Chapter 5
Screening for Anti-proteolytic Compounds

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Introduction

Dietary protein entering the rumen is broken down in an apparently uncontrolled way, resulting in ammonia formation and subsequent loss of N in the urine. The low efficiency of nitrogen retention represents a major economic loss, causes metabolic stress in the animal, and places a burden on the environment, by way of nitrogen-rich wastes. If a means of slowing the breakdown process at any of the individual steps can be identified, these problems would be decreased.

Many different microbial species, employing a range of proteolytic enzymes, carry out the initial step of protein breakdown. The variety of proteolytic microbes present has made rational manipulation of the initial proteolytic step impossible, and solutions have generally required treatment of the protein before feeding, by heating for example. Tannins have been explored as a means of decreasing protein breakdown, but they frequently impair other aspects of rumen fermentation, including fibre breakdown. New plant materials that bind to proteins and prevent their digestion, or preferably which inhibit the proteinases directly, yet are not otherwise detrimental, would be of enormous benefit to ruminant livestock production globally.

The subsequent processes of peptide and amino acid breakdown are carried out by more defined populations. The only methods available for altering these activities are dietary addition of antibiotics and ionophores, which suppress the growth of the bacteria responsible. Finding substances, which decrease ammonia formation, could lead to more acceptable ways of inhibiting the processes leading to ammonia formation in the rumen.

In this chapter, we describe methods that can be used to measure different aspects of the processes involved in degradation of protein by ruminal microorganisms in vitro. The methods described include the use of diazotized or radio-labelled protein

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substrates to assess the first step in proteolysis, a method for screening for anti-proteolytic plant compounds that involves a short-term incubation with a complex, protein rich substrate, and an inhibitor in vitro assay of the rate and extent of ruminal protein degradation.

Screening for Antiproteolytic Plant Compounds Using Diazotized or Radio-Labelled Proteins

Diazotized Proteins

Diazotized or radio-labelled protein substrates can be used to assess the first step in proteolysis, i.e. conversion of polypeptide to smaller, acid-soluble peptides. A longer incubation with other protein substrates is described which, unlike the aforementioned methods, incorporates to a degree any adaptation of the microbial community to additives; both the initial proteolysis and the overall end products are measured, by polyacrylamide gel electrophoresis and ammonia/branched chain fatty acids, respectively.

Preparation of Azoproteins

This method works well for soluble proteins, but less well for insoluble proteins, particularly heterogeneous protein supplements because the surface tends to become labelled more than internal regions of particles (see [32]). The method is also less suitable for grazing animals due to background colour derived from the forage. The preparation of azoproteins is based on the paper of Tomarelli et al. [28]. Alternatively, and recommended, azocasein and azoalbumin can be purchased from Sigma (A2765 and A2382, respectively).

To prepare the azoproteins, dissolve 10 g of substrate protein (casein is usually used, but other proteins such as bovine serum albumin can be used as well) in 100 mL of 4% $NaHCO_3$ in distilled water. Prepare diazotized sulfanilic acid by dissolving 0.26 g of sulfanilic acid in 20 mL of 0.25 M NaOH. Stir on ice and gradually (over 5 min) add 0.35 g sodium nitrite. Then add 2 mL of 5 N HCl, stir for 2 min, and add 2 mL of 5 M NaOH. Within 5 s of adding the NaOH, mix the diazotized sulfanilic acid solution to the substrate protein solution and stir on ice for 1 h. Add to dialysis tubing (e.g. Visking). Dialyze against distilled water for 24 h at 4°C, changing water four times. Freeze and freeze-dry resulting dialyzed solution.
Caution: *The diazotizing solution should not touch the skin – wear gloves! The HCl and NaOH solutions are hazardous.*

Measurement of Proteolysis

Make up 2-mg/mL azocasein in 0.1 M potassium phosphate buffer, pH 7.5. Take sample of rumen liquor and use fresh (This method is not suitable for frozen

samples!). Strain the rumen liquor through 4 layers of muslin cloth. Set up four numbered plastic 10-mL polypropylene centrifuge tubes for each sample that is to be analysed, containing:

2 mL of azocasein solution [1]
2 mL of azocasein solution + 1 mL 25% trichloroacetic acid [2]
2 mL 0.1 M potassium phosphate buffer pH 7.5 [3]
2 mL 0.1 M potassium phosphate buffer pH 7.5 + 1 mL 25% trichloroacetic acid [4]
Equilibrate in water bath adjusted at 39°C

At $t = 0$, add 2 mL of strained rumen digesta to all tubes. Continue to incubate at 39°C. After 1 h[1] at 39°C, add 1 mL 25% trichloroacetic acid to [1] and [3].

Transfer the tubes to ice water and then centrifuge at 5000 g for 30 min. [Or transfer the tubes to a cold room (4°C) for about 48 h and then centrifuge.]

