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Trypsin inhibitors, antinutrients or bioactive compounds? a mini review

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Abstract

Trypsin inhibitors are proteins found in plant-based foods, mainly legumes and cereals. They have traditionally been described as anti-nutrients since their consumption leads to lower protein digestibility along with pancreatic hypertrophy. Given the problems which can arise, there are various technologies used in food processing which help reduce trypsin inhibitors to safe levels. It has also been described that trypsin inhibitors can be related with beneficial effects for human health. The present review seeks to evaluate the evidence about trypsin inhibitors' health benefits in both *in vitro* and *in vivo* studies.

Keywords: Kunitz inhibitor; Bowman-Birk inhibitor; Food processes; Legumes; Cereals.

1. Introduction

Antinutritional factor or antinutrients are compounds present within foods, which can decrease efficiency of proteins and minerals from human diet, generally plant-based (Elizalde et al., 2009). Many of these compounds are the result of plants' defense mechanisms against their surrounding environment (López-Moreno et al., 2022). Legumes can contain lectins related with altered intestinal functions, phytates which in some cases can inhibit iron absorption, and oxalates which can inhibit calcium absorption (Petroski and Minich, 2020). Other antinutrients, such as tannins in grapes and green tea, can inhibit digestive enzymes and eventually inhibit iron absorption, and goitrogenic substances present in broccoli, cabbage, cauliflower, and sprouts can cause hypothyroidism in specific situations (Manzoor et al., 2021). Because of this, anti-nutrients are traditionally considered to be compounds which are harmful for human health (López-Moreno et al., 2022), leading some people to opt for decreasing their plant-based food intake (Petroski and Minich, 2020). However, it has been reported that at low concentrations, some of them can have positive health benefits (Thakur et al., 2019). In this regard, López-Moreno et al. (2022) mentioned anti-tumor effects, anti-diabetic effects from lectins, antioxidant effects, anti-cholesterolemic effects, and antidiabetic effects from phytates. Das et al. (2022) also mention that lectins have antioxidant and anti-tumor properties, and can modulate blood sugar levels.

Protease inhibitors are another group of anti-nutrients. Within this group, the most reported are the trypsin inhibitors, mainly present in legumes and cereals (Sarwar Gilani et al., 2012). Trypsin inhibitors can inhibit the activity of the pancreatic enzyme trypsin, leading to reduced protein digestion and absorption via the formation of non-digestible compounds (Avilés-Gaxiola et al., 2018). As with other anti-nutrients, there is evidence showing beneficial effects, such as the anti-cancer properties of protease inhibitors (Das et al., 2022). However, there is still a lack of clarity about trypsin inhibitors' role as an anti-nutrient or as a bioactive compound. The purpose of this review is thus to give an overall perspective on trypsin inhibitors, ranging from the contents, inhibitor types present in foods, and the technologies available to decrease them during food processing up to the scientific evidence related to *in vitro* and *in vivo* studies which help with understanding their toxic or beneficial effects in humans.

2. Trypsin inhibitors in food

Trypsin inhibitors are a group of protease inhibitors which are present in plant-based foods, such as legumes, cereals, and some other vegetables (Sarwar Gilani et al., 2012). They drew significant attention during the 1970s and 1980s due to their interference with growth and digestion in animals (Sharma, 2021). They are characterized by reducing the biological activities of proteolytic enzymes such as trypsin and chymotrypsin, interfering with protein digestion and causing pancreatic disorders (Li et al., 2017).

Both trypsin and protease inhibitors are joined to their substrates via different mechanisms to form complexes (Oliveira de Lima et al., 2019). These can act through competitive inhibition, competitive inhibition assisted by exocytosis joining with a secondary site different from the active protease site, and also through irreversible inhibition where protease catalyzes the activation of its respective inhibitor (Jmel et al., 2021).

Protease inhibitors in plants intervene in protecting vegetable tissue from elicitors (viruses, bacteria, and fungi) and predators (animals) (Velísek, 2014). Trypsin inhibitors' content is highly varied in foods. Soy has the highest concentration, ranging between 8.6 and 48.2 mg/g of sample or 20.3 to 122.6 mg/g of protein (Sharma, 2021). Avilés-Gaxiola et al. (2018) shows trypsin inhibiting activity in various foods, where we can note that the lowest trypsin inhibiting activity is from black beans while the highest is with soy. It is also apparent that the inhibiting activity can vary by different food variety. For example, sweet lupines had greater inhibiting activity than bitter lupines (Embaby, 2010).

