ENZYMATIC HYDROLYSIS OF EXTRUDED SOYBEAN MEAL AT HIGH SUBSTRATE CONCENTRATIONS

Anton Yu. Sharikov*, Anna S. Sereda, Elena V. Kostyleva, Irina A. Velikoretskaya and Victor A. Polyakov

All-Russia Research Institute of Food Biotechnology, Samokatnaya str. 4b, 111033 Moscow, Russia

Extrusion as a pretreatment before enzymatic hydrolysis of soybean meal is an effective technique to eliminate antinutritional properties of the main thermostable soy proteins glycinin and β-conglycinin for production of feed ingredients with enhanced properties. In terms of economic efficiency, biotechnological processes are preferable to carry out at high substrate concentrations. The aim of the investigation was to evaluate the influence of high substrate concentrations in the range of 26 - 32% and enzyme dosages (0.4 - 3.1 PU/g) on efficiency of hydrolysis of extruded toasted soybean meal with bacterial protease. The results showed that maximum degree of hydrolysis was 42.1% at the enzyme dosage of 3.6 PU/g and at the substrate concentration of 29.0%. The increase in the substrate concentration had a strong effect on the deterioration of dynamic viscosity of the hydrolysates from 0.2 to 5.82 Pa·s. A combination of extrusion cooking at 120ºC and enzymatic treatment with “Protolad B” protease enabled hydrolysis of glycinin and β-conglycinin to peptides with molecular mass below 15 kDa.

KEY WORDS: soybean meal, β-conglycinin, glycinin, extrusion cooking, protease

INTRODUCTION

Soybean meal (SBM) is one of the most economically effective sources of valuable protein in animal feed. However, the main soybean proteins – glycinin and β-conglycinin – exhibit antigenic properties and have a negative effect on the immune system (1, 2). Due to the compact structure and presence of disulphide bonds, they are thermostable, and are only partially denatured in the conventional process of SBM toasting. Complete denaturation of thermostable proteins can be provided by extrusion cooking which comprises a combination of moisture, pressure, temperature, and mechanical shear processing factors. Denaturation of β-conglycinin and glycinin during extrusion does not eliminate their allergenic properties caused by the characteristic amino acid sequences, but improves their accessibility for proteases action (3, 4). Marsman et al. (4) showed that only a combination of extrusion cooking with subsequent enzymatic hydrolysis using proteases allowed eliminating all glycinin and β-conglycinin subunits in toasted SBM (TSBM). It is

* Corresponding author: Anton Yu. Sharikov, All-Russia Research Institute of Food Biotechnology, Samokatnaya str. 4b, 111033 Moscow, Russia, e-mail: anton.sharikov@gmail.com
important to note that most of the investigations on SBM enzymatic hydrolysis were performed at substrate concentrations of lower than 10% (4-6). This may be explained by the challenging rheological properties of SBM/water suspensions at high substrate concentrations due to the characteristic property of glycamin and β-conglycinin to form gels through molecules aggregation caused by interactions of sulfhydryl and hydrophobic groups (7). Gelation impairs technological properties of the reaction mixture, impedes mass transfer, and reduces the effectiveness of the enzymatic hydrolysis.

At the same time, substrate concentration is one of the key economic factors of biotechnological processes (8). Hydrolysis at high solids reduces heating requirements, water losses, and energy consumption associated with water removal processes such as evaporation, concentration, drying. Volumetric productivity of the plant may be increased through more effective utilization of equipment.

The aim of this research was to evaluate the influence of high substrate concentrations (SC) and enzyme dosages (ED) on efficiency of extruded TSBM (ESBM) hydrolysis and completeness of glycamin and β-conglycinin elimination.

**EXPERIMENTAL**

**Material**

Commercial TSBM with crude protein content of 50% (dry matter basis) and 5.5% moisture content was used for the experiments. Enzyme preparation of *Bacillus licheniformis* protease – Protolad B (Mikrobioprom Ltd., Ukraine) with proteolytic activity 350 PU/g was used for protein hydrolysis. Proteolytic activity of Protolad B was assayed according to method developed by Sigma-Aldrich (9) using casein as a substrate.

