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Soybean Trypsin Inhibitor Activity - a review

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Abstract: Soybean (*Glycine max*) is one of the most important source of protein for food and feed globally. Complete utilization of protein in human and animal digestion can be reduced by certain trypsin inhibitory (TI) substances present in fresh soybeans. Two classes of protease inhibitors (polypeptides) are found in soybeans: Kunitz trypsin inhibitor (KTI) and Bowman-Birk trypsin inhibitor (BBI). KTI is primarily responsible for its trypsin inhibitory activity, while BBI has been reported to have nutraceutical properties. The content of TI in soybean depends on the soybean genotype, environmental factors and/or applied technology, especially nitrogen fertilization. The content of TIA in soybeans can be between 18.6 and 74.8 mg inhibited trypsin/g soybean meal, with an average value of 45.9 mg. Genotypes with values above 12 mg/g flour can be called "high KTI" genotypes, and those with content below 6 mg/g flour can be called "low KTI". Researchers have studied the possibilities of reducing these inhibitors. Of these, the most used are thermal methods (boiling, autoclaving, microwave treatment and heating in the oven), but also germination-sprouting leads to a decrease in TI and also fermentation. In this review we present the most important results in the determination of trypsin inhibitors and ways to reduce their content.

Keywords: feed, food, soybean, trypsin inhibitors.

Introduction

The quality of soybean (*Glycine max*) seed is determined by its constituents, which are important for sustaining the nutritional value

of the soybean. Of these, protein is the most important, providing 60% of the world's vegetable protein. Usually, there is a negative correlation between protein and oil content (Wijewardana et al., 2019). Soybeans also rank first in the world as a source of oil (Garima et al., 2020). However, there are also anti-nutritional substances in soybeans, such as lipoxygenase, tannins, trypsin inhibitors (TI), and phytates. In particular, TI interferes with the amino acid hydrolysis reactions of trypsin and chymotrypsin, strongly inhibiting protein digestion (Park et al., 2023). TI are protease inhibitors, which reduce the availability of trypsin - the human pancreatic enzyme, essential for human nutrition, but also for many monogastric (non-ruminant) animals. Therefore, the presence of TI in animal feed causes digestive disorders, pancreatic enlargement and liver damage in monogastric animals. TI makes up about 6% of the total soy protein (Park et al., 2023).

Fresh soybeans cannot be used in human consumption and as animal feed without a heat treatment that leads to the antitrypsin inactivation (McNiven et al., 1992; Vollmann et al., 2003; Peric et al., 2009).

About trypsin inhibitors

Two major classes of protease inhibitors are found in soybeans, 2 polypeptides (water-soluble proteins): Kunitz trypsin inhibitor (KTI) has a molecular weight of 21 Kda that exists as a monomer, consisting of 181 amino acids linked by two disulfide cysteine residues, and Bowman-Birk trypsin inhibitor (BBI), a low molecular weight, 7-8 Kd compound of 71 amino acids (Tan-Wilson and Wilson, 1986; Peric et al., 2009; Kumar et al., 2019; Khan et al., 2022; Park et al., 2023). KTI have a common structure composed of 12 anti-parallel β-strands separated by irregular loops (Xu et al., 2020) and is thermolabile, due to the presence of only 2 disulfide bonds, and BBI, due to the presence of 7 disulfide bonds, is considered relatively stable to heat (Kumar et al., 2019). The disulfide bonds in TI increase their stability at high temperatures and decrease the availability of processed soy foods (Chen et al., 2014; Park et al., 2023).

In the process of trypsin inhibition, KTI plays a larger role compared to BBI (Pesic et al., 2007; Park et al., 2023) and has been considered to have harmful effects on human health (Liener, 1994),

while BBI it has been reported to have nutraceutical properties (Wallo et al., 2007). While KTI inhibits only trypsin, BBI inhibits both trypsin and chymotrypsin and elastase (Seidl şi Liener, 1972; Ikena şi Norioko, 1986; McNiven et al., 1992; Cid-Gallegos et. al. 2022).