3. Trypsin inhibitor types

Trypsin inhibitors' activity is mainly attributed to two polypeptides: the Kunitz trypsin inhibitor and the Bowman-Birk inhibitor (Kumar et al., 2018).

3.1. Kunitz-type inhibitors

Kunitz-type inhibitors were the first protease inhibitor to be isolated and characterized (Savage and Morrison, 2003). They are characterized by having molecular weights between 18 and 22 kDa, a primary structure composed of 181 amino acid residues with 2 disulfide bridges stabilized by four cysteine residues which, upon breaking, cause a loss of inhibiting activity (Oliveira de Lima et al., 2019). The joining points, where the inhibitor interacts with the trypsin, are the residues of arginine amino acids where an inhibitor molecule interacts with a trypsin molecule (Velísek, 2014), which occurs because of a competitive inhibition mechanism resulting in hydrolysis of the peptide links between the residues of the reactive site of the inhibitor or substrate (Savage and Morrison, 2003). Likewise, it has been described that the Kunitz-type trypsin inhibitor from *Erythrina caffra* seeds can bind and inhibit tissue plasminogen activator (Onesti et al., 1991).

Kunitz-type inhibitors are primarily responsible for the total inhibiting activity of trypsin, and are considered damaging to human health. However, given the presence of only two disulfide links, they are thermolabile, so that thermal treatment can reduce their activity (Kumar et al., 2018). This group mainly contains soy trypsin inhibitors, which are the ones related with the damaging health effects of soy on human health (Kumar et al., 2019).

Kunitz inhibitors are generally proteins which plants use as a defense mechanism due to their various activities, including antibacterial and antifungal properties. They also act as a defense against predatory insects. However, it has also been reported that they act against inflammation, coagulation, thrombosis, and cancer (Bonturi et al., 2022).

3.2. Bowman-Birk type inhibitors

Bowman-Birk type inhibitors have a relative molecular weight of around 6–10 kDa, a greater number of disulfide bridges (Velísek, 2014), and show specificity sites of inhibition, one at Lys 16-Ser 17 against trypsin and the other at Leu 43-Ser 44 against chymotrypsin (Birk, 1985). The presence of 7 disulfide bridges makes them more heat-resistant than Kunitz-type inhibitors (Guerrero-Beltrán et al., 2009), as well as being proteolysis resistant and nontoxic. They are reportedly beneficial in treating various pathological states (Gitlin-Domagalska et al., 2020).

They are found in various species of monocotyledonous grass family including wheat (*Triticum aestivum*), rice (*Oryza sativa*) and barley (*Hordeum vulgare*), and among legumes such as soy (*Glycine max*), garbanzos (*Cicer arietinum*), common beans (*Phaseolus vulgaris*), lentils (*Lens culinaris*) and peas (*Pisum sativum*) (Clemente et al., 2011).

4. Reduction techniques in food

Given that consuming foods with trypsin inhibitors can interfere with protein digestibility (Vagadia et al., 2017), there is a wide range of methods and technologies available for their reduction. Physical processes described include heat treatment, extrusion, ultrasound, high hydrostatic pressure, soaking, gamma radiation and ultrafiltration, chemical processes such as using acids and bases, reducing agents and the use of functionalized copolymers, and biological processes such as germination and fermentation (Avilés-Gaxiola et al., 2018).

Table 1 shows the use of various technologies to reduce trypsin inhibitors in legumes. Other technologies have been described as well, including dielectric barrier plasma discharge which can reduce trypsin inhibitors by 86.1% in a soy drink exposed to 51.4 W for 21 min (Li et al., 2017). However, using microwaves and boiling processes with specific parameters can cut trypsin inhibitors by up to 100%.