Carefully[2] remove 2.0 mL of supernatant into another tube containing 2.0 mL of 0.5 M NaOH. Measure A_{440} of this solution.

Calculation of Proteolytic Activity

Make up the following tubes for calibration with 0.2-mg/mL azocasein (a 10-fold dilution of the stock solution used as substrate (Table 5.1)):

Table 5.1

0.2 mg/mL azocasein (mL)	Buffer (mL)	0.5 M NaOH (mL)
0	2.0	2.0
0.1	1.9	2.0
0.2	1.8	2.0
0.3	1.7	2.0
0.4	1.6	2.0
0.5	1.5	2.0

Measure A_{440}. Draw a standard curve of A_{440} vs. concentration of azocasein (i.e. 0–0.025 mg/mL). From the best fitting straight line (i.e. linear regression), calculate the extinction coefficient (E) in units of A_{440} of a 1-mg/mL solution of azocasein.

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[1] Exactly. Time may vary depending on the activity of ruminal liquor. Calculate how much of the azocasein has been digested – if it is more than half, the assay should be repeated for a shorter time. Or, if there is insufficient colour generated, extend the incubation time. Azoalbumin is hydrolysed more slowly.

[2] This is the step where the greatest error can occur. Some of the part-digested azocasein floats on the meniscus in some samples. The pipette should be submerged gently through this layer before drawing the 2-mL volume. When withdrawn, some of the part-digested azocasein often clings to the pipette tip. Take care not to touch the recipient tube with the tip in order to minimize contamination with this material.

Note that different batches of azocasein have different E values

The proteolytic activity of ruminal digesta is then calculated from the absorbance read in the four numbered tubes (see *Measurement of proteolysis*) as follows:

Absorbance of digested azocasein at time t = $[A_1 - A_2 - A_3 + A_4]$
Concentration of azocasein digested (mg/mL NaOH solution) = $[A_1 - A_2 - A_3 + A_4]/E$
Concentration of azocasein digested (mg/mL TCA extract) = $2 \times [A_1 - A_2 - A_3 + A_4]/E$
Dilution of ruminal digesta was 2 mL to a final volume of 5 mL in TCA extract, so:
Concentration of azocasein digested (mg/mL strained rumen digesta) = $2.5 \times 2 \times [A_1 - A_2 - A_3 + A_4]/E$
Therefore, proteolytic activity (mg azocasein hydrolysed/h per mL of ruminal digesta) = $(5 \times [A_1 - A_2 - A_3 + A_4])/(E \times t)$

Radio-Labelled Proteins

The use of radiolabelled proteins overcomes many of the limitations of the azocasein assay, in that there is minimal interference from chromogenic compounds in plant materials, including forages.

Note: *All procedures should be carried out using gloves and protective clothing and in a designated laboratory. Strict rules for the use and disposal of radioactive material should be observed. The half-life of ^{14}C is 5730 years – any spillage or inappropriate disposal will leave a long legacy of hazard.*

Preparation of ^{14}C-Formaldehyde-Labelled Proteins

The method is based on the reductive methylation of protein using formaldehyde and sodium borohydride. The result is a tiny structural modification of the protein that has no effect on its susceptibility to proteolytic digestion. Either ^{14}C-formaldehyde or ^{3}H-sodium borohydride can be used. The latter is much cheaper, but its use is much less widely reported.

For the application of the method to measuring the proteolytic activity of ruminal digesta, see [31].

Note: *This method works well for soluble proteins, but less well for insoluble proteins, particularly heterogeneous protein supplements because the surface tends to become labelled more than internal regions of particles.*

Dissolve 0.1 g of casein sodium (Sigma) in 10 mL of 0.2 M sodium borate buffer pH 9.0. Make up fresh 0.5 mg/mL $NaBH_4$ and a solution of 0.01% (0.1 mg/mL) formaldehyde. The stock solution is 37% (w/v) and has a specific gravity of 1.09 g/mL, so use 0.25 mL/L of water. Chill the casein solution on ice.

Add 10 μL of stock 1 μCi/μL ^{14}C-formaldehyde to 50 μL of the 0.01% formaldehyde (add more radioactivity if required; this method should give about 40,000 dpm per mg casein). On ice, add 0.15 mL of $NaBH_4$ solution to the casein solution, mix, and after a few seconds add the diluted ^{14}C-formaldehyde. Incubate on ice for 30 min, then dialyse overnight at 4°C and freeze dry. Redissolve the freeze-dried material in 10 mL of water and count 50 μl in duplicate and calculate the specific radioactivity (D_S) in dpm/mg. This solution can then be used as the basis of the substrate solution in proteinase assays. You should aim to dilute the radioactive casein with unlabelled casein to give a count of about 40,000 dpm per mL in the solution added to the assay mixture.

Measurement of Proteolysis

This protocol is small-scale in order to minimise the use of radioactivity. On this scale, it may not be suitable for the testing of small quantities of plant samples in powder form. However, it will be excellent for the testing of liquid extracts. For testing solids, it may be necessary to scale-up ten-fold.

