



SHORT COMMUNICATION

Development of an *In vitro* Assay for Evaluating Protease-mediated Protein Solubility in Soybean Meal

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Key words:

Antinutritional factors, *in vitro* assay, protease, protein solubility, soybean meal

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Cite this Article:

Cardoso, C.D.N., T.V. Jensen and J.S.C de Souza, 2026. Development of an *in vitro* assay for evaluating protease-mediated protein solubility in soybean meal. Int. J. Poult. Sci., 25: 209-212.

Abstract

Background and Objective: Soybean meal is a major protein source in animal nutrition but its nutritional value may be limited by antinutritional factors and processing conditions affecting protein solubility. This study aimed to develop and apply an *in vitro* assay to evaluate the effects of proteolytic enzymes on protein solubility in soybean meal and to assess the potential interaction between enzymes under controlled conditions.

Materials and Methods: Soybean meal samples were incubated with trypsin, an exogenous commercial protease (Sfericase), or their combination under standardized conditions. The assay involved controlled enzymatic hydrolysis followed by separation and quantification of the soluble protein fraction using total nitrogen analysis by the combustion (Dumas) method. Protein solubility was determined before and after enzymatic treatment.

Results: The baseline protein solubility of untreated soybean meal was 7.73%. Enzymatic treatment with trypsin or the exogenous protease increased protein solubility to 14.68% and 16.70%, respectively. The combined treatment resulted in a protein solubility of 26.69%, indicating a greater increase than that observed with either enzyme alone and suggesting a potential complementary effect between the enzymes under the conditions tested.

Conclusion: The proposed *in vitro* assay provides a controlled approach for screening protease efficacy and investigating enzyme interactions in feed ingredients. The method may serve as a useful tool for evaluating enzymatic strategies aimed at improving protein solubility and, potentially, protein utilization in animal feed.

INTRODUCTION

Soybean meal is one of the most widely used protein sources in animal nutrition. However, its nutritional value may be affected by antinutritional factors, particularly trypsin inhibitors, as well as by processing conditions that alter protein structure and solubility¹. Although, heat treatment is commonly used to reduce antinutritional activity, excessive processing may decrease protein solubility and digestibility, ultimately affecting nutrient utilization².

Exogenous proteases have been increasingly used in animal feeds to improve protein utilization through the hydrolysis of complex proteins into smaller peptides and amino acids, potentially enhancing protein solubility and digestibility^{3,4}. In addition, some proteases may contribute to the degradation of antinutritional protein fractions present in soybean meal³.

Reliable *in vitro* methodologies are valuable tools for screening enzyme efficacy and evaluating enzyme-substrate interactions under controlled conditions before undertaking

more complex *in vivo* studies⁵. However, practical and reproducible assays specifically designed to quantify protease-mediated changes in soybean meal protein solubility remain limited.

Therefore, the objective of this study was to develop and demonstrate an *in vitro* assay capable of quantifying changes in soybean meal protein solubility following enzymatic treatment. The assay was evaluated using trypsin, an exogenous commercial protease and their combination as model treatments.

MATERIALS AND METHODS

Soybean meal was obtained from a commercial producer in southeastern Brazil. The exogenous protease used was a sfericase (ProAct 360™, Novonesis) and trypsin from porcine pancreas (Type IX-S) was purchased from Sigma-Aldrich (CAS 9002-07-7).

Soybean meal samples were milled using an IKA A11 basic mill and sieved to obtain a particle size of 850 µm. Dry matter content was determined in duplicate using a moisture analyzer (Mettler Toledo HB43-S) at 105 °C.

Protein content was determined by total nitrogen analysis using the combustion method (Dumas method) with a LECO FP 628 nitrogen analyzer. The procedure was based on ISO 16634-1:2008⁶. Calibration was performed using ethylenediaminetetraacetic acid (EDTA) as a standard and a nitrogen-to-protein conversion factor of 6.25 was applied.

Enzyme extracts were prepared by dispersing 1 g of enzyme product in 10 mL of extraction buffer (100 mM acetate buffer containing 5 mM Ca²⁺, 0.01% Tween 20 and 0.01% BSA, pH 5.0). The mixture was stirred at 500 rpm for 1 hrs at room

temperature and centrifuged at 3000×g for 10 min at 5 °C. The supernatant was collected and stored under refrigerated conditions until use.

An *in vitro* assay was developed to evaluate protein solubility following enzymatic hydrolysis under controlled conditions. Briefly, 2.25 g of soybean meal (dry basis) was weighed into pre-weighed 50-mL tubes. Enzyme solutions were added to achieve a final dosage of 500 mg enzyme/kg substrate (dry matter basis). Control samples received ultrapure water. All treatments were evaluated in triplicate independent experimental units (n = 3).

