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Methods and procedures for reducing soy trypsin inhibitor activity by means of heat treatment combined with chemical methods

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Abstract. We have developed a new procedure for reducing soy trypsin inhibitor activity by means of heat treatment combined with chemical methods, through which soy trypsin inhibitor activity decreases to the tenth or twentieth part of the original value. We determined the optimal concentration of the applied chemicals (hydrogen-peroxide, ammonium-hydroxide) as well as the optimal temperature and duration of the treatment. The chemical procedure combined with heat treatment results in lower energy consumption as compared to the original heat treatment methods.

1 Introduction

Soy has a high protein and energy content, and it is a widely consumed food ingredient due to its low price range and especially its high lysine content. It

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has recently become highly popular as, besides its excellent content values, it has been used efficiently for the treatment of various chronic diseases and different types of cancer (*Vagadia et al.*, 2017). Unfortunately however, soy also contains a great number of substances (protease inhibitors, phytic acid, isoflavones) that limit the use of soy owing to their adverse physiological effects. Inactivation of trypsin inhibitors, harmful enzymes, and bioactive components, increasing protein quality, improving texture, colour, and smell, enhancing functionality and digestibility are all decisive factors of the production of soy products (*Csapó* & *Csapóné*, 2006).

Trypsin and chymotrypsin inhibitors form inactive complexes with trypsin and chymotrypsin in the small intestine, thus inhibiting the hydrolysis and bioavailability of dietary proteins. Their presence stimulates an increased pancreatic activity, which may eventually cause pancreatic enlargement followed by pancreatitis. These inhibitors can be inactivated by heat treatment. Their thermal stability depends on molecular size and the number of disulphide bridges. One of the most stable trypsin inhibitors is the Bowman–Birk inhibitor found in soybean, with a molecular mass of 7,900 Da, consisting of 71 amino acids and containing 7 disulphide bridges. Dry-heated at 105 °C in aqueous solution, it can retain most of its activity even after ten minutes of boiling (*Csapó* & *Csapóné*, 2006). The Kunitz-type trypsin inhibitor, also isolated from soy, is markedly more thermolabile; it is irreversibly denatured at 90 °C, its molecular mass is 21,500 Da, it is made up of 181 amino acids, and it contains two disulphide bonds (*Kunitz*, 1947). Nevertheless, in acid environment, it is resistant to digestive enzymes, which is why it does not dissolve either in the stomach or later in the small intestine, following the enzymatic digestion (*Astwood et al.*, 1996).

The specificity of protease inhibitors is extremely varied – it can comprise different proteases and isodynamic enzymes as well. On the other hand, we have knowledge of inhibitors that neutralize two types of enzymes, e.g. trypsin and chymotrypsin – such is the Bowman–Birk inhibitor. Protease inhibitors are formed in floras first of all, but there are also a few animal and microbial variants. Among them, the best known is the ovomucoid and the ovoinhibitor found in egg white, which retain some of their activity during ordinary cooking but do not inhibit the human trypsin. Recognizing the adverse health effects of inhibitors made it necessary to limit the food inhibitor content. This has been made especially necessary by the widespread use of soy products, crude soybeans being one of the most relevant products containing antinutrients. In our country, only those ingredients can be used for direct human consumption whose trypsin inhibitor activity is under the value of 10 per mg (*Csapó* &

Csapóné, 2006).

Heat-sensitive antinutrient substances include trypsin and chymotrypsin inhibitors, which are proteins in terms of their composition and are primarily formed in legume seeds as well as in cereal grains and potatoes. Their molecular mass is 6–64 kDa. A single plant may contain several types of antinutrient substances that differ in molecular mass, isoelectric point, and thermal stability.

Serine protease inhibitors occur naturally in large numbers and diverse forms, and they inhibit protein catabolism by the inactivation of serine protease enzymes (*Silverman et al.*, 2001; *Rawlings et al.*, 2004). In the largest quantities, they can be found in cattle pancreas, egg, lima bean, and soy (*Zhou et al.*, 1989). Every inhibitor is a substrate analogue, which, linked to the enzyme, forms an inactive complex with the latter, inhibiting protein hydrolysis to amino acids.