Make up a stock 2-mg/mL ^{14}C-labelled casein (approx. 80,000 dpm/mL, but a higher specific activity can be used) in 0.1 M potassium phosphate buffer pH 7.5. Dilute the radio-labelled casein solution with 2 mg/mL unlabelled casein in 0.1 M potassium phosphate buffer pH 7.5. Remove sample of rumen liquor and use fresh. This method is not suitable for frozen samples. Strain through 4 layers of muslin cloth.

Set up two micro-centrifuge tubes for each sample that is to be analysed, containing:-

100 μL of ^{14}C-labelled casein [1]
100 μL of ^{14}C-labelled casein + 50 μL 25% trichloroacetic acid [2]
Equilibrate in water bath at 39°C

At t = 0, add 100 μL of strained rumen digesta to all tubes. Continue to incubate at 39°C. After 1 h[3] at 39°C, add 50 μL 25% trichloroacetic acid to [1]. Transfer the tubes to ice water then centrifuge at 12,000 g for 10 min [Or transfer the tubes to a cold room (4°C) for about 48 h and then centrifuge].

Carefully[4] remove 100 μL of supernatant into a scintillation vial. Add scintillation fluid and measure ^{14}C. In addition, count 2 × 50 μl of the ^{14}C-labelled casein solution added to tubes 1 and 2. Calculate mean as D_S dpm/50 μL = 20 × D_S/mL = 20/2 × D_S dpm/mg casein = 10 × D_S dpm/mg casein.

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[3] Exactly. Time may vary depending on the activity of ruminal liquor. Calculate how much of the casein has been digested – if it is more than half, the assay should be repeated for a shorter time.

[4] This is the step where the greatest error can occur. Some of the part-digested casein floats on the meniscus in some samples. The pipette should be submerged gently through this layer before drawing the 100 μl volume. When withdrawn, some of the part-digested casein often clings to the pipette tip. Take care not to touch the recipient vial with the tip in order to minimize contamination with this material.

Calculation of Proteolytic Activity

The proteolytic activity of ruminal digesta is then calculated as follows (subscripts to D refer to the respective tube numbers, see *Measurement of proteolysis*):

Dpm of digested casein at time t	$= [D_1 - D_2]$ dpm/100 μL supernatant
	$= 10 \times [D_1 - D_2]$ dpm/mL supernatant
	$= 2.5 \times 10 \times [D_1 - D_2]$ dpm/mL rumen digesta
	$= 25 \times [D_1 - D_2]$ dpm/mL rumen digesta
But specific radioactivity of casein	$= 10 \times D_S$ dpm/mg casein
So, concentration of casein digested	$= 25 \times [D_1 - D_2]/(10 \times D_S)$ mg/mL reaction mixture
Therefore, proteolytic activity of ruminal digesta.	$= (2.5 \times [D_1 - D_2])/(DS)$ mg casein hydrolysed/h per mL

Screening for Antiproteolytic Plant Compounds by Short-Term Incubation with a Complex, Protein Rich Substrate

This screening method is based on short-term batch incubation as described by Mauricio et al. [20]. It is thus small enough to work with small amounts of plant samples (ca. 1.5 g DM) and quick enough to process sets of 15–20 samples per experiment. The plant material to be tested is added to a standardized, protein rich substrate. Over a period of 10–12 h with repeated sampling the disappearance of soluble substrate protein as well as the release of branched SCFA and ammonium are monitored as proteolysis-specific parameters. Total SCFA release gives supplementary information on general fermentation. If undisturbed parallels are included and incubation time is extended, gas production and 24 h digestibility can be optionally determined alongside. As shown in Selje-Assmann et al. [26, 27] this experimental approach is able to detect immediate effects, such as the precipitation of dietary protein by tannins, as well as slower effects mediated by modification of the microbial activity. When monensin, a well-established inhibitor of ruminal proteolysis, was added to this system, the effects described in the literature could be reproduced. On one hand, this validated the experimental approach. On the other hand, monensin could be introduced as an external standard to correct for the variability in biochemical responses associated with variations in the rumen fluid inoculum.

Incubation

Substrates

The substrate was composed to resemble a concentrate rich ruminant diet. Maize silage served as roughage component, barley grain as energy supplement, and a combination of soybean meal (Sigma-S9633) and BSA (bovine serum albumin)

Table 5.2 Substrate composition for batch incubations

Component (mg/bottle)	Negative control	Positive control	Treatment
Maize silage	450	450	300
Barley grain	225	225	225
Soybean meal	150	150	150
BSA	10	10	10
Monensin[a]	–	11.5 μl (ad 3 μM)	–
Test plant in optional parallels	–	–	150
PEG	450	45	450

[a]A stock solution (14 mg/mL) in ethanol is prepared freshly prior to each experiment and is added immediately after filling in the buffered rumen fluid.