The reaction mixture was adjusted to 15% dry solids using citrate-phosphate buffer (pH 6.65), vortexed and incubated at 40 °C for 4 hrs in a rotisserie incubator at 25 rpm. The reaction was stopped by placing the tubes in an ice bath.

After incubation, samples were centrifuged and the residual solids were washed four times with ultrapure water, followed by a final wash with 70% ethanol. The samples were dried at 60 °C for 48 hrs, ground and analyzed for nitrogen content using the Dumas method as described above.

Protein solubility was calculated based on the difference between the initial and residual protein content.

Data were analyzed by one-way ANOVA and treatment means were compared using Tukey's test, with significance set at p<0.05 (JMP 16, SAS Institute).

RESULTS

The proposed *in vitro* assay successfully quantified differences in protein solubility among enzymatic treatments applied to soybean meal (Fig. 1). The method demonstrated

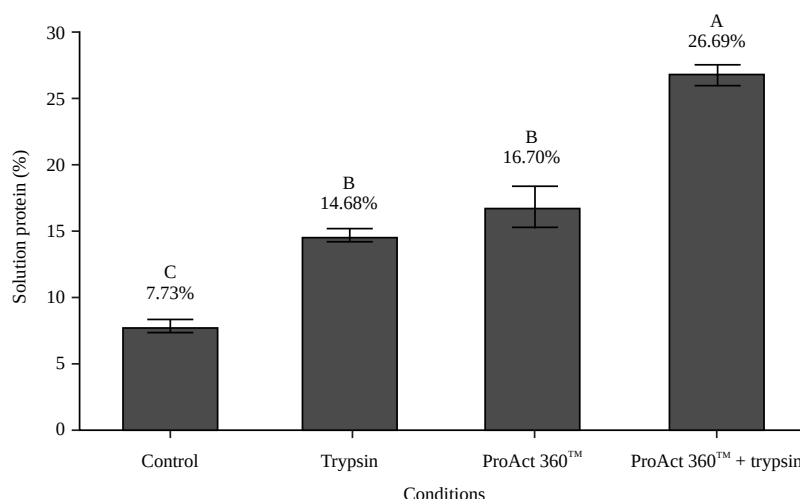


Fig. 1: Effect of enzymatic treatments on soybean meal protein solubility as determined by the developed *in vitro* assay. Values represent Mean ± SD (n = 3). Bars with different letters differ significantly (p<0.05)

sufficient sensitivity to distinguish among treatments, indicating its suitability for evaluating proteolytic effects under controlled conditions. The low coefficients of variation observed across treatments (2.9-9.4%) demonstrate good experimental precision and further support the suitability of the assay for comparative evaluation of protease-mediated changes in protein solubility.

The baseline protein solubility of untreated soybean meal was $7.73\% \pm 0.58$ ($n = 3$), reflecting the limited solubility of native proteins under the conditions tested. Enzymatic treatments increased protein solubility, with measurable differences depending on the enzyme applied.

Addition of trypsin resulted in a significant increase ($p < 0.05$) in protein solubility to $14.68\% \pm 0.42$ ($n = 3$), whereas treatment with the exogenous protease yielded a solubility of $16.70\% \pm 1.57$ ($n = 3$). No statistically significant difference was observed between these two treatments, indicating comparable effects under the assay conditions.

The combined application of trypsin and the exogenous protease resulted in a further increase in protein solubility to $26.69\% \pm 0.81$ ($n = 3$), which was significantly higher than that observed for all other treatments ($p < 0.05$). This finding indicates that the assay is capable of detecting the effects of combined enzymatic treatments and provides a useful framework for evaluating potential interactions between proteases.

Overall, the results demonstrate that the *in vitro* assay can effectively capture variations in protein solubility resulting from different enzymatic treatments and may be applied as a screening tool for assessing protease functionality in soybean meal.

DISCUSSION

The present study describes the development of an *in vitro* assay designed to quantify changes in soybean meal protein solubility following enzymatic treatment. The results demonstrated that the method was sufficiently sensitive to discriminate among treatments, indicating its suitability as a screening tool for evaluating protease activity under controlled conditions.

Both trypsin and the exogenous protease increased protein solubility relative to the untreated control, confirming that the assay can detect proteolytic effects relevant to feed applications. Proteolytic enzymes hydrolyze complex protein structures into smaller peptides and amino acids, thereby increasing protein solubility and facilitating subsequent digestion processes⁴.