**Extrusion cooking**

A co-rotating twin-screw extruder Werner & Pfeiderer Continua 37 (Stuttgart, Germany) was used for treatment of TSBM. The screws diameter was 37 mm with ratio of the screw length to its diameter (L/D) of 27:1. The screw configuration was as follows: 160 mm forwarding twin lead screw (FTLS) with 40 mm pitch, 2 × 10 mm kneading segments (KS), 120 mm FTLS with 40 mm pitch, 3 × 10 mm KS, 40 mm FTLS with 40 mm pitch, 15 mm reverse screw (RS) with 15 mm pitch, 160 mm FTLS with 40 mm pitch, 2 × 10 mm KS, 210 mm FTLS with 30 mm pitch, 2 × 10 mm KS, 60 mm FTLS with 20 mm pitch, 15 mm RS with 15 mm pitch, 120 mm FTLS with 20 mm pitch. The die had two openings each 2.5 mm in diameter. The barrel temperatures were 25°C in the feed zone, 50°C in the central zone and 120 – 160°C at the die. The screw speed and feed rate were kept constant at 300 rpm and 14 kg/h, respectively. Moisture content of feed material in the process of extrusion cooking was adjusted to 20.0%. After drying, the extruded samples were maintained at room temperature and ground in a laboratory hammer mill with 1 mm sieve openings.
Hydrolysis

Ground TSBM and ESBM samples were hydrolyzed by protease at 50°C, pH 6.1±0.1 (natural pH of SBM/water suspension without adjustment) for 5 hours. The substrates were mixed with water phase containing Protolad В in proper concentration, the reaction mixtures were stirred vigorously and then incubated without agitation until the end of the process. The hydrolyzed mixtures were heated to 85°C and incubated at this temperature for 10 min for protease inactivation. The resulting hydrolysates were dried at 55°C to constant weight for subsequent analyses.

Substrates and hydrolysates characterization

Crude protein content in TSBM and ESBM was measured according to Kjeldahl method using Turbotherm TT-625 digestion unit (Gerhardt, Germany) and DL 15 titrator (Mettler Toledo, Switzerland). Soluble protein concentration in hydrolysates was measured according to Lowry et al (10).

Protein solubility (PS) was determined as percentage of soluble protein content in relation to the total protein content of the sample. The degree of protein hydrolysis (DH) was determined as the percentage of content of protein soluble in 10% (w/v) trichloroacetic acid (TCA) in relation to the total protein content of the sample (11, 12).

Completeness of proteinaceous antinutritional factors (ANF) hydrolysis was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-Protean Tetra System (BioRad, USA). Samples were prepared as follows: 20 mg of dry hydrolysates were taken to Eppendorf tubes and extracted in 0.125 mL of SDS buffer for 1 h at constant stirring, then diluted 5-fold in the same SDS buffer with mercaptoethanol and incubated for 15 min at 100°C.

Determination of the rheological properties

Dynamic viscosity (DV) was measured by a sine-wave viscometer SV-10 (AND, Japan) with frequency of 30 Hz in 45-mL sample container (13). Rheological properties were also determined using a CT-3 Texture Analyzer (Brookfield Engineering, USA) equipped with a 4.5 kg load cell. The diameters of the back extrusion container and disk were 40 and 34 mm, respectively. Tests were carried out to a depth of 20 mm at 1 mm/s penetration speed. The maximum force was taken as a measurement of media firmness (14).

Experiment design and statistical analysis

The first step of the experiment was to determine the optimal extrusion conditions, i.e. optimal temperature of the die. In this step SC of 25% and ED of 2 PU/g were used for hydrolysis of TSBM after extrusion. At the next stage of the experiment, TSBM was extruded at the chosen temperature and then hydrolyzed at various SCs and EDs. SC and ED were used as independent variables (factors) for central composite rotatable design
The ranges of factor values in this investigation were determined on the basis of preliminary research (not shown). Each variable in coded and real values is shown in Table 1. Protein solubility, degree of hydrolysis, dynamic viscosity, and firmness were investigated as response parameters of the design.

Student's t-test was carried out to determine the significance of the regression coefficients. The Fisher's variance ratio test was used for evaluation of the adequacy of the fitted regression models.

The regression coefficients estimation and statistical analysis were performed in accordance with Lazic (15) using Scilab 5.5 (Scilab Enterprises, France).

Table 1. Coded and real values of variables used in the CCRD

<table>
<thead>
<tr>
<th>Coded value</th>
<th>Real value</th>
<th>Substrate concentration, %</th>
<th>Enzyme dosage, PU/g of substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>-α</td>
<td>26.0</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>-1</td>
<td>26.9</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>29.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>31.1</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>32.0</td>
<td>3.6</td>
<td></td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

Effect of extrusion temperature on enzymatic elimination of glycinin and β-conglycinin