(Sigma-A9647) as protein supplement. The proportions are given in Table 5.2. With our materials, the crude nutrient composition of the control substrate was: CA 3.9%, CP 16.9%, EE 1.9%, NDF 31.3%, ADF 23.1% and ADL 3.5% DM.

If entire plant material (i.e. dried, ground (green) biomass) was to be tested, it was added to replace an equivalent amount of maize silage, as this resulted in the lowest changes of crude nutrient composition. Routinely, 150 mg of test material were added; series of 30–200 mg of test material were successfully assayed for dosage effects. If purified extracts are to be evaluated, addition of the extract without reducing the maize silage may be a more suitable strategy [15]. This should be decided by the lowest interference with crude nutrient composition.

A negative control shows the proteolytic activity of a given inoculum under standard conditions. A positive control shows the inhibitory effect of monensin, relative to the negative control. Treatments can be evaluated relative to the negative control, or relative to the effect achieved by monensin.

The contribution of tannins to any observed effects on proteolysis can be evaluated if the experimental design is amended by additional parallels of all controls and treatments including polyethylene glycol (PEG) [26].

All substrate components (except monensin, which is added as stock solution immediately after inoculation) are weighed directly into 100 mL serum bottles, which are then pre-warmed prior to the addition of buffered rumen fluid.

Donor Animals and Preparation of Inoculum

For the screening system described here, the donor animals of rumen fluid (fistulated cattle or sheep) should be adapted to a concentrate-rich diet. If hay-fed animals are used as donors, the kinetics of fermentation are slower and total incubation time and sampling points need to be adapted. Rumen fluid is collected prior to morning feeding by manually squeezing liquid from the feed mat into pre-warmed thermos flasks. Again, if rumen fluid is withdrawn from the liquid phase by pump, kinetics are likely to be slower due to lower microbial density.

Experimental Design

Only incubations run with different inocula can be considered true replicates. This can be achieved by using different donor animals within the same experiment, or by subsequent experiments using the same donor animal. The lowest variation should be expected when the rumen fluid of two or three donors is mixed to provide a single inoculum, and experiments are repeated on different days.

In any case, positive and negative controls as well as blanks (bottles without any substrate) need to be included for every inoculum. Every treatment is incubated in 3 parallels, one of which is kept closed for exact gas readings, while the other two are designated for repeated sampling. Incubations run for 12 h, with samples being taken repeatedly from the same bottle after 1, 6, 8, and 10 h.

Thus, the total number of bottles to prepare is $b = (9 + 3 \times T) \times I$, where I = number of inocula, T = number of treatments respective additives to be tested, and the 9 resulting from triplicates of blank, positive and negative control. Substrates should be weighed into the bottles a day ahead. Collection of rumen fluid, inoculation, incubation (12 h) and processing of samples will add up to a total duration of ca. 16 h for the actual experiment.

Incubation Medium

The incubation medium (Table 5.3) can be considered as "artificial saliva", providing the buffer capacity and minerals to maintain favourable conditions for microbial fermentation. It is prepared freshly before each incubation and is pre-warmed and reduced before rumen fluid is added. Reduction is achieved by bubbling the solution with CO_2-gas for several hours, and by addition of the reducing solution (Table 5.4) shortly before the incubation. Reduction is indicated by the dye Reazurin, which changes from blue (over pink) to colourless.

Table 5.3 Composition of incubation buffer

Components	MW	Final conc.	Amount
Ammonium bicarbonate	60.1	13.5 mM	4.06 g
Sodium bicarbonate	84.0	86.5 mM	36.33 g
Di-Sodium hydrogen phosphate	142.0	5.5 mM	3.91 g
Potassium di-hydrogen phosphate	136.1	9.5 mM	6.46 g
Magnesium sulphate ($\times$ 7 H_2O)	246.5	0.5 mM	0.62 g
Micro-minerals (stock solution[b])		0.020%	1000 µL
Resazurine (1%)		0.001%	500 µL
dH_2O			4190 mL
Reducing solution		6%	310 mL
Total volume			4500 mL
Rumen fluid to be added		10%	500 mL
Total volume[a]			5000 mL

[a]The final volume of 5 L allows for the incubation of ca. 15 treatments plus blanks and controls in triplicates. It can be adjusted to the experimental design.
[b]The composition of the micro-minerals stock solution is given in Table 5.4.