Inhibitors may take on a great number of diverse, varying roles in nature. They inhibit the proteolytic activity of serine proteases once these have fulfilled their role in various parts of the organism, while in the seeds of different legumes and plants they inhibit the protein digestion of the insects consuming the seeds. So, they actually constitute an integral part of the plant's defence mechanism against the animal pests consuming them. In the case of consuming improperly heat-treated or crude soybean, the trypsin inhibitor found in high concentrations in soybean contributes to increased pancreatic activity, while, if consumed in large quantities over a long period of time, it may cause pancreatitis and reduce animals' feed intake, but the Bowman–Birk trypsin inhibitor can also play a role in preventing cellular proliferation and combating cancer (*Kennedy*, 1998).

The trypsin inhibitor of cattle pancreas is made up of 158 amino acids and contains three disulphide bridges. Both in cattle and in humans, it inhibits trypsin, chymotrypsin, and plasmin activity, but it does not inhibit elastase activity in pigs (*Huber et al.*, 1971). The glycoprotein of hen's ovomucoid egg white is made up of 186 amino acids (*Stadelman & Owen*, 1995), and it functions as a trypsin inhibitor. It contains three tandem domains, each of which having three disulphide bridges (*Salahuddin et al.*, 1985; *Cooke & Sampson*, 1997). The ovoinhibitor in the egg white has five binding sites, inhibiting cattle trypsin, chymotrypsin, and pig elastase activity (*Begum et al.*, 2003). Ovomucoid and ovoinhibitor make up 11% and 15% of the egg white respectively (*Gertler & Ben-Valid*, 1980; *Kinoshita et al.*, 2004).

Kunitz- and Bowman–Birk-type protease inhibitors (BBI) are present in the largest quantities in soybean. The BBI molecular mass is 87 kDa, which,

having independent binding sites, is highly bound to trypsin and chymotrypsin alike. The Kunitz inhibitor's molecular mass is 20.1 kDa, has a single binding site, is highly bound to trypsin, but it only slightly inhibits chymotrypsin. The Kunitz inhibitor was first prepared in pure form by Kunitz in 1945 – it is a single-chain protein made up of 181 amino acids, having two disulphide bridges (*Steiner*, 1965; *Koide & Ikenaka*, 1973; *Kim et al.*, 1985), and inhibiting mainly trypsin and slightly chymotrypsin and plasmin (*Naninga & Guest*, 1964; *De Vonis Bidlingmeyer et al.*, 1972). By a trypsin-like mechanism, it inhibits other enzymes as well, but it has no effect whatsoever on metallic, acidic, and thiol proteases.

Trypsin inhibitor and the enzyme form a stoichiometric complex, which has the ability to split the inhibitor between the arginine and isoleucine. Enzyme inhibition is reversible and pH-dependent – the dissociation of the enzyme-inhibitor complex releases the inhibitor in its initial form (*Ozawa & Laskowski*, 1966; *Finkenstadt & Laskowski*, 1967).

The trypsin-chymotrypsin Bauman–Birk inhibitor is a monomer protein with 71 amino acids and containing seven disulphide bridges (*Kay*, 1979). It has a separate binding site for trypsin and another one for chymotrypsin. It has a one to one binding with protein, that is, one molecule of inhibitor inhibits one molecule of enzyme. This inhibition is not competitive, the inhibitor being able to form tertiary structure with both enzymes (*Birk*, 1985). One mg of inhibitor is able to inhibit 0.5 mg of trypsin and 1 mg of chymotrypsin.

DiPietro and *Liener* (1989) carried out a heat treatment of the Kunitz inhibitor, the Bowman–Birk inhibitor, and crude soybean extract at 100 °C, and they established that the soybean extract rapidly lost its enzyme inhibitor activity, the Kunitz inhibitor added to the soybean extract could only become inactive with difficulty, while the Bowman–Birk inhibitor remained mostly active in the soybean extract even after a rather long period of heat treatment. Taking a look at the clear solution of the inhibitors, a rather rapid inactivation at a temperature ranging between 75 °C and 90 °C was observed with both inhibitors, which contradicts the results obtained in relation to the soybean extract. According to Kassell's research (1970), besides soy, all the other legumes contain trypsin in significant quantities, but the soy inhibitor was found to contain by far the largest quantities.

High temperatures and steam injection technique were also applied by many in an attempt to inactivate the trypsin inhibitor. Blanching was adopted as well, while, adopting preliminary heat treatment, they also achieved successes at temperatures below 100 °C (*Yuan & Chang*, 2010), and the high temperature–short time period (137 °C, 70 seconds) technique paid off, too

(*Rouhana et al.*, 1996). *Kwok et al.* (1993) treated soymilks of various pH (2.0, 6.5, 7.5) and established that trypsin inhibitor is less stable at a higher pH level as compared to a lower one. At higher temperatures (143 °C, 154 °C), pH level had much less effect on activity.