Marsman et al. (4) reported that extrusion and the following hydrolysis with Neutrase enzyme preparation provided elimination of thermostable allergy-causing glycinin and β-conglycinin in TSBM. The results confirming the benefits of extrusion pretreatment for soybean proteins hydrolysis were also obtained by other authors (16-18). However, there is a lack of information about the influence of extrusion temperature level on completeness of the subsequent enzymatic hydrolysis of all glycinin and β-conglycinin subunits. Glycinin consists of basic polypeptide B (~20 kDa) and acidic polypeptide (~38 kDa) linked by a disulfide bond. β-Conglycinin is a trimeric glycoprotein composed of three types of subunits: α' (72 kDa), α (68 kDa), and β (52 kDa) (1). The completeness of glycinin and β-conglycinin elimination is usually estimated by the absence of the corresponding bands on SDS-PAGE electrophoregram. To define the optimal extrusion temperature TSBM was extruded at different die temperatures ranging from 120 to 160°C, while the moisture content, screw speed, and feeding rate were kept constant. Samples extruded at different temperatures and unextruded TSBM were subjected to hydrolysis by 2 PU/g Protolad B, for 5 hours, at a substrate concentration of 25%.

The results of SDS-PAGE (Fig. 1) show that the changes in tertiary structure of TSBM proteins during extrusion promoted their hydrolysis by protease. This was confir-
med by the absence of the pronounced bands corresponding to proteins with a molecular weight above 15 kDa in ESBM hydrolysates in contrast to TSBM hydrolysates, where the bands corresponding to antinutritional proteins subunits remained visible. However, a weak band corresponding to a protein with molecular weight of about 20 kDa was observed on SDS-PAGE of hydrolysates of ESBM obtained at 140–160°C. It probably belongs to the residual amount of unhydrolysed subunit B of glycinin (Fig. 1, lanes 5-7). A decrease of extrusion temperature to 120–130°C led to a more complete degradation of soy proteins to peptides with molecular weight under 15 kDa (Fig. 1, lanes 3, 4). For further experiment, the most energy-saving temperature mode (120°C) providing complete soy proteins hydrolysis into short peptides, was chosen.

**Figure 1.** SDS-PAGE of processed TSBM. Lane M, molecular weight markers 100, 70, 55, 35, 25 and 15 kDa; lane 1, hydrolyzed TSBM; lane 2, TSBM; lanes 3,4,5,6,7, hydrolyzed ESBM, previously extruded at 120, 130, 140, 150, 160°C, respectively

**Influence of the substrate concentration and protease dosage on the ESBM protein hydrolysis and rheological properties of the slurries**

Enzymatic hydrolysis at high solids is a promising way to enhance effectiveness of technological processes. It allows using bioreactors with increased productivity by reducing specific energy, water and materials costs. For this reason it was important to investigate the enzymatic hydrolysis of proteins in ESBM at high substrate concentrations, where gelation of the main soy proteins occurs, that impairs rheological properties and impedes mass transfer. On the basis of earlier studies (data not shown) we have found that the most dramatic change in the rheological properties of ESBM/water suspensions was observed at SCs close to 30%. Therefore, at the next stage of the experiment, the effect of SC in the range of 26–32% and proteolytic EDs on ESBM hydrolysis rate and rheological properties of the slurries was studied using response surface methodology. The final experimental data are presented in Table 2.

For the two independent variables, a second order polynomial regression model has been proposed as:
where ‘y’ is the response; ‘β₀’ is a constant; ‘βᵢ’, ‘βᵢᵢ’, and ‘βᵢⱼ’ are linear, quadratic and interaction regression coefficients, respectively; ‘Xᵢ’ and ‘Xⱼ’ are values of independent parameters.