Several studies have reported increases in soybean meal protein solubility following enzymatic hydrolysis. Microbial fermentation combined with enzymatic treatment has been

shown to increase the proportion of soluble proteins and peptides by disrupting complex protein structures and reducing antinutritional factors⁴. Similarly, Pettersson and Pontoppidan³ described the potential of feed proteases to enhance the nutritional value of soybean meal through protein degradation and improved accessibility of nutrients. The increases in protein solubility observed in the present study are consistent with these previously reported effects of proteolytic enzymes on soybean proteins. Although, different methodologies and response variables were used across studies, the direction of the response was similar, supporting the biological relevance of the developed assay.

In addition to improving protein solubility, enzymatic treatments have been associated with the degradation of soybean protein fractions that are less susceptible to digestion. *In vitro* enzymatic hydrolysis of soybean meal incubated with trypsin and pancreatin increased the content of rapidly digestible crude protein and decreased the content of slowly digestible and resistant crude protein⁵. Recent investigations have demonstrated that protease supplementation can modify protein structure and increase the release of soluble peptides, thereby facilitating subsequent protein utilization. In an *in vitro* simulation of the gastrointestinal tract with endogenous enzymes, protease supplementation increased digestibility from 83-89% to 91-92%⁷.

The ability of the present assay to detect these changes suggests that the methodology is sufficiently sensitive to capture biologically relevant proteolytic effects.

The greater protein solubility observed when trypsin and the exogenous protease were combined may reflect the action of enzymes with different substrate specificities. Similar complementary effects have been described in studies evaluating combinations of proteolytic activities, in which the hydrolysis of different protein fractions resulted in enhanced overall protein degradation³. Although the present study was not designed to quantify enzymatic synergy, the assay successfully detected differences among treatments and therefore provides a useful platform for investigating potential enzyme interactions.

An important aspect of the proposed methodology is that its results are consistent with the expected behavior of soybean meal proteins subjected to enzymatic hydrolysis. The progressive increase in protein solubility from the untreated control to the individual enzyme treatments and finally to the combined treatment supports the biological relevance of the assay response. Therefore, in addition to providing analytical discrimination among treatments, the assay generated results that agree with current knowledge regarding protease-mediated modification of soybean proteins. Additionally, this

methodology was designed to evaluate the isolated effects of the exogenous proteases tested, while minimizing interference from associated endogenous enzymes.

A major advantage of the proposed assay is its simplicity and reproducibility. *In vitro* approaches are widely used in feed research as practical tools for enzyme screening because they allow evaluation under standardized conditions while minimizing the biological variability inherent in animal studies⁸. Consequently, this method may be useful for comparing proteases, optimizing enzyme inclusion levels and assessing ingredient or processing factors that influence protein solubilization.

Nevertheless, the results should be interpreted within the limitations of *in vitro* systems. Protein solubility represents an indirect measure of protein utilization and does not fully reflect *in vivo* digestibility or animal performance. Soybean meal contains antinutritional factors that may affect nutrient utilization under practical feeding conditions and these complex physiological interactions are not fully captured by laboratory assays^{1,9}. Therefore, the assay should be considered a preliminary screening tool rather than a direct predictor of nutritional responses. While the magnitude of solubility changes cannot be directly compared with values reported in studies using different analytical methods, the overall response pattern observed in the present work is consistent with the literature describing increased soybean protein solubilization following enzymatic hydrolysis^{5,7}.

Unlike conventional protein digestibility methods, the present assay estimates protease-mediated protein solubilization by quantifying residual protein after controlled enzymatic hydrolysis and recovery of the insoluble fraction. *In vitro* pepsin digestibility assays are widely used for rapid evaluation of protein quality and feed ingredients; however, they do not fully reproduce the complexity of *in vivo* digestive processes and ingredient interactions^{10,11}. Overall, the developed *in vitro* assay successfully discriminated among enzymatic treatments based on their isolated effects on soybean meal protein solubility, demonstrating its suitability as a tool for protease screening.

CONCLUSION

This study describes a practical and reproducible *in vitro* assay capable of detecting changes in soybean meal protein solubility following enzymatic treatment. The method successfully discriminated among enzymatic treatments and demonstrated good experimental precision, supporting its application as a screening tool for evaluating protease functionality and potential enzyme interactions in feed ingredients.

The proposed approach offers a precise, efficient and relevant tool for evaluating factors affecting protein solubility in soybean meal. By leveraging such methodologies, researchers and feed manufacturers can better understand enzyme-substrate interactions, optimize enzyme application and support precise diet formulation to develop higher-quality and more digestible feed products.

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