The fermentation of soybean meal by rumen microbes in vitro reveals different kinetic features for the inactivation and the degradation of trypsin inhibitor protein

Ellen M. Hoffmann*, Stephan Muetzel, Klaus Becker

Department of Aquaculture Systems and Animal Nutrition, Institute for Animal Production in the Tropics and Subtropics (480), University of Hohenheim, Fruwirthstr. 12, D-70593 Stuttgart, Germany

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Abstract

Soybean meal is a widely used protein supplement in animal feeds. When fed to monogastrics, thermal pretreatment (roasting) is needed to destroy antinutritional components. The major antinutritive compound of soybeans is trypsin inhibitor, a protein of 21 kDa molecular mass. Ruminants can tolerate this compound due to the microbial fermentation in the rumen rendering the inhibitor ineffective. This process was monitored under controlled conditions in an in vitro fermentation system. Raw soybean meal was added at different levels to a basal roughage and incubated with bovine rumen fluid. Samples were taken at regular time intervals up to 24 h of incubation. The samples were assayed enzymatically for trypsin inhibitor activity, and parallel to this the proteolytic degradation of trypsin inhibitor was documented qualitatively and quantitatively through polyacrylamide gel electrophoresis and densitometric methods. It was shown that trypsin inhibitor is both inactivated and degraded in the rumen, but with different kinetic characteristics. Its concentration in the soluble fraction showed a more pronounced initial increase than the apparent activity, and the time of complete degradation lagged 1–2 h behind the complete loss of activity. Protein concentration and activity were related to each other by calculating specific trypsin inhibitor activity. As this parameter showed a rapid, presumably exponential decay with the onset of fermentation, inactivation must be due to a mechanism preceding and not directly related to proteolysis.

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* Corresponding author. Tel.: +49-711-459-3176; fax: +49-711-459-3702.
E-mail address: ehoffman@uni-hohenheim.de (E.M. Hoffmann).

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1. Introduction

Trypsin inhibitor is the major antinutritional component in many legumes, e.g. soybean. It is a small protein (21 kDa) which inhibits the digestive enzyme trypsin with very high specificity and thereby impairs digestive functions in the lower gut. It is sometimes referred to as the “Kunitz” type of inhibitor to distinguish it from other similar, but less specific activities (Richardson, 1980; Susmel et al., 1995). Heat treatments denaturing the inhibitor protein are used to make soybeans suitable to feeding monogastric animals. Ruminants, however, tolerate the intake of, e.g. raw soybean meal, which thus must be mediated by the microbial fermentation preceding the digestion in the lower tract.

Only few studies have been undertaken so far on the fate of trypsin inhibitor in the rumen, all of which focused on activity assays, but nevertheless talked about its ruminal degradation (Dixon and Hosking, 1992; Simovska et al., 1991; Susmel et al., 1995). Inactivation, however, should be strictly defined as the loss of biological activity, and may thus occur while the protein is still present. Degradation, on the other hand, describes merely the physical disappearance of the protein due to proteolytic breakdown, without implications for the biological activity of the remaining fraction. Both processes may be expected to overlap during microbial fermentation. The activity assay used in the other studies was therefore complemented by methods of protein analysis to quantify the trypsin inhibitor concentration in the samples. The experiments were carried out in an in vitro fermentation system to allow regular sampling and quantitative recovery of products. Using this approach we individually characterized the kinetics of inactivation and degradation of trypsin inhibitor, and then related the two parameters to each other.

2. Materials and methods

2.1. In vitro fermentation

A fistulated Hinterwaelder cow, kept on a hay diet and fed twice a day, served as donor animal. A short time batch incubation system was inoculated with rumen fluid taken prior to morning feeding. The liquid was withdrawn manually by squeezing material removed from the feed mat, and was filtered through a 100 μm nylon net. The filtrate was mixed with two volumes of carbonate-buffered incubation medium according to Menke et al. (1979). The basal roughage substrate was young maize stover, supplemented with three levels of raw soybean flour (type I, Sigma S-9633), i.e. 2, 4, and 10 mg/ml in a total substrate ratio of 20 mg/ml. Incubations were run up to 24 h. Following a preliminary test with 2 mg soybean/ml only, two independent experiments were carried out with all three inclusion levels. At 1 h intervals small aliquots were withdrawn from the same fermentation vessel. The samples were centrifuged (10 min, 10,000 × g, 4°C) to remove microorganisms and residual feed particles. The supernatants were analyzed as described in Section 2.2.