5. Trypsin inhibitors anti-nutrients or compounds with beneficial properties

Constant consumption of food with high trypsin inhibitor contents can lead to excessive digestive enzyme secretion and pancreatic hypertrophy, along with decreased or delayed growth (Savage and Morrison, 2003). Since trypsin is rich in sulfurous amino acids, a large amount of them is needed for greater trypsin synthesis, deteriorating other metabolisms which require sulfurous amino acids and leading to weight loss (Das et al., 2022). *In vivo* studies mostly done on rats showed lower protein digestibility, along with pancreatic pathologies or lower growth; these appear in Table 2.

Despite the aforementioned effects, depending on trypsin inhibitors' application beneficial effects can occur. Trypsin inhibitors have been described as being used in obesity treatments due to their action on satiation-relate mechanisms (Oliveira de Lima et al., 2019). Gitlin-Domagalska et al. (2020) mention that Bowman-Birk inhibitors have immunomodulating activities, along with anti-inflammatory and chemopreventive properties. According to Clemente et al. (2011) these can reach the large intestine in an active form, as they can resist acidic conditions such as proteolytic enzymes' action. Table 3 shows different studies using cell cultures, animals and humans describing possible anti-inflammatory and anti-cancer activities from purified trypsin inhibitors.

Table 1. Extant technologies for deactivating trypsin inhibitors in legumes

Method	Raw materials	Treatment conditions	Trypsin inhibitor inactivation (%)	Advantages	Disadvantage
Thermal treatment	Peas	Microwaves (2,450 MHz, 4 min)	100	High efficiency.	Energy demand.
	Mung bean	Boiling (100 °C for 90 min)	100	Standardized process.	Reduced solubility.
	Garbanzo	Soaked cooked seeds (95 °C for 1 h)	88.4	Scalable process.	Decreased lysine, tryptophan, and sulfurous amino acids.
	Lentil	Cooking (80 °C for 1 min)	95.6	Increases aromatic amino acids.	Mineral loss (Na, Ca and Mg).
	Soy	Baking (200 °C for 20 min).	67.3	Improves gelling capacity.	Reduced B vitamins.
Extrusion	Peas	Prior cooking and humidifying processes at 120 °C, opening nozzle 55 mm, velocity 380 rpm.	58.9	Reduces total phytate and tannin levels	Reduces emulsion activity.
	Garbanzo	Prior cooking and humidifying processes at 120 °C, nozzle diameter 55 mm, velocity 380 rpm.	91.8	Raises protein digestibility.	Pre-processing may be needed.
	Fava	Prior cooking and humidifying processes at 120 °C, nozzle diameter 55 mm, velocity 380 rpm.	53.7		
	Soy	Dry extrusion at 150 °C	95		
	Soy	Wet extrusion at 150 °C	60		
	Lentil	Wet extrusion between 140–180 °C.	> 93.0		
Ultrafiltration	Garbanzo	Filter at 50 kDa	9.47	Selective method, with no toxic residues on the final product.	Energy demand.
Ultrasound	Soy	20 kHz, 3.3 s pulses for 20 min	55	Selective method.	Energy demand.
High hydrostatic pressure	Soy	550 MPa, 65 °C for 15 min (prior treatment with 0.5% bicarbonate of sodium)	76	Prolongs shelf life.	Must be combined with other methods for peak effectiveness.
	Common bean	600 MPa and 60 °C for 60 min	84	Conserves and improves organoleptic characteristics.	
	Peas	600 MPa at 60 °C.	3.1	Reduces phytic acid and lectin content, maintaining thermolabile compounds.	
Gamma radiation	Soy	8 kGy	38.7	Improves isoflavone, phenol and anthocyanin content.	Reduced vitamin C.
	Soy	5 kGy	63.3		
Soaking	Soy	96 h	35,0	Simple and profitable.	Long process

Table 1. Extant technologies for deactivating trypsin inhibitors in legumes - *(continued)*

