Effect of germination and lactic fermentation on the trypsin inhibitor content of soybean (*Glycine max*.)

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Abstract

The aim of this study was to investigate the urease activity evolution in soybeans processed by germination and lactic fermentation; evaluation of residual urease activity is used as an indicator for an appropriate processing of soybeans, as concerned the activity of trypsin inhibitor. After the germination for 4 days at 25°C, a reduction with 35.79% of urease activity occurred in soybeans compared to those unprocessed. Lactic fermentation was conducted for 24, 48, 72 or 96 hours by immersing soybeans into a fermentative medium with Lactobacillus (0.8x10⁸ CFU/ml), supplemented with 1, 3 and 5% saccharose. It was observed an improvement of nutritional value of soybeans as related to urease activity decrease to levels similar to those obtained by heating procedures. The dynamics of urease activity by germination and fermentation of soybean seeds showed that these processes are appropriate and effective for the improvement of soybeans nutritional value and they can be applied as biotechnological techniques for obtaining functional foods from soybean.

Keywords: soybean, germination, lactic fermentation, antinutritional factors, trypsin inhibitor, urease activity, nutrition, health

1. Introduction

Many investigations have been conducted on soybean during the years, since it is a unique dietary source of isoflavones which display a diversity of biological activities and reduce the risk of some chronic diseases. Soybean is distinctive in that it has a high content of isoflavones which allegedly diminish the risk of cancer, cardiovascular disease, and osteoporosis, and also alleviate menopausal symptoms; besides isoflavones soybean (*Glycine max*) also contains protease inhibitors, lectins, and antifungal proteins, which have important biological activities [11]. Unprocessed soybean seeds contain the three main protein inhibitors: Kunitz, Bowman-Birk and trypsin glycine-rich inhibitor. These compounds affect the trypsin activity; interact with protein metabolism and negative influence the animal growth. Studies on animals shown that a high level of trypsin inhibitors in rats diet determined the increasing of pancreatic enzymes and decreasing of growth rate; but the trypsin inhibitors exerted in vivo and in vitro anticancer effects [2]. Trypsin inhibitor may inhibit breast cancer with 50% and also the skin, bladder, colon, lung, pancreas or oesophageal cancer. Furthermore, it inhibits some cancer stimulating factors or prevents the conversion of normal cells into carcinoma cells [6]. But these beneficial effects of protein inhibitors are exerted on very low levels in soybean seeds; in high
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doses those compounds can reduce protein digestion, affecting animal growth and health. It is well known that heating significantly reduces the activity of trypsin inhibitor; so the most used/applied techniques for trypsin inhibitor reduction are based on heat: boiling, autoclaving, [9] or roasting [4]. In these cases of heat processing of soybean seeds, a maximum of 85% decrease in trypsin activity was noticed. The exhaustive research on the Kunitz inhibitor and Bowman-Birk inhibitor from soybean has been conducted in order to find the best technology or processing that decrease the activity of trypsin inhibitor but to preserve the bioactive compounds find in soybeans. Food processing by soaking, germination or/and fermentation greatly influences the nutritive value of legumes. The evolution of trypsin inhibitor from a mix of soybean and lupine was investigated after its germination at 25°C for 72 hours followed by a hydrolysis with pepsin. The results showed a good reduction of urease activity (an indicator of trypsin inhibitor activity): 12.31% decreasing by germination and 100% by hydrolyse [7]. Germination for 72 hours at 25°C determined the reduction of the trypsin inhibitor activity in soybean with 20.91% compared to ungerminated seeds and with 5.48% compared to those germinated only 48 days under the same conditions [3]; in mung bean, the decreasing was 22.4% compared to control samples, while autoclaving (121°C/ 15 minutes) determined a 89.48% reduction of this parameter [9]. Similar results were obtained on chickpea by germination when the trypsin inhibitor activity decreased with 33.95% by germination, 83.86% by autoclaving, 82.27% by simple boiling [4]. Germination of soybean seeds for 4 days at 25°C determined the decreasing of urease activity with 29.31% compared to ungerminated seeds [12]. Germination can degrade both KSTI and the major Bowman-Birk soybean trypsin inhibitor; the degradation would be enhanced if germination lasted more than 4 days. Moreover, germination increased the in vitro digestibility of the proteins, as the degraded proteins from germinated soya beans are better digested than the proteins in their native form. Fermentation was not studied so intense related to its effect on trypsin inhibitor activity evolution, but there are studies that underline the importance of fermentation on the improvement of nutritive quality of beans The level of trypsin inhibitor decreased with 42.30% by bean fermentation with Pediococcus acidilactici inoculum compared to those unprocessed [1] with 29.3% in bean natural fermentation [13] and with 65.76% through germination followed by fermentation with Lactobacillus reuteri [7]. In this study, we investigated the effect of combining of two biotechnological processes as germination for 4 days at 25°C with natural lactic fermentation on the trypsin inhibitor activity in soybeans (Glycine max.).