2.2. Trypsin inhibitor assay

The method of Kakade et al. (1969), in a downscaled modification, was used to determine trypsin inhibitor activity. The enzyme (trypsin T-4665), substrate (N-benzoyl-arginine-p-
nitroanilide, BAPNA, B-4875), and purified inhibitor (T-9003) were obtained from Sigma. According to the supplier 1 unit (u) of trypsin activity is defined to produce an absorbance change of 0.001 per min at pH 7.6 at 25°C using N-benzoyl-l-arginine ethyl ester as substrate. Briefly, triplicates of 200 μl sample taken from the in vitro fermentation were combined with 200 μl assay buffer (50 mM Tris–HCl, 20 mM CaCl₂, pH 8.2), and 1 ml of substrate solution (40 mg BAPNA/ml dissolved in dimethyl sulfoxide, freshly diluted 1/100 in assay buffer). Then 40 μl of trypsin solution (0.2 mg/ml in 0.01 M HCl) containing 66 units of enzyme activity were added to two of the three parallels, and the vials were incubated (20 min, 37°C). A control in which the sample was replaced by assay buffer was run with each set of samples. The reaction was stopped by addition of 200 μl acetic acid (30%, v/v), the vials were centrifuged (10 min, 10,000 × g, 4°C) and the supernatant transferred to a cuvette. Absorbance was read at 410 nm. The blanks that did not receive trypsin solution were used to correct for intrinsic color of the samples. The net absorbance was related to the uninhibited control and expressed as relative inhibition in percentage, or as total activity in trypsin units inhibited per ml sample.

2.3. Protein analysis

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970). Samples (100 μl) taken from the in vitro fermentation were diluted with the same volume of denaturing buffer (final concentration: 62.5 mM Tris–HCl pH 6.8, 2% SDS, 5% mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and boiled for 2 min. These extracts were separated on a polyacrylamide gel (15% acrylamide/bis-solution 37.5:1 (Biorad)), run for 10 min at 60 V, 10 min at 80 V, and 20 min at 100 V. Gels were fixed for 1 h in sulfosalicylic acid (10%), and stained with Coomassie Brilliant Blue according to Neuhoff et al. (1985).

Stained gels were recorded with a digital camera system (DIANA 1.6, Raytest GmbH) and analyzed using the AIDA 2.31 software (Raytest GmbH). To calibrate the quantification by image analysis, standards of purified trypsin inhibitor protein (Sigma T-9003) were applied to the same gel.

3. Results

3.1. Trypsin inhibitor activity

3.1.1. Variability between assays and incubations

There was little variation in the inhibitor activity determined for the same sample in independent enzyme assays, as shown in Fig. 1a for the 2 mg/ml inclusion level. Between different incubations variation was much higher, such that complete inactivation for the lowest soybean inclusion was achieved between 4 and 6 h. The highest standard deviation, due to this time shift in individual kinetics, occurred at 4 h. However, as shown in Fig. 1b, even at the points of maximum standard deviation the profiles obtained for the different inclusion levels were clearly separated, i.e. the dosage effect exceeded by far the variation between independent incubations.
3.1.2. Total inhibitor activity

It is also evident from Fig. 1b that the samples achieving 100% relative inhibition were limited by the assay conditions. Therefore, these samples were diluted to resolve their total activity. Under the same assay conditions as before, serial dilutions up to a factor of 1/200, covered the full range of 0–100% inhibition of the trypsin activity in the assay. Three examples from an incubation with 10 mg soybean/ml are shown in Fig. 2. A logarithmic regression was fit over the central part of the dilution curves to determine the sample concentration at 50% inhibition. The total inhibitory activity of the undiluted sample was estimated from this value (Fig. 3).