Method	Raw materials	Treatment conditions	Trypsin inhibitor inactivation (%)	Advantages	Disadvantage
	Black-eyed peas	22 h at room temperature, sample: water of (p/v)	18.2		Loss of water-soluble proteins and other components including minerals.
	Common bean	2 h at room temperature, sample: water of 1:5 (p/v)	19.4		
	Peas	2 h at room temperature, sample: water of 1:5 (p/v)	19.8		
Acids and bases	Soy	NaOH of 1 % at 74 °C for 15 min	63	Decreases required TI inactivation temperature.	If not processed correctly, chemical substances can remain in the final product.
Germination	Black bean	3 days at 25 °C	88.2	No energy demand.	Long process, low inactivation output
	Garbanzo	3 days at 25 °C in darkness	34	Improves natural compound content.	
	Common bean	5 days at 25 °C in darkness	19.2		
Fermentation	Common bean	Lactobacillus fermentum at 37 °C for 3 days	38	Fat content reduced.	Long process
	Soy	Aspergillus oryzae for 5 days	89.2	Decreased phytic acid content.	
	Soy	Lactobacillus plantarum for 5 d	99.2	Scalable process.	
	Soy	Lactobacillus acidophilus for 2 d	82.6	No excessive energy requirements.	

Source: Adapted from Avilés-Gaxiola et al. 2018.

Table 2. Trypsin inhibitors' effects as anti-nutrients

Sample	Trypsin inhibitor	Study type	Time	Dose	Result	Reference
Unheated soy flour extract (1); TI-free soy flour extract (2)	AIT 1 = 125.1 U/mg protein; AIT 2 = 12.9 U/mg protein	*In vivo*: Male rats, 21 days old	20 days	Around 10% of the diet was soy flour extract	Less growth in the group of rats fed with unheated soy flour extract.	Kakade et al. (1973)
Pea flour 1 (H1); Pea flour 2 (H2); Pea flour 3 (H3); Pea flour 4 (H4)	AIT H1 = 1.5 U/mg dry material; AIT H2 = 8.7 U/mg dry material; AIT H3 = 1.8 U/mg dry material; AIT H4 = 7.4 U/mg dry material	*In vivo*: Rats	11 days	10 g dry matter + 150 mg of N	Direct relation between inhibiting activity, decreased protein digestibility, and biological value of protein.	Hedemann et al. (1999)
Raw soy flour	Not indicated	*In vivo*: Male Wistar rats	36 weeks	–	No symptoms present at 24 weeks, but pancreatic cancer emerged at 36 weeks.	McGuinness et al. (1987)
Fat-free soy flour: 1. Raw; 2. Toasted; 3. Overtoasted; Isolated soy protein; 1. Raw, high in TI; 2. Raw, high in TI; 3. Raw, medium TI; 4. Raw, low in TI; 5. Heated, low in IT; 6. Heated, low in IT	Fat-free soy flour: 1. 57.7 mg/g protein; 2. 16.0 mg/g protein; 3. 9.3 mg/g protein; Isolated soy protein; 1. 35.8 mg/g protein; 2. 29.4 mg/g protein; 3. 20.4 mg/g protein; 4. 7.0 mg/g protein; 5. 6.1 mg/g protein; 6. 3.2 mg/g protein	*In vivo*: Male Wistar rats, 21 days old	28 days	10 and 30% of protein in diet	Diets with higher TI concentrations increased pancreatic pathology rates.	Gumbmann et al. (1986)
Soy trypsin inhibitor	Not indicated	*In vivo:* Male Sprague-Dawley rats, 14 days old	14 weeks	1.0 g/100 g food + 9 or 30 µg Zinc	Regardless of dietary zinc level, diets with soy TI caused pancreatic hyperplasia and/or hypertrophy.	Ellwood et al. (1994)
Raw and toasted soy	Not indicated	*In vivo*: Golden hamster, 6 weeks old	28 days	Diet with 55% raw or toasted soy flour	Short-term trophic effects appeared in the pancreas (significant pancreatic weight increases). Raw soy did not favor pancreatic cancer development in treated hamsters.	Herrington (1994)
Bowman-Birk Inhibitor	Not indicated	*In vivo*: Humans, 15 men and 6 women, ages between 19 and 37 years	55 min	4 mg of BBI/mL of pancreatic juice	Inhibition of around 95% of trypsin and chymotrypsin activities. BBI doubled or tripled production of trypsin, chymotrypsin, elastase and amylase enzymes.	Liener et al. (1988)

TIA: Trypsin inhibition activity, U: Units of trypsin inhibition activity, TI: trypsin inhibitor, BBI: Bowman-Birk Inhibitor.