Table 2. The experimental design and trial results

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Solids concentration (%)</th>
<th>Enzyme dosage (PU/g)</th>
<th>PS (%)</th>
<th>DH (%)</th>
<th>DV (Pa·s)</th>
<th>Firmness (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26.9</td>
<td>0.9</td>
<td>37.33</td>
<td>32.56</td>
<td>0.40</td>
<td>3.29</td>
</tr>
<tr>
<td>2</td>
<td>31.1</td>
<td>0.9</td>
<td>34.98</td>
<td>33.34</td>
<td>3.32</td>
<td>7.43</td>
</tr>
<tr>
<td>3</td>
<td>26.9</td>
<td>3.1</td>
<td>42.98</td>
<td>40.25</td>
<td>0.20</td>
<td>1.69</td>
</tr>
<tr>
<td>4</td>
<td>31.1</td>
<td>3.1</td>
<td>40.16</td>
<td>37.44</td>
<td>2.46</td>
<td>6.47</td>
</tr>
<tr>
<td>5</td>
<td>26.0</td>
<td>2.0</td>
<td>40.36</td>
<td>37.86</td>
<td>0.20</td>
<td>1.88</td>
</tr>
<tr>
<td>6</td>
<td>32.0</td>
<td>2.0</td>
<td>38.72</td>
<td>35.98</td>
<td>5.82</td>
<td>10.52</td>
</tr>
<tr>
<td>7</td>
<td>29.0</td>
<td>0.4</td>
<td>29.10</td>
<td>27.04</td>
<td>2.42</td>
<td>6.61</td>
</tr>
<tr>
<td>8</td>
<td>29.0</td>
<td>3.6</td>
<td>42.68</td>
<td>42.10</td>
<td>0.69</td>
<td>3.82</td>
</tr>
<tr>
<td>9</td>
<td>29.0</td>
<td>2.0</td>
<td>39.12</td>
<td>37.63</td>
<td>1.69</td>
<td>4.53</td>
</tr>
<tr>
<td>10</td>
<td>29.0</td>
<td>2.0</td>
<td>40.78</td>
<td>36.36</td>
<td>1.82</td>
<td>5.53</td>
</tr>
<tr>
<td>11</td>
<td>29.0</td>
<td>2.0</td>
<td>40.32</td>
<td>38.06</td>
<td>1.21</td>
<td>5.12</td>
</tr>
<tr>
<td>12</td>
<td>29.0</td>
<td>2.0</td>
<td>40.80</td>
<td>37.00</td>
<td>1.80</td>
<td>5.55</td>
</tr>
<tr>
<td>13</td>
<td>29.0</td>
<td>2.0</td>
<td>42.00</td>
<td>38.05</td>
<td>2.04</td>
<td>5.00</td>
</tr>
</tbody>
</table>

PS – protein solubility; DH – degree of protein hydrolysis; DV – dynamic viscosity

On the base of each data set, regression coefficient for second order polynomial models and their statistical significance at 95% confidence were calculated (Table 3). The check of the lack of fit of the obtained regression models proved that all models were adequate with 95% confidence level.
Table 3. Regression coefficients and model adequacy evaluation

<table>
<thead>
<tr>
<th>Terms</th>
<th>PS (%)</th>
<th>DH (%)</th>
<th>DV (Pa·s)</th>
<th>Firmness (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>39.9534</td>
<td>10.81</td>
<td>65.8231</td>
<td>26.6162</td>
</tr>
<tr>
<td>SC</td>
<td>-0.4458</td>
<td>0.50</td>
<td>-5.3475</td>
<td>-2.5458</td>
</tr>
<tr>
<td>ED</td>
<td>9.8648</td>
<td>19.52</td>
<td>2.4933</td>
<td>-2.0697</td>
</tr>
<tr>
<td>SC²</td>
<td>-a</td>
<td>-a</td>
<td>0.1081</td>
<td>0.0632</td>
</tr>
<tr>
<td>ED²</td>
<td>-1.6129</td>
<td>-1.12</td>
<td>-0.2087</td>
<td>-0.1750</td>
</tr>
<tr>
<td>SC×ED</td>
<td>-a</td>
<td>-0.39</td>
<td>-0.0709</td>
<td>0.0700</td>
</tr>
<tr>
<td>Adequacy of the regression model&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R²</td>
<td>0.90</td>
<td>0.92</td>
<td>0.91</td>
<td>0.94</td>
</tr>
</tbody>
</table>

<sup>a</sup> insignificant at p<0.05  
<sup>b</sup> at significance level p<0.05  

PS – protein solubility; DH – degree of protein hydrolysis; DV – dynamic viscosity

Graphical interpretations of the estimated equations are shown in Figure 2. The character of contour lines for PS and DH equations shows that protease dosage was a more significant variable for these response parameters, especially in the local domain of low enzyme dosages. The increase of ED improved PS and HD. A maximum PS value in the investigated range of factor values was 42.98% at 26.9% solids and 3.1 PU/g protease dosage. The maximum DH was 42.1% at the starlike point with maximum enzyme dosage of 3.6 PU/g and 29.0% solids. The minimal ED (0.4 PU/g) provided 29.1% and 27.04% values for PS and DH, respectively.

Graphical analysis of contour plots for rheological properties of the hydrolysates (Figure 2) shows that DV and firmness correlate increasingly with the substrate concentration. The maximum DV and firmness values were observed at a starlike point of factorial space at 32.0% solids and 2.0 PU/g protease dosage and accounted to 5.82 Pa·s and 10.52 N, respectively. The increase in the substrate concentration from 26% to 32% resulted in a sharp increase of DV from 0.2 to 5.82 Pa·s. This indicated that the selected factor range was a domain of significant deterioration of rheological properties of the hydrolysates. This effect represents a challenging factor for developing a technological process in terms of selection of mixers and pumps for the process line. The increase of enzyme dosage had a slight effect on decrease of DV.
The efficiency of proteinaceous ANF elimination was estimated using SDS-PAGE analysis of the hydrolysates related to all points of factor space by the disappearance of protein bands with molecular mass above 15 kDa. Results of SDS-PAGE are shown in Figure 3.