Van der Ven et al. (2005) studied trypsin inhibitor activity in minced meat, where 30% of the meat was replaced by soy preparations (concentrate, isolate, textured flour). Heat treatment carried out at 70, 80, 90, and 100 °C for 15 minutes reduced trypsin inhibitor content to such a degree that caused no more indigestion in consumers. Under similar conditions, the components of the developed meat-structure model provided better protection for the trypsin inhibitor than during heat treatment in aqueous solution.

Johnson et al. (1980) used direct steam injection to produce soymilk out of soybean extract. Carrying out their experiments within the temperature range of 99–154 °C, they found that at a pH value of 6.7 with every 11 °C temperature rise trypsin inhibitor activity was halved. At this pH level, at 154 °C, and in 40 seconds, the same amount of decrease in activity was obtained as at 99 °C in 60 minutes. At an increasing pH level (pH = 9.5), activity declined at an even faster pace.

There has been a recent spread of modern techniques that do not follow traditional procedures but almost always entail heat treatment. Such is, for instance, the ohmic heating treatment, during which electric current goes through foodstuffs and the generated heat produces its effect (*Wang et al.*, 2007). Dielectric heat treatment is widely adopted in the industry, during which all types of frequencies were used from radio waves (42 MHz) to microwaves (2450 MHz). Microwave treatment following soaking inhibited the trypsin inhibitor with high efficiency, while not causing any significant changes in the composition of the soybean (*Hernandez-Ifante et al.*, 1998). Applying a treatment of 2500 MHz for 30 minutes, *Zhong et al.* (2015) managed to inactivate a significant amount of the trypsin inhibitor. In a layer of 30 cm, the pre-soaked soybean was heated by microwave to 120 °C, which resulted in the inactivation of 93% of the trypsin inhibitor (*Petres et al.*, 1990).

Radio frequency treatment is also a dielectric heat treatment technology, which is a lot less time-consuming than traditional heat treatment, and, combined with high temperatures (80–120 °C), they were able to reduce trypsin activity in a short amount of time (90–120 s) to 7–12% of the original value (*Vearasilp et al.*, 2005). In the course of infrawave treatment, energy is transferred at a low temperature to the treated foodstuff, during which inhibitor activity declines but food components are not damaged. The treatment improves water adsorption, reduces cooking time and the concentration of antin-

utrients (*Kayitesi et al.*, 2013). *Abu-Tarboush* (1998) used gamma radiation to reduce the activity of defatted soy flour inhibitor, during which he was able to reduce the inhibitor content by 34% with a radiation intensity of 10 kGy, while there was a 4% increase regarding the digestibility of the nutrients.

Applying an ultrasonic treatment at 20 kHz for 20 minutes, *Huang et al.* (2008) achieved a 55% decrease of inhibitor activity, a decrease influenced by ultrasound amplitude and treatment duration. They established that inactivation in the inhibitor's construction was caused by the digestion of disulphide bridges and various structural changes. Extrusion is another widely used method in food industry, being able to significantly reduce inhibitor quantity to safe levels while protein quantity suffers no changes at all during the procedure (*Clarke & Wiseman*, 2007). Adopting instantaneous heat treatment at 150 °C, during extrusion, inhibitor activity decreased under 5% of the initial value.

Haddad & Allaf (2007) developed a procedure for reducing inhibitor activity, during which the soaked soybean was heated to appropriate temperature (170 °C), and, alternating pressure values of 8×10^5 and 50×10^2, they were able to neutralize 94% of the inhibitor in one minute and 99% of it in six minutes. *Osela et al.* (1997) used fluidized bed, which is suitable not only for the drying of the crop but also to inactivate the inhibitor. Adopting this procedure, they were able to carry out the entire inactivation process at 140 °C in 10 minutes.

A common trait of the chemical methods used to inactivate the trypsin inhibitor is that they somehow digest the disulphide bridges. The activity of soy protease inhibitor can be reduced with Escherichia Coli or the nicotinamide adenine dinucleotide phosphate (NADP)-thioredoxin system found in plants, which contains NADP, thioredoxin, NADP-thioredoxin reductase, reduced lipoic acid, and dithiothreitol. The system is targeting the Kunitz and the Bowman–Birk inhibitors, whose 45% and 32%, respectively, are dissolved during a 1–2-hour treatment at 30 °C (*Jiao et al.*, 1992).