Table 3. Trypsin inhibitors' effects as bioactive compounds

Sample	Study type	Time	Dose	Result	Reference
Purified Kunitz and Bowman-Birk type soy inhibitors	Cell culture (human ovarian cancer cell line HRA)	24 h	0 - 10 µM	KTI, but not BBI, could inhibit cell impassivity, at least via suppression of the signal cascade from uPA.	Kobayashi et al. (2004a)
Bowman-Birk black soy inhibitor	Cell culture (human nasopharyngeal carcinoma cell lines CNE-2 and HNE-2)	-	0.71 µg/mL	KTI presented inhibiting activity against the inverse transcriptase from HIV-1, with immunostimulant activity and inhibition of tumor cell growth.	Fang et al. (2010)
Kunitz-type black soy inhibitor	Cell culture (Breast cancer MCF-7 cells and hepatoma HepG2 cells)	-	35 µM	BBI from black soy presented anti-proliferation activity against breast cancer cells and hepatoma cells, as well as inhibiting HIV-1 inverse transcriptase.	Ho and Ng (2008)
BBI	*In vivo*: Humans (men and women over 21 yrs)	16 weeks	8,000 UIC/day	Soy extract could be associated with disease regression in ulcerative colitis patients without apparent toxicity or adverse side effects.	Lichtenstein et al. (2008)
Interalpha inhibitor (KTI)	*In vitro* using serum	-	3.5 % of protein in serum	Possible anti-inflammatory activity, relevant in local inflammation sites	Okroj et al. (2012)
Inter-α-trypsin inhibitor	*In vivo:* Mice	-	100 µg/mL	Decreases tissue inflammation in a murine pulmonary lesion model	Garantziotis et al. (2007)
Urinastatin	*In vivo*: Humans	14 days	150 000 U	Protects against cisplatin-induced nephropathy.	Umeki et al. (1989)
Soy trypsin inhibitors	*In vivo:* Humans (women)	60 min	60 mg IBB or 180 mg KTI	Protects the pancreas and the pancreatic conduct from premature trypsinogen activation.	Reseland et al. (1996)
Aprotinin (KTI)	*In* vivo: Sheep	3 h	280 mg	No reduction of peri-operational bleeding measured by drainage or hemoglobin loss	Ohri et al. (2001)
Bikunin	*In vivo*: 7-week rats	7 days	30 mg/kg	Possible anti-metastatic activity in humans	Kobayashi et al. (2004b)
Trypsin inhibitor of soy + genistein	*In vivo*: 7-week rats	2 h	100 mg/kg	Anti-inflammatory activity	Sadeghalvad et al. (2019)
Bowman-Birk soy inhibitor	Cell culture (Normal human prostate epithelial cells (PrEC), 267B1, BRF-55T, 267B1/Ki-ras, LNCaP, and PC-3 cells)	8 days	100 µg/mL	May be a useful agent for treating prostate ailments.	Kennedy and Wan (2002)

KTI: Kunitz-type trypsin inhibitor, BBI: Bowman-Birk inhibitor, uPA: urokinase plasminogen activator, UIC: Units of chymotrypsin activity.

6. Conclusions

Trypsin inhibitors' presence in foods raises concern among consumers due to their well-known anti-nutritional effects, shown via *in vivo* studies with rats. However, the existence of a wide range of technologies with adequate parameters for its decrease could ensure the harmless consumption of foods with trypsin inhibitors, both domestically and industrially. There is also evidence to consider trypsin inhibitors as bioactive compounds, following studies on cell cultures and animal models, and highlighting the anti-cancer effects (with a particular emphasis on Kunitz-type inhibitors). Despite trypsin inhibitors' potential importance for consumer health, there are apparently no reports on toxicological parameter such as NOAEL or LOAEL. One potential starting point would be to standardize methodologies for trypsin inhibitor measurements in food and establishing toxicological parameters for frequently consumed foods and products which can cause problems among high-risk populations like children and the elderly. While evidence shows the potential role of trypsin inhibitors as an anti-nutrient or a bioactive compound, we cannot conclude on a specific effect (whether toxic or beneficial) among humans related with the trypsin inhibitors present in foods. The effect considered depends on the doses used in the studies, and the use of inhibitors which are purified or contained in a food matrix. The concentrations needed to achieve one effect or another still remain to be seen.

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