Table 5.4 Reducing solution

Components	MW	Final conc.	Amount
Cysteine HCl	52.9	0.118 M	1.938 g
NaOH (1 M)	40.0	0.040%	12.40 mL
Na_2S	240.2	0.026 N	1.938 g
dH_2O to make total volume			310 mL

Table 5.5 Micro-minerals stock solution

Components	MW	Final conc.	Amount
Calcium chloride $2 \times H_2O$	147.0	0.45 M	3300 mg
Manganese chloride $4 \times H_2O$	197.9	0.25 M	2500 mg
Cobalt chloride $6 \times H_2O$	237.9	0.02 M	250 mg
Ferric trichloride $6 \times H_2O$	270.3	0.15 M	2000 mg
dH_2O to make total volume			50 mL

Note: *For reasons of time management, the buffer, without reducing solution and rumen fluid, can be prepared a day ahead and gassed for 2–3 h with CO_2. It can then be pre-warmed over night. In the next morning, prior to the collection of rumen fluid, the reducing solution is prepared and added, and gassing continues while collecting the rumen fluid.*

Inoculation

When the buffer is completely reduced, one volume of filtered (100-μm) rumen fluid is added to 9 volumes of buffer, to make up the "buffered rumen fluid". Three 1 mL samples of this are taken for subsequent analysis (see "Sampling section below"). Aliquots of 75 mL are then dispensed in the pre-warmed serum bottles containing the substrate. Routinely, all treatments are run in triplicate; three bottles without any substrate serve as blanks (to monitor gas production arising just from the inoculum rather than the incubated substrate). Monensin solution (Table 5.6) is pipetted in the designated controls. The headspace is flushed with CO_2 gas; the bottles are closed with rubber stoppers and incubated at 39°C.

Gas Reading

As fermentation sets on, gas is released into the headspace of the bottle and builds up a pressure. A syringe needle with an attached pressure transducer is inserted through the rubber stopper at regular intervals to measure the current pressure. Once

Table 5.6 Monensin stock solution

Components	Amount
Monensin	3.5 mg
dH_2O	250 μL

the value has been recorded (along with the time of measurement), the transducer is detached and the pressure is released through the open syringe needle. Finally, the needle is removed and pressure can build up again until the next measurement point. Routinely, pressure is measured after 1, 2, 3, 4.5, 6, 8, 10 and 12 h (where applicable: prior to sampling). Further measurements are necessary if incubation time is extended to 24 h. A calibration series is used to convert pressure data to gas volume.

In order to obtain the calibration curve shown in Fig. 5.1, serum bottles were filled with 75 mL water, warmed to 39°C (i.e. the incubation temperature) and closed with rubber stoppers. Defined gas volumes of 5, 10, 15, 20 ... to 40 mL were injected into four parallel bottles each with calibrated, gas-tight syringes. The pressure was then measured as described above. This calibration should be done with the specific equipment available in the lab when introducing the methodology. The same calibration curve can then be used in subsequent experiments.

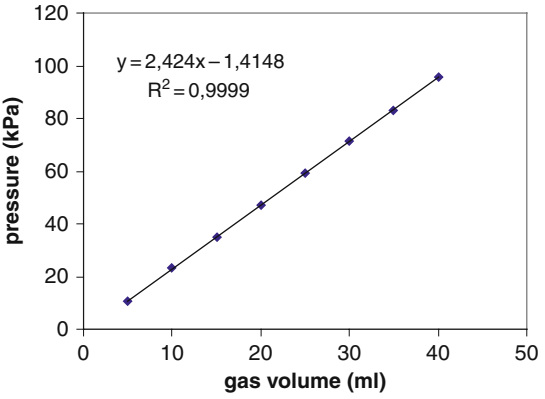

Fig. 5.1 Example of a calibration curve to convert pressure to gas volume

Calculation of Gas Production Rate and Cumulative Gas Production

All pressure readings are converted to mL gas by the calibration curve; the average gas volume produced in the blanks is calculated at each time point, and all measurements are corrected by their corresponding blank.

The exact incubation time since the previous gas reading is determined for each bottle (from time data recorded along with the pressure measurement), and net gas production is related to the hours of incubation to express data as gas production rate (mL/h)

Net gas production is added up over the entire incubation period, and the sum is related to the amount of substrate incubated in the respective bottle and expressed as cumulative gas production (mL/g).

If bottles with and without PEG are incubated, gas production of the sample + PEG is set to 100%, and inhibition in the sample without PEG is expressed relative to this.

Note: *Exact gas measurements can only be taken from undisturbed bottles, which have never been opened for sampling. Gas readings from sampling bottles can only serve as a rough indicator for the speed of fermentation.*

Sampling

After 1, 6, 8, 10 and 12 h samples are withdrawn repeatedly from the designated bottles. After recording and release of gas pressure, the stoppers are removed, and aliquots of 1 mL volume are pipetted into prepared sampling tubes (e.g. 1.5 mL Eppendorf cups) kept on ice to stop the fermentation process. To ensure the withdrawal of homogeneous samples, a stirbar is inserted into the bottle, and contents are vigorously stirred while pipetting; wide bored tips have to be used to avoid plugging by feed particles. The stir bar remains inside the bottle for subsequent samplings. The headspace is flushed again with CO_2 gas, bottles are closed again with the same stopper, and incubation continues.