Total trypsin inhibitor activity decreased rapidly and steadily with fermentation time, until a background level was reached after 5, 7, and 11 h with 2, 4, and 10 mg soybean/ml, respectively. This background must be due to unspecific trypsin inhibition, as it was also observed in rumen fluid samples without any addition of trypsin inhibitor. As there was no purification of the samples apart from the removal of particles by centrifugation, the
Fig. 2. Serial dilutions of samples with inhibitory capacities exceeding the assay limits (e.g. 0, 4 and 6 h of an incubation with 10 mg soybean/ml). They were used to determine the dilution factor at 50% relative inhibition.

rumen liquid may contain phenolics (Makkar et al., 1997), or other soluble compounds of animal, bacterial or plant origin, which negatively affect the trypsin reaction in the assay. The background level remained almost constant over the analyzed incubation period. During the first hour of incubation, a slight increase in the total trypsin inhibitor activity was observed for the higher inclusion levels. This can be explained by the concentration of trypsin inhibitor protein in the samples as described in Section 3.1.3.

3.1.3. Trypsin inhibitor concentration

At the starting point of incubation the samples showed the typical soybean protein pattern. The supernatants analyzed here did not contain microbial proteins at a detectable level. Trypsin inhibitor was the major protein band in the pattern, and was easily identified by coelectrophoresis with the corresponding molecular weight standard. The gel shown in Fig. 4 was loaded with samples from an incubation with 4 mg soybean/ml. While most
of the larger soybean proteins were rapidly degraded, the intensity of the trypsin inhibitor band seemed to increase in the initial period. Once it reached a maximum after ca. 3 h, it decreased steadily and was no longer detectable after 7 h.

Standards of purified trypsin inhibitor were applied to the same gel to calibrate quantification by image analysis. This allows precise quantification of the trypsin inhibitor concentration in the range between 0.1 and 1.2 mg/ml. Quantification verified that the kinetics described earlier with maxima after a few hours of incubation also applied to the other inclusion levels (Fig. 5). The initial increase in protein concentration is due to slow solubility of the trypsin inhibitor in rumen liquid, since a trypsin inhibitor band was detected in the pellets until the maximum was reached. Once all of the protein had dissolved, its

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**Fig. 4.** Protein electrophoresis of the samples taken from an in vitro fermentation containing 4 mg/ml soybean meal. MW: molecular weight standards; TI: trypsin inhibitor; the sample lanes (0–8 h) are labeled with the respective incubation time. The TI standards contained 0.1, 0.3, 0.6 and 1.2 mg protein/ml.

**Fig. 5.** Trypsin inhibitor concentration during in vitro fermentation with rumen fluid at three different soybean inclusion levels.
Fig. 6. Specific trypsin inhibitor activity during in vitro fermentation with rumen fluid at two different soybean inclusion levels (with preliminary exponential regression curves).

concentration declined steadily. The time of complete degradation always lagged 1–2 h behind the complete loss of activity.

3.1.4. Specific activity

From the inhibitory activity and the concentration of trypsin inhibitor protein in a given sample the specific activity, i.e. trypsin units inhibited per μg trypsin inhibitor protein, was calculated. Specific activity decayed rapidly immediately after the onset of fermentation. In Fig. 6 only the results for 4 and 10 mg soybean/ml are shown, because trypsin inhibitor concentrations <0.1 mg/ml were not included in the calculations, and therefore the lowest inclusion level did not yield sufficient data points. In all our experiments these data could be approximated by exponential regression curves with correlations higher than 0.9. However, the exponential decay of specific activity at this stage is just a hypothesis that needs further statistical validation.