Sessa et al. (1988) used sodium bisulphite to inactivate the trypsin inhibitor content of flattened and whole soybeans. Following a 2-hour heat treatment at 65 °C in a 0.1 molar solution, they managed to inhibit 94% of the soy's initial trypsin inhibitor content. The excess sodium bisulphite was removed by extraction.

In a model medium, *Sessa et al.* (1990) successfully applied the combination of L-ascorbic acid and copper sulphate to inactivate the Kunitz and the Bowman–Birk trypsin inhibitors. However, when they treated soy flour under similar conditions, most of the inactivating effect did not occur, which

they could attribute to the reactions occurring between the ascorbic acid and other components of the soy. In a model medium, the inactivating effect was accounted for by the pro-oxidant effect of the ascorbic acid and copper ions, which digested the trypsin inhibitor structure. In a model system as well as in a lyophilized, alkaline soymilk extract, *Sessa & Ghantous* (1987) used sodium metabisulphite and glutaraldehyde, separately and in combination, to inactivate the Kunitz trypsin inhibitor of the soy. The glutaraldehyde treatment reduced only 60–75% of the trypsin inhibitor activity, which was not significantly changed even by increasing the temperature from 25 °C to 75 °C. Under similar circumstances, the sodium metabisulphite treatment resulted in 96% loss of trypsin inhibitor activity. The two reagents were more efficient in the inactivation process when used together as compared to their separate use.

Huang et al. (2004) performed an experiment with tea polyphenols and established that these are able to inactivate the Kunitz inhibitor at 30 °C in 30 minutes. *Lei et al.* (1981) managed to accelerate trypsin inhibitor inactivation through heat treatment by adding cysteine. During a heat treatment at 80 °C for 10 minutes at a pH level of 9.0, they used 2.5 mM of cysteine to inhibit 90% of the trypsin inhibitor activity. This significant decrease attained at a relatively low temperature during the treatment can be explained by the digestion of disulphide bridges. A thiol–disulphide exchange takes place between the inhibitor protein and the free sulfhydryl groups of the cysteine, in which the inhibitor is inactivated.

Taking a look at the changes in the amino acid composition of complete soybean according to crude protein content, *Csapó & Henics* (1990) established that the increasing protein content is accompanied by a decrease of the protein's biological value, that is, there is no change in the concentration of the essential amino acids, whereas that of the non-essential amino acids increases. Following these observations, *Varga-Visi et al.* (2005, 2006, 2009a, 2009b, 2009c) investigated the effect of the various treatments on the antinutrient substances of the soy as well as the degree of racemization taking place during the time of the heat treatment. They concluded that during optimal heat treatment, when trypsin inhibitor content falls below 10% of its initial value, amino acid racemization is not significant and no relevant changes occur in the soy protein composition.

After an analysis of literature data, the structure and chemical properties of trypsin inhibitors can be summarized as follows. The molecular mass of the Kunitz-type trypsin inhibitor derived from soybean is 21,000 Da, and its isoelectric point is pH = 4.5; regarding the structural characteristics: it includes the two disulphide bridges and does not contain a free sulfhydryl group.

Its N-terminal amino acid is aspartic acid, while its C-terminal amino acid is leucine. Trypsin inhibitor derived from 1 mg of soybean inhibits 1.5 mg of trypsin stoichiometrically, reversibly, and in a pH-dependent reaction, forming a trypsin–inhibitor complex. During this reaction, it blocks the enzymatic activity of the trypsin, and, besides trypsin, it also inhibits chymotrypsin and other proteolytic enzymes.

The molecular mass of the Bowman–Birk trypsin inhibitor found in soy is 7,900 Da, it is made up of 71 amino acids, and it contains seven disulphide bridges. After dry-heating at 105 °C in an aqueous solution and boiling for ten minutes, it is still able to retain much of its activity. It is highly bound both to trypsin and chymotrypsin with independent binding sites. It has a one to one binding with protein, that is, one molecule of inhibitor inhibits one molecule of enzyme. This inhibition is not competitive, the inhibitor being able to form tertiary structure with both enzymes. One mg of inhibitor is able to inhibit 0.5 mg of trypsin and 1 mg of chymotrypsin.

The trypsin inhibitor derived from soybean is thermolabile, sensitive to high pH levels and protein precipitation reagents. However, it dissolves faster under stable acidic conditions, in an alkaline environment. Regarding the structure and characteristics of the trypsin inhibitor and in terms of inactivation, the most important features are as follows:

- it contains disulphide bridges,
- the active structure dissolves rapidly in alkaline environment, and
- it is sensitive to high pH levels.