Despite the high viscosity and adverse rheological properties of the reaction mass, almost all modes of hydrolysis provided complete hydrolysis of β-conglycinin and glycine in ESBM with formation of the peptides with molecular weights under 15 kDa. Lane 7, corresponding to the minimum ED (0.4 PU/g), was characterized by the more intense staining, which may be explained by the presence of residual peptides with a molecular weight above 15 kDa, but lacked pronounced bands corresponding to subunits of the main anti-nutritional soybean proteins. In general, it can be assumed that Protolad B provides a sufficient hydrolysis of anti-nutritional soy proteins at high ESBM concentrations (26-32%).

**Figure 2.** Effect of solids concentration (%) and enzyme dosage (PU/g) on protein solubility (a), degree of hydrolysis (b), dynamic viscosity (c), and firmness (d)
Figure 3. SDS-PAGE of hydrolysed ESBM. Lane M, molecular weight markers 66.2, 45.0, 35.0, 25.0, 18.4, 14.4 kDa; lanes 1-8 corresponding to treatments 1-8 in Table 2, respectively; lane 9 corresponding to treatment 10

CONCLUSION

The present study demonstrated a potential for combining of extrusion cooking technology and enzymatic hydrolysis at high substrate concentrations for processing of toasted soybean meal and elimination of its antinutritional proteins. The results obtained indicated that the extrusion cooking at 120–130°C was an appropriate pretreatment for proteolytic hydrolysis of the extruded substrate. TSBM extruded at 120°C underwent easily enzymatic treatment with protease at 50°C and pH 6.0 within 5 hours at solids up to 32%. The observation of CCRD results and corresponding contour plots pointed out to the effect of substrate concentration on sharp deterioration of rheological properties of the hydrolysates. It was shown that enzyme dosage was more significant factor for the degree of hydrolysis and protein solubility compared to solids concentration. The results of SDS-PAGE of the hydrolysates indicated complete hydrolysis of glycinin and β-conglycinin fractions. The developed technology allows the obtaining of high quality protein feedstuff based on toasted soybean meal for animals with intolerance to glycinin and β-conglycinin.

Acknowledgement

This research was supported by Grant of President of Russian Federation MK-5743.2015.4.
REFERENCES


ENZIMSKA ХИДРОЛИЗА ЕКСТРУДИРАНЕ СОЈИНЕ САЧМЕ ПРИ ВИСОКИМ КОНЦЕНТРАЦИЈАМА СУПСТРАТА

Антон Ју. Шариков, Ана С. Середа, Јелена В. Костиљова, Ирина А. Великорецкаја и Виктор А. Пољаков

Сверуски научно-истраживачки институт прехранбене биотехнологије, ул. Самокатнаја 4б, 111033 Москва, Русија

Термостабилне, тешко хидролизирајуће сојине белачевине глицинин и β-конглицинин имају имуногени утицај и негативно утичу на организам животиња. Комбинација екструзионе предобраде и ензимске хидролизе сојине сачме представља ефикасан начин одстрањивања антинутритивних својстава глицинина и β-конглицинин, са циљем добијања хранива са побољшаним својствима. Један од кључних фактора који одређују економску ефикасност биотехнолошких процеса је концентрација супстата. Циљ овог рада био је проучавање утицаја дозирања ензимског препарата и концентрације супстата, која се кретала у границама 26-32%, на ефикасност хидролизе белачевина екструдата тосираних сојине сачме као и на реолошке карактеристике хидролизата. Истраживањем је утврђено да је максимални степен хидролизе износио 42,1% при највећој концентрацији протеазе (3,6 PU/g) и при концентрацији супстата од 29%. Повећање концентрације супстата доведио је до повећања вискозности суспензије од 0,2 до 5,82 Pa.s. Комбинација претходног экструдирања на 120°C са даљом обрадом бактеријском протеазом „Протолад Б“ омогућује хидролизу глицинина и β-конглицинин до пептида са молекулском масом мањом од 15 kDa.

Кључне речи: сојина сачма, β-конглицинин, глицинин, екструдирање, протеаза

Received: 27 May 2016.
Accepted: 30 September 2016.