An overview of the sample processing is given in Fig. 5.2. The samples are centrifuged (10 min, 10,000 g, 4°C) and supernatant and pellet are carefully separated. An aliquot of 50 µl of the supernatant is transferred to a fresh vial and frozen at –20°C for determination of soluble protein under native conditions.

Another 50 µl of the supernatant are mixed with 50 µl of double strength Laemmli-buffer (2 × LBF, Table 5.17) and boiled for 3 min to generate denatured samples of soluble protein. Pellets are re-suspended in 1 mL of Laemmli-buffer (1 × LBF), boiled for 5 min to generate denatured samples of insoluble protein. Both of the denatured samples are backups for protein determination by dot blot (see "Quantitative protein analysis by dot blot assay") and, if desired, subsequent qualitative analysis by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (see "Qualitative protein analysis by SDS-PAGE" below).

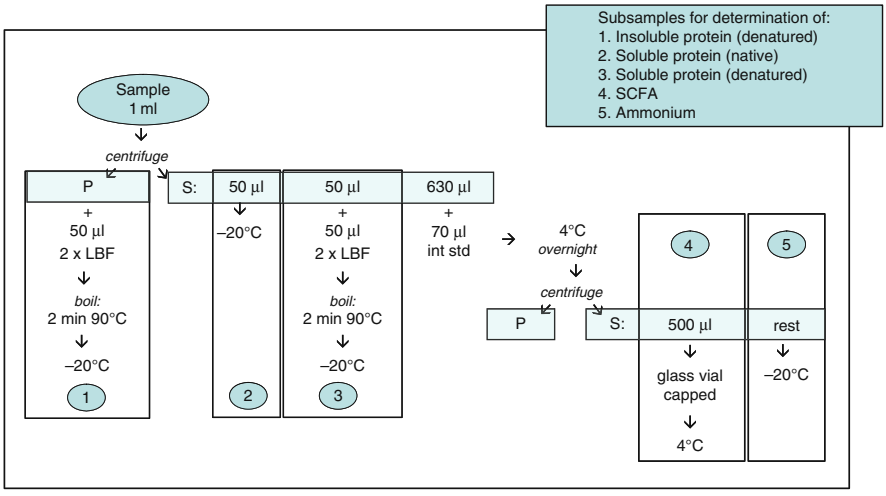

Fig. 5.2 Flow diagram of sample processing

Another aliquot of 630 μl of the supernatant is transferred into a fresh vial and 70 μl of internal standard (int std, Table 5.8) are added. These samples are kept at 4°C over night to precipitate soluble proteins. They are centrifuged again (10 min, 10,000 g, 4°C) to remove the precipitate. 500 μl of the acidified, de-proteinized supernatant are transferred into glass vials, sealed with serum caps, for SCFA analysis. The remaining supernatant is transferred into a fresh vial, frozen at –20°C, and kept for determination of ammonium.

Note: *Due to the complex sampling scheme and the large number of samples to process all cups and vials should be prepared and labelled ahead of the experiment.*

The amount needed is:
sampling bottles $= b_s = (4 + 2 \times t) \times I$
glass vials $= b_s \times t \times a$
sampling cups $= b_s \times t \times 5 \times a$
(where: t = no. of sampling times, a = no. of aliquots, I = no. of inocula)

One set of cups is needed for collecting the original 1 mL aliquots; after centrifugation, the pellet is left in these cups and re-suspended in 1 × LBF. Three sets of cups are needed for sharing the first supernatant as indicated; one of these sets should be prefilled with 2 × LBF. Another set of cups is needed to collect the supernatant after the second centrifugation.

Optional: 24 h Digestibility

If applicable, digestibility is determined in undisturbed incubation bottles set aside for gas reading only. Incubation time is extended to 24 h, which usually requires 1 or 2 additional gas readings to avoid high pressure in the gas space. At the end, all bottles are transferred to an ice bath to stop fermentation, and the contents are emptied quantitatively into pre-weighed nylon bags (50 μm pore size, e.g. from Bar Diamond Inc. Parma, ID, USA). The nylon bags are held by glass beakers to collect the filtrate, any particulate matter is held back in the bags. Analytical samples are withdrawn from the filtrate as described in previous section. After this, the bottles can be rinsed with distilled water and residues are combined in the respective bag. The bags are closed by folding the upper edge, excess liquid is gently squeezed from the bags, and they are hung up using a fold back clamp until drained completely. The bags are dried overnight at 100°C and weighed to determine apparent digestibility. Dry bags are heat-sealed and boiled for 1 h in NDS (Table 5.7), rinsed several times in distilled water and dried again to determine in vitro true digestibility. The mass difference of original residue and NDS-boiled residue can be taken as a rough estimate of microbial mass.