The method adopted by *Lei et al.* (1981) appears to be the most promising one, the researchers being able to accelerate trypsin inhibitor inactivation through heat treatment by adding cysteine. This significant decrease attained at a relatively low temperature during the treatment can be explained by the digestion of disulphide bridges. A thiol–disulphide exchange takes place between the inhibitor protein and the free sulfhydryl groups of the cysteine, in which the inhibitor is inactivated.

The rest of the procedures introduce some substance into the system which was not there before and which needs to be removed subsequent to the treatment. Cystine/cysteine amino acids are natural components of soy, wherefore, even if a certain amount of "reagent" is retained, no further problems are caused; moreover, it increases the amino acid content of the soy, already low in sulphur. During our experiments, we were also trying to follow this path,

except that for the splitting of disulphide bridges we did not intend to apply reduction and thiol-disulphide exchange but oxidation.

Our experiments thus aimed at elaborating a method and procedure for reducing soy trypsin inhibitor activity by means of heat treatment combined with chemical methods. In practice, crude soybean can be used only to a very limited degree due to antinutrients, while the widely applied heat treatment is a costly solution. As most of the currently adopted procedures are highly energy-consuming and the quality of the end-product also leaves much room for improvement, we tried to obtain oxidation with hydrogen-peroxide combined with heat treatment to reduce soy trypsin inhibitor activity, expecting significant energy savings. Since literature data suggested higher inhibitor instability in an alkaline environment, we combined hydrogen-peroxide treatment with ammonium hydroxide, expecting a better efficiency.

Based on our pre-trials, it seems certain that chemical treatment (ammonium hydroxide, hydrogen-peroxide) is conducive to significant energy savings, and the biological value of the obtained heat-treated soy protein declines to a lesser degree during the procedure owing to less heat exposure. Pre-trials have been successfully performed, and we are about to report on their results in our article.

2 Methods and materials

Measurement of trypsin inhibitor and urease enzyme activity and of the degree of heat treatment

We have verified the efficiency of inactivation methods in two ways: determining urease enzyme activity and measuring trypsin inhibitor content. We can estimate trypsin inhibitor activity with the much more easily measurable urease enzyme activity as the two enzymes have very similar heat sensitivity, and, having analysed the measurement results of several hundred samples, we found a linear relationship between the degree of trypsin inhibitor and urease enzyme activity.

Determination of the trypsin inhibitor activity of minced and flattened soy products as well as of samples heat-treated with extruder was performed according to the EN ISO 14902 (2002) standard. The determination is based on that the inhibitors are extracted from the substance under examination, followed by the measurement of the degree to which in the solution containing the extract, incubated with trypsin, p-nitroaniline is released from the benzoyl-L-arginine-p-nitroanilide substratum (L-BAPA).

When estimating urease enzyme activity (*Csapó & Csapóné*, 2010a), we added 50 cm^3 of phosphate buffer (pH = 7.5) and 50 cm^3 of buffered urea solution (30 g urea/1,000cm^3 phosphate buffer) to two times 1,000 mg of soybean meal. After mixing, incubation was performed at 35 °C for 30 minutes, and then the pH of the solutions was measured right away. The bigger the difference in pH was between the solution containing the substratum and the one not containing it, the more ammonia was released as an effect of the urease enzyme. Determination of the degree of heat treatment based upon the measured difference in pH level is as follows: crude, not heat-treated: ΔpH = 1.7–2.5; partially heat-treated: ΔpH = 0.2–1.7; highly heat-treated (toasted): ΔpH = 0.0–0.2. In the examination of the degree of heat treatment (*Csapó & Csapóné*, 2010b), we applied the cresol red dye binding test.

Pre-trials

Since protein hydrolysis and the splitting of disulphide bridges are dependent on concentration, temperature, and time, we set up the following experiments. As the trypsin inhibitor is less stable in an alkaline environment and as digestion of disulphide bridges can also take place through oxidation, we started our experiments with these two chemicals, using the hydrogen-peroxide independently and the mixture of hydrogen-peroxide and ammonium-hydroxide. As a first step, we intended to have a look into whether hydrogen-peroxide digests disulphide bridges at all, whether inhibitor activity decreases upon treatment, and whether oxidation performed under alkaline conditions is any step forward compared to simple oxidation. When testing for the chemicals' effects and optimizing the concentration, we made use of 100 g of flattened soybean (dry matter content [hereinafter DM]: 92%) in each of our experiments (*Table 1*).