4. Discussion

Although our activity assay was based on the same method as previous works, it differed from those in several respects especially in the preparation of samples. Simovska et al. (1991) incubated soybean meal in sacco, which resulted in loss of soluble protein due to dilution in the rumen, as suggested by Susmel et al. (1995). The latter authors therefore used a closed in vitro system, as we did in the present work. However, Susmel et al. (1995) incubated pure soybean meal and harvested the complete fermentation residue. After drying it, they extracted trypsin inhibitor with NaOH, and thus strictly followed the published procedure for the analysis of foodstuffs (Smith et al., 1980). We tried to maintain conditions that are closer to the in vivo situation, by using soybean meal on a basal roughage diet, and analyzed the soluble protein fraction only. This procedure is justified by the concept that only in the dissolved state can a protein actually exert its biological function. The solvent, i.e. incubation buffer versus NaOH, did not affect the trypsin reaction in the assay, but the solubility of trypsin inhibitor in the incubation medium influenced the results as described. Trypsin inhibitor is well soluble at higher pH and ionic strength than encountered under physiological
rumen conditions (Smith et al., 1980). The extraction with NaOH as suggested by Smith et al. (1980) takes further advantage of the loosening of cell wall structures under alkaline conditions to facilitate the release of trypsin inhibitor from ground tissue fragments. In our situation this has to be achieved by the fermentation of cell walls, which takes considerably more time. Well in line with these considerations are the kinetics observed for purified trypsin inhibitor incubated in vitro, which does not require liberation from a substrate. Here, the maximum concentration was measured at 0 h, followed by a steady decline (data not shown).

In the experiments presented here we did not check the pellets for residual trypsin inhibitor activity, and it may therefore be argued that any of this protein which reaches the lower gut in the unsoluble state may be liberated there and perhaps inhibit trypsin activity. However, since an insoluble fraction of trypsin inhibitor persisted for a maximum of ca. 3 h (at the highest, already unphysiological inclusion level), the magnitude of this effect should be neglectable. Still, this question will be addressed in further work.

The variability between the trypsin inhibitor activity profiles observed in independent experiments was expected due to well-known variations in the microbial activity of the inocula. An external standard to correct for this (as used, e.g. when measuring gas production) was not available for this test. Even without normalization the dosage effects resulting from the different soybean inclusion levels by far exceeded the variation between incubations. Anyhow, to avoid a blurring of effects due to averaging between incubations, the experiments were evaluated individually in the further analyses.

The effects “inactivation” and “degradation” were separated for the first time by our approach using a differentiated analytical methodology. This made it possible to determine the specific activity of trypsin inhibitor. For the specific activity of the purified trypsin inhibitor used as control in protein analysis the manufacturer reported that 1 mg inhibits 20,000 units of trypsin activity; this converts to an inhibitory capacity of 20 u/μg and compares well with the maximum value of 8 u/μg determined in our samples at 0 h. If physical degradation was the only process mediating the observed loss of total activity during incubation, specific activity should remain constant as long as any protein is detectable. This was clearly not the case. On the contrary, a rapid, exponential decay of specific activity was observed during fermentation. The hypothesis that the decay might follow exponential kinetics, should be reevaluated with more suitable sets of data, i.e. sufficiently high inclusion rates, more replicates and more frequent sampling in the early fermentation period. The data presented here are sufficient to conclude that inactivation must be due to a different, much faster biochemical mechanism than proteolysis.

Nevertheless, trypsin inhibitor is degraded as well, though much more slowly than most of the other soybean proteins. This point by itself has some implications for the nutritional utilization of trypsin inhibitor. If it bypasses rumen fermentation and reaches the lower gut in an inactivated form, it should be degraded as any other protein and contribute to the supply of valuable sulfo-amino acids.

5. Conclusion

Our work shows for the first time that trypsin inhibitor is functionally inactivated as well as physically degraded during ruminal fermentation. Inactivation proceeds much faster than
degradation. If any of the trypsin inhibitor protein bypasses fermentation it is most probably already inactivated when it reaches the lower gut. The results support the view that heat treatment is not mandatory when feeding legume grains to ruminants.

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