2. Materials and methods

Materials
Considering the nutritive and functional potential of soybean seeds and also the inconvenience of its sensorial proprieties in raw consumption, we have focused our research on the possibility of improving these characteristics by biotechnological processing via germination and lactic fermentation. We have used soybean seeds from the local market, germinated under controlled conditions and introduced them into a lactic fermentation media represented by a wheat bran extract.

Methods
Germination
Soybean seeds (Glycine max L.) were washed with tap water, rinsed twice with distilled water and sterilized by immersing for 10 minutes into a sodium hypochlorite solution 1%. Then the seeds were three times rinsed with distilled water and gently dried at 40°C;
ungerminated seeds were kept for analysis. The washed seeds were soaked in distilled water for 12 hours at 25°C, 1:4 (w:v), then washed and placed on a water-soaked paper filter to germinate for 96 hours at 25°C.

**Fermentation**

The wheat bran fermentative extract was purchased on the local market; the pH and total viable cell counting was determined before fermentation starts (the medium had pH 2.5 and, 0.8x10^8 CFU/ml *Lactobacillus*). Germinated soybean seeds were immersed into the fermentative extract with a 1:3 (w:v) ratio and for the improvement of fermentation 1, 3 or 5% saccharose was added in the medium. The seeds were fermented at 35°C for 24, 48, 72 and 96 hours, then washed and dried gently at 40°C for 12 hours. The dried germinated and fermented soybean seeds were milled (0.4mm) and kept at 4°C for determinations.

**Urease activity**

The evaluation of residual urease activity is used as an indicator for an appropriate processing of soybeans, as concerned the activity of trypsin inhibitor: the highest urease activity means a highest trypsin inhibitor level is present [8].

**Reagents**: hydrochloric acid 0.71 N; solution of sodium hydroxide 0.71 N, Phosphate buffer containing, per 1000 ml, 4.45 g of disodium phosphate (Na₂HPO₄·2H₂O) and 3.40 g of monopotassium phosphate (KH₂PO₄), freshly-prepared urea in buffer (30 g of urea/1000 ml of phosphate pH 6.9-7.0).

**Apparatus**: high sensitivity pH-meter (0 702 pH) with magnetic stirrer; water bath fitted with thermostat set at 30 ºC exactly; test-tubes with ground-glass stoppers, 150 × 18 mm.

**Procedure**: 0.2 g of milled soybean so that it passes through a sieve with a mesh of 0.2 mm were placed in a test tube with a ground-glass stopper and 10 mL of urea loading agent was added. The tube was immediately covered, vigorously shacked and placed in a water bath set at 30 ºC exactly and shacked again vigorously. It was maintained in these conditions for 30 minutes; after that, 10 mL of 0.1 N hydrochloric acid was added immediately, cooled rapidly to 20 °C and transferred quantitatively to a titration vessel. The mix was titrated immediately and rapidly to pH 4.7 with the 0.1 N sodium hydroxide solution by electrometry.

A blank test was carried out as follows: 0.2 g sample were placed into a test tube with a ground-glass stopper, 10 mL of 0.1 N hydrochloric acid was added immediately, cooled rapidly to 20 ºC and transferred quantitatively to a titration vessel. It was maintained in these conditions for 30 minutes; after that, 10 mL of 0.1 N hydrochloric acid was added immediately, cooled rapidly to 20 ºC and transferred quantitatively to a titration vessel. The mix was titrated immediately and rapidly to pH 4.7 with the 0.1 N sodium hydroxide solution by electrometry.