Optimization of treatment temperature

During the pre-trial phase, it turned out that it is not worth working with ammonium-hydroxide and hydrogen-peroxide concentrations of more than 1.5–3% and that the combination of the two chemicals gives the best result – in what followed, we carried out our experiments with 2.5% ammonium-hydroxide (decimal dilution of the concentrated solution) and 2.5% hydrogen-peroxide. In this experiment, the solution added to the 100 mg of soy contained the ammonium-hydroxide and hydrogen-peroxide as well (*Table 2*).

The treated soy obtained subsequent to pre-trials was immediately cooled down to room temperature, desiccated with the help of a ventilator, and the trypsin inhibitor activity of the samples was determined.

Table 1: Pre-trial set-up

Sample indication	Treatment	Duration (hour)	Temperature (°C)
1.1.	DM + 20 cm^3 25% ammonium-hydroxide	1	100
1.2.	DM + 20 cm^3 25% ammonium-hydroxide + 20 cm^3 25% hydrogen-peroxide	1	100
1.3.	DM + 20 cm^3 distilled water	1	100
1.4.	DM + 20 cm^3 12.5% ammonium-hydroxide	1	100
1.5.	DM + 20 cm^3 12.5% ammonium-hydroxide + 20 cm^3 12.5% hydrogen-peroxide	1	100
1.6.	DM + 20 cm^3 3.0% ammonium-hydroxide	1	100
1.7.	DM + 20 cm^3 3.0% ammonium-hydroxide + 20 cm^3 3.0% hydrogen-peroxide	1	100
1.8.	DM + 20 cm^3 1.5% ammonium-hydroxide	1	100
1.9.	DM + 20 cm^3 1.5% ammonium-hydroxide + 20 cm^3 1.5% hydrogen-peroxide	1	100
1.10.	DM + 20 cm^3 0.2% ammonium-hydroxide	1	100
1.11.	DM + 20 cm^3 0.2% ammonium-hydroxide + 20 cm^3 0.2% hydrogen-peroxide	1	100

Table 2: Heat optimization experiment – set-up

Sample indication	Treatment	Duration (hour)	Temperature (°C)
2.1.	DM + 20 cm^3 distilled water	1	20
2.2.	DM + 20 cm^3 reagent*	1	20
2.3.	DM + 20 cm^3 distilled water	1	40
2.4.	DM + 20 cm^3 reagent*	1	40
2.5.	DM + 20 cm^3 distilled water	1	60
2.6.	DM + 20 cm^3 reagent*	1	60
2.7.	DM + 20 cm^3 distilled water	1	80
2.8.	DM + 20 cm^3 reagent*	1	80
2.9.	DM + 20 cm^3 distilled water	1	100
2.10.	DM + 20 cm^3 reagent*	1	100

* Solution of 2.5% ammonium-hydroxide and 2.5% hydrogen-peroxide

Establishment of the optimal temperature was followed by determining the optimal duration of the treatment.

Optimization of treatment duration

As a starting-point of the experiments aimed at time optimization, we opted for 2.5% ammonium-hydroxide and 2.5% hydrogen-peroxide and a temperature of 100 °C. We set up the experiment as follows (*Table 3*).

Table 3: Experiment set-up for optimizing the duration of heat treatment

Sample indication	Treatment	Duration (hour)	Temperature (°C)
3.1.	DM + 20 cm^3 distilled water	20	1000
3.2.	DM + 20 cm^3 reagent*	20	100
3.3.	DM + 20 cm^3 distilled water	40	100
3.4.	DM + 20 cm^3 reagent*	40	100
3.5.	DM + 20 cm^3 distilled water	60	100
3.6.	DM + 20 cm^3 reagent*	60	100
3.7.	DM + 20 cm^3 distilled water	120	100
3.8.	DM + 20 cm^3 reagent*	120	100

* Solution of 2.5% ammonium-hydroxide and 2.5% hydrogen-peroxide

3 Results and discussion

Due to the structure of the trypsin inhibitor, we attempted to reduce its activity in three ways:

- splitting the disulphide bridges with oxidation,
- splitting the disulphide bridges with reduction,
- hydrolysis of the carrier protein under alkaline conditions, and
- the combination of the above methods.

