations (9,27). The commercial feasibility of these procedures remains to be evaluated. Moist heat either with or without thiols presumably denatures the inhibitors by causing rearrangement of disulfide bridges which are important in maintaining the integrity of reactive sites.

Protease inhibitors generally have a lower molecular weight than major plant proteins. This can be exploited during the preparation of soy protein isolates with decreased protease inhibitor levels either by precipitation under specific conditions or by ultrafiltration. The aim of these procedures would be to separate the small molecular weight protease inhibitors from the larger molecular weight major component of soy protein. Commercial feasibility remains questionable for these processes because total protein yield may be decreased. Mild protease treatment of soy protein isolates can also decrease trypsin inhibitor levels (Burns, unpublished data).

### CONCLUSIONS

Protease inhibitors in some unprocessed plant foods such as soybean, are of nutritional, physiological and toxicological concern in rats. The trypsin inhibitor levels found in raw soybeans are substantially decreased by the processing conditions used to isolate soy proteins and those used in the further manufacture of human foods from these protein preparations. The resulting low levels of trypsin inhibitors are not of nutritional importance in experimental animals or humans.

Low levels of trypsin inhibitor may be of toxicological concern in the Wistar rat because in some, but not all, studies they have been shown to develop preneoplastic pancreatic lesions when fed heated soy flour diets for 2 years. (However, two other animal species did not develop pancreatic lesions under similar conditions.) Further research is required to determine mechanisms and ultimately to evaluate the effects of low dietary trypsin inhibitor levels on human pancreatic physiology. Food processing conditions could be employed to decrease trypsin inhibitor levels below those already found in certain foods. The potential benefit of further removal of protease inhibitors from processed plant foods for human consumption needs to be critically evaluated in relation to the cost of the additional processing and the contribution of non-processed foods to the total consumption of protease inhibitors.

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