Urease activity was estimated by the amount of ammonium nitrogen liberated per 1 g of product per minute at 30 ºC from a solution of urea, by using the formula:

\[
\text{urease activity} = \frac{1.4 \cdot (V_1 - V_2)}{30 \cdot E} \quad g \text{N} / g \text{sample} \cdot \text{min} \cdot 30^\circ \text{C}
\]

\[V_1 = 0.1 \text{ N sodium hydroxide solution volume used for samples titration, ml;}
\]
\[V_2 = 0.1 \text{ N sodium hydroxide solution volume used for blank titration, ml;}
\]
\[1.4 = \text{nitrogen equivalent to 1mL, 0.1 N sodium hydroxide solution;}
\]
\[30 = \text{time in contact with urea, minutes.}
\]
3. Results and discussions

The purpose of this work was to determine the influence of biotechnological processing by germination and lactic fermentation on the trypsin inhibitor activity of soybean seeds (Glycine max).

We have investigated the urease activity in soybean seeds germinated at 25°C for 4 days, followed by fermentation at 35°C for 24, 48, 72, 96 hours, by changing the level of saccharose adding in the fermentative medium, to establish if it influences the fermentation process and the trypsin inhibitor activity in soybean.

The results of experimental work are presented in table 2.

Table 1. Urease activity of soybean seeds processed by germination and fermentation *

<table>
<thead>
<tr>
<th>% saccharose</th>
<th>Time, hours</th>
<th>S</th>
<th>SG</th>
<th>1%</th>
<th>3%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
<td>2.073±0.036</td>
<td>1.331±0.015</td>
<td>1.107±0.012</td>
<td>1.086±0.010</td>
<td>1.007±0.066</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>1.044±0.023</td>
<td>0.987±0.016</td>
<td>0.808±0.019</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>-</td>
<td>-</td>
<td>0.546±0.009</td>
<td>0.518±0.016</td>
<td>0.386±0.006</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>-</td>
<td>-</td>
<td>0.392±0.005</td>
<td>0.339±0.007</td>
<td>0.285±0.008</td>
</tr>
</tbody>
</table>

* Each value represents mean ± SD (standard deviation) (n=3) and is significantly at p<0.05.

It can be easily observed that germination for 4 days at 25°C of soybeans determines the reduction of urease activity with 35.79% compared to unprocessed legume seeds.

Germination improves the nutritional quality of bean proteins by reducing the trypsin inhibitor activity thanks to its hydrolyse (during germination, the activity of hydrolytic enzymes become more intense). Consequently, soybean proteins become more digestible with an increased susceptibility to enzymatic attack [9, 13].

In our study, fermentation was following germination as a biotechnological process for the improvement of soybean seeds quality and nutritive value. We have investigated the influence of the level of saccharose in the fermentative wheat extract the urease activity of processed soybean seeds, and the results are shown in figure 1. From figure 1 it can be observed that urease activity is following a descending linear curve for all concentration of saccharose supplementation, during the fourth time period of processing. The maximum decreasing of urease activity of fermented soybean seeds occurred after 96 hours of fermentation. The value of urease activity was 0.392 g N/g·min·30C (for 1% saccharose), with
70.55% decreasing compared to germinated seeds and reached 0.285 g N/g·min·30C (for 5% saccharose), with 78.59% smaller than germinated seeds. It can be underlined that fermentation with 5% supplementation of saccharose is more effective in reducing urease activity, so represents a better way to improve soybean seeds nutritional quality. Considering the signification and equations for the urease activity of processed soybean seeds with the level of saccharose supplementation of the fermentative medium (figure 1), it can be concluded that the \( R^2 \) (squared Pearson) coefficient is higher than 0.92, and that shows a good definition of the model at a significance level of 5% \( (p < 0.5) \). Germination of soybeans followed by fermentation for 96 hours appeared to be a good processing technique for urease activity decreasing.

![Fermented 96 hours vs. germinated soybean](image)

**Figure 3.** Percentage decreasing of the urease activity in germinated and fermented seeds for 96 hours, compared to the value of germinated seeds.

The data presented in figure 3 are very suggestive and show that the influence of saccharose level of the fermentative medium on urease activity evolution, for the same time period is not so obvious: in germinated and fermented soybeans with 1% saccharose, the level of urease reduction was of 70.55% compared to germinated seeds, for those fermented into a medium with 3% saccharose with 4.08% bigger, and for 5% saccharose with 8.04% higher than 1% saccharose samples.