In fact, the natural role of these substances is precisely to prevent the degradation of proteins during storage, so that the grains do not lose their value. This protease inhibitor is considerably reduced in fermented soy products such as tempeh, miso, and natto, which are in the common diet of people in Southeast Asia (Kumar et al., 2019). On the other hand, lately it has been discovered that these protease inhibitor substances have anticancer effects, both in the initial and advanced stages (gastric, colorectal, breast and lung), antioxidants, regulate blood sugar, obesity, are useful in cardiovascular diseases, autoimmune, in inflammatory processes and reduces inflammation. The proposed mechanisms of action of the anticancer activity comprise several inhibitory effects at different molecular levels, i.e., transcription, post-transcription, translation, post-translation, and secretion of cancer cells (Kennedy, 1993; Cid-Gallegos et. al. 2022).

Antitrypsin content in soybeans is influenced by soybean genotype (Pesic et al., 2005), but also by the applied technology – especially nitrogen fertilization (Peric et al., 2009) (Vollmann et al., 2003). Peric et al. (2009) reveals that the antitrypsin content was lower when different fertilizer doses with nitrogen (N30, N60 and N90) were applied compared to the unfertilized variant.

Depending on the environment, the values of the total trypsin inhibitory activity (TIA) were between 69.5 and 104.8 mg g^{-1}. Nitrogen application caused an increase in seed protein content but resulted in a reduction of TIA by about 15% compared to the unfertilized experimental variant. Simultaneous application of nitrogen and sulfur in the form of ammonium sulfate also decrease TIA as that of nitrogen alone, although soybean protease inhibitors are rich in sulfur amino acids. Significant genetic variation in TIA was found in both the Kunitz inhibitor genotype class and the class without this inhibitor. The results suggest that TIA in soybean can be considerably modified by improving genotypes, but also by appropriate agronomic management (Vollmann et al., 2003).

Pesic et al. (2007) determined the concentration of KTI in 12 soybean genotypes and reported a range of 4.28–6.85 mg/g, with a mean value of 4.94 mg/g defatted meal.

Srebric et al. (2010), studied the variability of TIA in 7 soybean cultivars and reported contents between 13.20 and 33.93 mg inhibited trypsin/g.

In a study conducted on soy milk made from one soybean variety, Chen et al. (2014) reveals that 74% of the total TIA was attributed to KTI.

According to Kumar et al. (2019), who evaluated the TIA content of 102 soybean genotypes, values between 18.6 and 74.8 mg inhibited trypsin/g soybean meal, with an average value of 45.9 mg were obtained. In this study, 53.9% of all TIA is attributed to KTI. The concentration of KTI showed a wide genetic variation, ranging from 0.07 to 15.9 mg/g soybean meal, which corresponded to 1.0–79.8% of the total TIA. Genotypes with values above 12 mg/g flour were called "high KTI", and those with content below 6 mg/g flour, "low KTI". Recently, a low KTi genotype, Punjab1, was identified in Indian soybean germplasm (Kumar et al., 2019).

Appropriate agronomic management of the soybean crop could reduce antitrypsin content, which may open new possibilities for postharvest treatment and utilization of soybeans (Vollmann et al., 2003).

TIA determination methods

The content of trypsin inhibitors can be determined by several methods:

- ✓ the Erlanger method, with Na-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPA) as substrate, the absorbance being measured at 410 nm (Kakade et al., 1969; Hamerstrand et al., 1981; Peric et al., 2009);
- ✓ spectrophotometry according to the method proposed by Hammerstrand et al. (1981), which is the improved method described by Kakade et al., 1969. This method does not differentiate Kunitz trypsin inhibitor (KTI) from Bowman-Birk inhibitor. (Zhou et al., 2017; Kumar et al., 2018);
- ✓ densitometry makes it possible to quantify the specific KTI polypeptide, by polyacrylamide gel electrophoresis by running

the known amount of the standard for this polypeptide (Kennedy, 1993; Pesic et al., 2005; Kumar et al., 2018; Kumar et al., 2019);

- ✓ immunochemical methods - the enzyme-linked immunosorbent assay (ELISA) was also used to quantify protease inhibitors (Kennedy, 1993; Kumar et al., 2018);
- ✓ method with polyclonal antibodies for quantification of KTI and monoclonal antibodies for estimation of BBI in soybean seeds and products (Brandon et al., 2004).

Kumar et al. (2015) and Szmigielski et al. (2010) show that there are KTI-free soybean genotypes that have been developed by crossing common KTI-positive soybean genotypes with donor parents carrying the null allele of KTI. In some soybean varieties, the low activity of KTI may be due to mutation of the KTI3 gene or differences in the cis-regulatory sequences of this gene (Kumar et al., 2019).