Considering the examinations prior to our experiments, it has become obvious that reduction is complicated; it is difficult to get rid of the excess reagent and the by-products generated during reduction. Oxidation performed with hydrogen-peroxide does not generate by-products as water produced during oxidation evaporates in the drying process. Ammonium-hydroxide used in the alkaline treatment partly evaporates during the drying process and is partly adsorbed and thus increases the crude protein content on the surface of the

treated soybean; therefore, we went on to opt for the combination of these two chemicals.

Optimization of the chemical concentration

Regarding the optimization of the chemical concentration, the results obtained for urease enzyme activity and trypsin inhibitor quantity are included in *Table 4*. In each of the cases, heat treatment was carried out at 100 °C for 60 minutes.

Table 4: Results of experiments for the optimization of the chemical concentration

Sample indication	Urease enzyme activity (pH difference)	Trypsin inhibitor (TIU/mg)
1.1. DM + 20 cm^3 25% hydrogen-peroxide	0	0
1.2. DM + 20 cm^3 25% ammonium-hydroxide + 20 cm^3 25% hydrogen-peroxide	0	0
1.3.* DM + 20 cm^3 distilled water	1.48	128
1.4. DM + 20 cm^3 12.5% hydrogen-peroxide	0	0
1.5. DM + 20 cm^3 12.5% ammonium-hydroxide + 20 cm^3 12.5% hydrogen-peroxide	0	0
1.6. DM + 20 cm^3 3.0% hydrogen-peroxide	0.22	14.7
1.7. DM + 20 cm^3 3.0% ammonium-hydroxide + 20 cm^3 3.0% hydrogen-peroxide	0.15	5.9
1.8. DM + 20 cm^3 1.5% hydrogen-peroxide	0.44	33.9
1.9. DM + 20 cm^3 1.5% ammonium-hydroxide + 20 cm^3 1.5% hydrogen-peroxide	0.17	12.8
1.10. DM + 20 cm^3 0.2% hydrogen-peroxide	0.51	40.2
1.11. DM + 20 cm^3 0.2% ammonium-hydroxide + 20 cm^3 0.2% hydrogen-peroxide	0.21	15.3

* Control

Both the 25% and the 12.5% concentration of hydrogen-peroxide and combined treatment respectively reduced both urease enzyme and trypsin inhibitor activity to zero as compared to the control, where pH difference was 1.48 and inhibitor activity 128 TIU/mg. When the quantity of hydrogen-peroxide was reduced to 3%, pH difference decreased to 0.22 and TIU to 14.7, which is 12% of the control. Using the same concentration with combined treatment, pH difference dropped to 0.15 and TIU to 5.9, 5% of the initial value. When the concentration of hydrogen-peroxide was reduced to 1.5%, both pH difference

and TIU value increased, which was significantly improved by the combined treatment, where pH difference decreased to 0.17 and TIU value to 12.8, which is exactly 10% of the original value. Chemical concentration being reduced to 0.12%, combined treatment resulted in a pH difference of 0.21 and a TIU value of 15.3.

The obtained results show that heat treatment performed at 100 °C for 60 minutes with a combination of 1.5% hydrogen-peroxide and ammonium-hydroxide reduces TIU value to safe levels, while during control treatment, using water instead of the reagents, much of the trypsin inhibitor activity was retained. Therefore, in our further experiments, also considering a safe inhibitor reduction, we used a 2.5–2.5% chemical concentration.

Temperature optimization

During temperature optimization, the results obtained for urease enzyme activity and trypsin inhibitor content are included in *Table 5*.

Table 5: Experiment results for temperature optimization

Sample indication	Urease enzyme activity (pH difference)	Trypsin inhibitor (TIU/mg)
2.1. control	1.48	135.2
2.2. 20 °C	1.34	102.4
2.3. control	1.48	138.4
2.4. 40 °C	0.99	82.3
2.5. control	1.48	124.3
2.6. 60 °C	0.84	31.4
2.7. control	1.52	128.2
2.8. 80 °C	0.38	32.2
2.9. control	1.40	125.8
2.10. 100 °C	0.19	12.1

A one-hour chemical treatment at room temperature resulted in a minimal decrease of pH difference, while TIU value dropped by 24%. At 40 °C, TIU value dropped by 40%, at 60 °C and 80 °C, there was a 75% decrease, while heat treatment performed at 100 °C yielded a TIU value below 10% of the initial value. Thus, the experiment for temperature optimization demonstrated the following: decrease upon chemical treatment both at room temperature

and at 40 °C can be significant; 60 °C and 80 °C are not sufficient for a one-hour treatment; 100 °C is sufficient for the TIU value to be reduced to 10% and for urease enzyme activity to fall within the well-toasted range.