Urease activity in processed soybean seeds was also influenced by fermentation time and the results of the experiments are shown in figure 4.

In the first 24 hours of fermentation, urease activity had a linear decreasing for all fermented samples. Thus, for fermented soybeans, compared to germinated seeds, the decreasing of urease activity was of 16.83% (for 1% saccharose content in fermentative medium), 18.41% (for 3% saccharose) and 24.34% (for 5% saccharose added in medium) (figure 4 a).

Continuing the fermentation for 48 hours, urease activity decreased to a minimum level for samples fermented with 5% saccharose, 0.808 N/g·min·30C, which represents a 39.29% reduction compared to germination (figure 4 b).

The trend line is the same for prolonged fermentation to 72 and 96 hours, when a decreasing of urease activity is also occurred (figure 4 c, d).
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11309

1.331

1.086

1.007

1.107

\[
y = -0.0993x + 1.381
\]

\[
R^2 = 0.8506
\]

Urease activity, g N/g min 30°C

Soybeans fermented for 24 hours

SG 1% saccharose 3% saccharose 5% saccharose

Figure 4. Urease activity of germinated and fermented soybean seeds depending on fermentation time: a) 24 hours; b) 48 hours; c) 72 hours; d) 96 hours

Considering the signification and equations for the urease activity of processed soybean seeds with the level of saccharose supplementation of the fermentative medium (figure 4), it can be concluded that the \(R^2\) (squared Pearson) coefficient is higher than 0.68, and that shows a good definition of the model at a significance level of 5% (p<0.5).

For a more suggestive presentation of the influence of fermentation time on the urease activity in soybean seeds we have built figure 5; this represents the percentage decreasing of the urease activity in germinated and fermented seeds, with 1% saccharose compared to the value of germinated seeds.

**Figure 5.** Percentage decreasing of the urease activity in germinated and fermented seeds, with 1% saccharose compared to the value of germinated seeds
The data presented in figure 5 show that the influence of fermentation time is very important on urease activity evolution, for the same percentage addition of saccharose in the fermentative medium. In germinated and fermented soybeans for 24 hours, the level of urease reduction was of 17.45% compared to germinated seeds, after 48 hours with 4.7% bigger and continuing fermentation for 72 and 96 hours, urease activity increased with 33.06% and 53.32% respectively compared to 24 hours of processing.

We can affirm that lactic fermentation is very intense after 72 hours of fermentation with 1% saccharose and the urease activity is decreasing faster and reach the maximal reduction after 96 hours of processing.

![Figure 6. The evolution of urease activity of germinated and fermented soybean seeds with fermentation time](image1)

![Figure 7. The evolution of urease activity of germinated and fermented soybean seeds with saccharose adding in the fermentative medium](image2)

Figures 6 and 7 present the evolution of urease activity with fermentation time and saccharose content of fermentative medium. These data underline an intense reduction of urease activity by germination (35.79%) compared to unprocessed soybeans and this reduction is continuing by fermentation, when reaching the minimal value of urease activity after 96 hours of fermentation. The reduction of trypsin inhibitor activity by germination followed by fermentation might be explained by the increased activity of proteases that occur in processed soybean seeds (activated by germination or microbial proteases from *Lactobacillus*): proteases determine trypsin inhibitor hydrolyse and as a consequence improve the protein digestibility.

4. Conclusions

Germination of soybeans for 4 days at 25°C determines a good reduction of urease activity, (35.79%) which is unfortunately less than that achieved by heat processing (85% decreasing of the trypsin inhibitor activity) [3]. Changing the saccharose content of the fermentative medium did not induce a significant reduction of the urease activity in
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germinated and fermented soybean seeds, but increasing fermentation time is more effective on improving soybeans nutritional value. Our attempt to combine germination with lactic fermentation for a better decreasing of urease activity was successful because the results show that by double processing of soybeans the decreasing of urease activity is more obvious, especially after 72 and 96 hours of fermentation with 5% saccharose and reached 86.26% decreasing which is similar with that obtained by heating procedures. The advantage of using germination and lactic fermentation for the removal of trypsin inhibitor activity is that those two are biotechnological processes is protective for the other nutritional characteristics of soybean seeds (which are also increased – data not published here).

References