According to Kumar et al. (2018), 1 mg of KTI inhibited 2.51 mg of trypsin. Thus, the weight of KTI to the total TIA in soybeans calculation is established by multiplying the KTI content by 2.51 (Kumar et al., 2019).

Possibilities for reducing TIA from soybeans

There is a high interest in reducing the content of TIA in soybean seeds or soy milk, through different procedures: thermal, irradiation and germination.

Many studies have evaluated changes in TIA activity levels in soybean seeds following boiling, autoclaving, microwave treatment, and oven heating (Jourdan et al., 2007; Andrade et al., 2016). Investigations on changes in soybean KTI polypeptide concentration upon boiling and autoclaving are limited (Chen et al., 2014), and studies investigating the effect of microwave irradiation on this polypeptide are not available.

In experiments with soybean seed boiling, it was found that after boiling for 5 min, KTI was inactivated by 68.8%, as is also evident from the reduction of this polypeptide from 11.2 to 3.5 mg/g soy flour. Upon boiling for 10 min, KTI decreased from 11.2 to 2.7 mg/g soybean meal, i.e. 75.9% inactivation of this anti-nutritional inhibitor. Boiling for 15 min completely inactivated the KTI polypeptide (Kumar et al., 2019).

In the case of soy milk, Chen et al., 2014 revealed that 30 min of boiling was required to completely inactivate KTI.

By **autoclaving** (at 121°C and 15 psi) for 15 min, KTI and TIA were completely inactivated. Friedman et al. (1991) reported a 60.0 and 80.2% reduction of KTI in soybean meal samples autoclaved at 121°C for 10 and 20 min, respectively.

By treating soybeans with microwave irradiation, at different time periods, interesting results were obtained: at the time of 1 minute, the KTI concentration was reduced from 11.2 to 5.4 mg/g soybean meal, i.e. an inactivation of 51.8%, and in soaked seeds, KTI was reduced from 11.2 to 1.5 mg/g flour, i.e. 86.6% inactivation. Exposure of soaked seeds for 2 min to microwave irradiation resulted in a KTI reduction of 94.6 and 99.1%, respectively, i.e., a significantly greater KTI reduction effect than the 1 min time.

From the results obtained by Kumar et al. (2019) it can be concluded that the inactivation of KTI induced by microwave irradiation was proportional to the exposure time and the magnitude of inactivation was significantly ($P<0.05$) greater in soaked seeds than in dry seeds. The observed greater reduction in KTI concentration in soaked seeds than in dry seeds may be due to higher electric dipole shapes due to water molecules in the former case, which may lead to intense heat energy transfer to proteins.

Treatment by **heating** in an oven at 100°C for 40 minutes strongly decreases TI activity, but causes nutrient denaturation (Park et al., 2023).

The decrease in TIA content by **sprouting** was studied by Collins et al., 1976; Malomo et al., 2011. Estimation of KTI polypeptide during germination has been carried out in limited studies (Kumar et al., 2006; Dia et al., 2012). Kumar et al. (2006) reported only qualitative changes in KTI polypeptide during soybean germination, but the authors did not quantify changes in KTI concentration at different germination times. Dia et al. (2012) reported no significant degradation of the KTI polypeptide during germination (for 3 days at 25°C), but Kumar et al. (2019), in a germination performed at 28°C for 4 days, had caused a continuous decrease in the concentration of KTI, from 11.2 to 3.8 mg/g (66.1%) in soybean meal. The decrease of KTI due to germination may be due to the synthesis of proteases, as suggested by Papastoitsis and Wilson (1991), who demonstrated the role of K1 protease in the degradation of KTI during seed germination.

Conclusions

Among all treatments to reduce KTI in soybeans, boiling for 15 min., autoclaving for 15 min. (at 15 psi and 121°C), microwave irradiation for 2 min. and heating in an oven at 100°C for 40 min resulted in complete inactivation of KTI. Sprouting for 4 days induced a 66.1% reduction of this trypsin inhibitor.

Heat treatment methods are expensive and require special facilities. On the other hand, these heat treatments can destroy some nutritional qualities of soybeans. KTI reduction by sprouting is also a complicated method and cannot be applied in all cases. Therefore, the most effective method, but requiring a lot of plant breeding or genetic engineering work, would be to create soybean genotypes with a low KTI content. This is one of the objectives of researchers from the Agricultural Research and Development Station in Turda, Romania.

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