Analytical Procedures

SCFA Analysis by Gas Chromatography

SCFA are determined in an acidified, de-proteinized rumen fluid sample (see "sampling" section above) containing 10% (v/v) of internal standard. The sample is

Table 5.7 Neutral detergent solution (NDS)

Components	MW	Final conc.	Amount
EDTA	372.2	0.050 M	93.0 g
Sodiumtetraborate (10 H_2O)	381.4	0.018 M	34.0 g
SDS		3.0%	150.0 g
Monoglycolether		1.0%	50.0 mL
Sodium dihydrogenphosphate	142.0	0.032 M	22.8 g
dH_2O to make total volume			5000 mL

provided in a 1.5 mL glass vial, closed tightly by a serum cap. Samples are analysed in a gas chromatograph (e.g. GC 14A, Shimazu Corp., Kyoto, Japan) with a stainless steel column packed with GP 10% SP, 1000 1% H_3PO_4, Chromosorb W AW (Supelco Inc. Bellafonte, PA). The method was developed by [13].

To guarantee reliable measurements, internal as well as external standardization is used. Methylvaleric acid, which does not naturally occur in rumen liquid, is used as internal standard (Table 5.8). This serves as reference for the analytical device and needs to be present in every sample measured. Double distilled water containing 10% (v/v) internal standard is used to clear the column in regular intervals. For samples derived from the described incubation, two vials of water are inserted after every 15 vials of samples. An external standard (Table 5.9) is inserted once per run among the second half of the sample set.

The gas chromatography program automatically detects the individual SCFA-peaks and converts the peak area to concentration (μmol/mL). All readings are corrected for SCFA brought in with the inoculum. The net SCFA concentration can then be related to the amount of substrate present in each bottle (mol/g). The sum

Table 5.8 Internal standard for SCFA analysis

Components	Final conc.	Amount
Methylvaleric acid (100%)	1.0%	1.0 mL
Formic acid to make total volume		100 mL

Table 5.9 External standard (10x stock solution)

Components	MW	Final conc.	Amount
100% acetic acid	60.1	60.0 μmol/ml	3.03 g
100% propionic acid	74.1	30.0 μmol/ml	2.22 g
100% butyric acid	88.1	10.0 μmol/ml	0.81 g
100% valeric acid	102.1	1.0 μmol/ml	0.102 g
98% isobutryric acid	88.1	1.0 μmol/ml	0.090 g
100% isovaleric acid	102.1	1.0 μmol/ml	0.102 g
dH_2O to make total volume			100 mL

When diluting the stock solution (1/10) to the final concentration, 10% (v/v) internal standards are added.

of branched SCFA is considered a proteolysis-specific parameter and is expressed, either in absolute terms (μmol/g) or as proportion of total SCFA (%), relative to the negative and positive control.

Ammonium Determination by Phenol Hypochlorite Reaction

The assay is based on the method of Koroleff [17]. In alkaline solution, ammonium ions react with hypochlorite and form an intermediate product, monochloramine. If hypochlorite is in excess and nitroprusside is added as catalyst, this reacts further with phenol to form a dark blue dye, indophenol. The maximum absorbance of this dye occurs around 620–640 nm. Absorbance is proportional to the initial ammonium concentration.

This test has wide application in the analysis of water quality; other trivalent forms of nitrogen do not interfere with the assay. Turbidity or hydrogen sulphide, however, may disturb it. Therefore, when applied to rumen liquid, the sample has to be clarified by centrifugation and hydrogen sulphide has to be eliminated by acidification. Both are achieved in the sample preparation outlined above. The volumes given below were downscaled as compared to the original protocol, and the concentrations are adapted to measurements in 150 μl aliquots in a microplate reader.

A standard curve is prepared according to Table 5.10, using 0.5–5 mM $(NH_4)_2SO_4$ (i.e. 1–10 mM NH_4, respectively). Aliquots of 300 μl of phenol nitroprusside reagent (Table 5.11) are pipetted into a 1.5 mL vial, then 15 μl of standard or sample are added and mixed well. 15 μl of ddH_2O are added for a blank. All treatments should be prepared at least in duplicates. Finally, 300 μl of alkaline

Table 5.10 Pipetting scheme of calibration series for ammonium determination

$(NH_4)_2SO_4$ stock solution 10 mM (μl)	H_2O (μl)	Final conc. (mM)
100	900	1.0
200	800	2.0
400	600	4.0
600	400	6.0
800	200	8.0
1000	0	10.0

Table 5.11 Phenol nitroprusside

Components	Final conc.	Amount
Phenol*	10.0 mg/mL	2.5 g
Sodium nitroprusside	50.0 μg/mL	12.5 mg
dH_2O to make total volume		250 mL

The solution can be stored at 4°C for 1 month.
*Phenol is very hazardous compound. When handling it make sure to wear gloves and protective clothing and always work in a fume hood.