Time optimization

During time optimization, the results obtained for urease enzyme activity and trypsin inhibitor content are included in *Table 6.*

Table 6: Experiment results for time optimization

Sample indication	Urease enzyme activity (pH difference)	Trypsin inhibitor (TIU/mg)
3.1. control	1.62	135.2
3.2. 20 minutes	0.67	44.1
3.3. control	1.43	129.3
3.4. 40 minutes	0.19	14.3
3.5. control	1.49	125.8
3.6. 60 minutes	0.17	12.2
3.7. control	1.17	82.2
3.8. 120 minutes	0.08	6.8

The table data show that in 20 minutes' time TIU value decreases from 135.2 to 44.1, which amount to an almost 70% decrease. In 40 minutes, this decrease is close to 90%, while it takes an hour for the TIU to drop below 10% of the initial value. After a 120-minute heat treatment, TIU value did not drop significantly and stayed constant at around 8% of the original value. We can also infer from the table data that by increasing the duration of heat treatment the TIU values of the control sample decreased from 135.2 to 82.2.

Overview of the procedure's optimal parameters

An optimal chemical concentration is 1.5–5.0% aqueous solution of the hydrogen-peroxide and ammonium-hydroxide, while the optimal solution quantity is 20% of the soy's dry matter content. The optimal temperature is 100°C, and the optimal time for heat treatment is 60–120 minutes. The reason why we specify ranges and not exact parameters is that values can change depending on the sample preparation, the initial moisture content, and the used equipment. Based on the above, one possibility/method for reducing the soybean's

trypsin inhibitor content and urease enzyme activity is as follows: Mix one tonne of flattened, air-dried soy with a 200 l solution containing 1.5–2.5% hydrogen-peroxide and 1.5–2.5% ammonium-hydroxide. Consequent upon gas evolution, an expansion occurs, wherefore the capacity of the apparatus has to be designed so that it can hold 2.0–2.5 times the soy's volume foreseen for the treatment. Subsequent to gas evolution, the initial volume returns to its original value in 10 minutes' time. After leaving it for 15–20 minutes, a heat treatment of 60–120 minutes at 100°C causes the soybean's urease enzyme activity and trypsin inhibitor content to fall below the tenth of their initial values.

We cannot evaluate our results in the light of the literature as we have not found a single reference at anyone using hydrogen-peroxide or ammonium-hydroxide to inactivate soy trypsin inhibitor content. Treatment performed using these two chemicals gives rise to several questions. What happens to the hydrogen-peroxide that is not used up in oxidation? During heat treatment, it most likely transforms into oxygen and water, wherefore no harmful substance is retained in the soybeans following such a heat treatment.

Does it lead to oxidation in the case of valuable soy components, sulphurous amino acids, vitamin E, and unsaturated fatty acids? As trypsin inhibitors occur in high concentrations in the soybean shell, the outer shell, and directly underneath it, it is not probable that the oxidative transformation would be a significant one. In the case of amino acids, these transformations – based on our examinations – are negligible; however, we did not assess the oxidation of fatty acids and vitamin E, which is why we have no data on these.

The question might be raised as to what happens to excess ammonium. In 60 minutes, it partially evaporates, and a small part of it may be bound to soy components via surface adsorption. If we regard soy as animal feed, especially the dairy feed of high-yielding cattle, then it causes no problem at all since microorganisms inhabiting the rumen can use both ammonia and non-protein nitrogen for protein synthesis. Nevertheless, finding answers to these questions is a task for the future.

Summary

Owing to its high trypsin inhibitor content, crude soybean meal can only be used with certain restrictions for human consumption and animal feeding. To reduce and optimize its trypsin inhibitor content, certain heat treatment procedures and more or less successful physical and chemical methods were developed. These procedures mostly entail high energy consumptions, while

the end-product's quality also leaves much to be desired. The abovementioned urged us to try out the chemical method using hydrogen-peroxide–ammonium-hydroxide combined with heat treatment to reduce soy trypsin inhibitor activity, expecting a significant amount of energy savings. We determined the optimal concentration of the chemicals applied, the optimal temperature and duration of treatment, in the course of which soy trypsin inhibitor activity decreased to tenth–twentieth part of the original value. The procedure results in lower energy consumption as compared to the original heat treatment methods.

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