Table 5.12 Alkaline hypochlorite

Components	Final conc.	Amount
NaOH	10.0 mg/mL	2.5 g
Sodium hypochlorite	0.84% (v/v)	2.1 mL
dH_2O to make total volume		

The solution can be stored at 4°C for 1 month.

hypochlorite reagent (Table 5.12) are added and the mixture is incubated for 30 min at 37°C. After the colour has fully developed, absorbance is read at 625 nm.

Absorbance measured in unknown samples is converted to ammonium concentration by the respective calibration curve. In contrast to cumulative parameters such as gas or SCFA concentration, ammonium concentration reflects the current balance between release by fermentation and uptake by microbes.

Quantitative Protein Analysis by Dot Blot Assay

This assay is based on the method of Neuhoff [23], as modified by Hoffmann et al. [14]. It works in the presence of SDS, but due care has to be taken that the SDS concentration of the samples is the same as that in the standards used for calibration. Denaturation is recommended only if qualitative analysis of protein patterns by SDS-PAGE is to follow, or if the insoluble protein pellet is to be quantified.

The protein concentration in the native supernatant can be determined without further processing.

Dot Blot Procedure

A calibration series is prepared with BSA as shown in Table 5.13.

A cellulose acetate membrane (Sartorius 12200) is placed in a holder (Fig. 5.3), i.e. between two plates with an application grid of 84 holes (ca. 1 cm in diameter), and fixed by inserting two pins. The holder can be manufactured from any kind of

Table 5.13 Pipetting scheme of calibration series for protein determination

BSA stock solution 2 mg/mL (µl)	H_2O (µl)	(2×) buffer[a]	Final protein conc.
20	180	200	0.1
40	160	200	0.2
80	120	200	0.4
120	80	200	0.6
160	40	200	0.8
200	0	200	1.0

[a]The components of the RPT-buffer do not affect the staining intensity; therefore water can be used for native calibration standards; for denatured samples, 2× Laemmli-buffer has to be used, as SDS affects the staining with amido black.

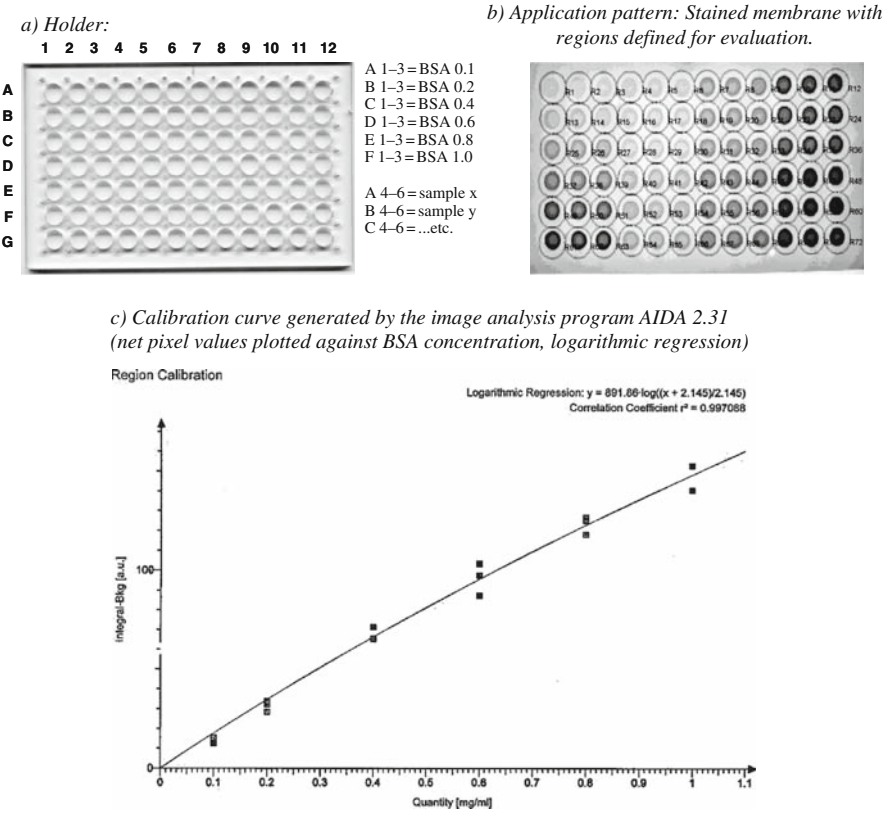

Fig. 5.3 Illustration of holder, application pattern and a stained membrane. (**a**) Holder; **(b)** Application pattern: Stained membrane with regions defined for evaluation; (**c**) Calibration curve generated by the image analysis program AIDA 2.31 (net pixel values plotted against BSA concentration, logarithmic regression)

inert plastic and guarantees, that within the cavities, the membrane does not touch the support and the samples can be quantitatively applied. An application pattern is designed to define each sample by its position on the grid. Triplicates of 2 μl of standard or sample are applied very slowly in the centre of the cavities. The protein concentration should be in the range of 0.1–1.0 mg/mL. Gilson/Eppendorf pipets or 2 μl glass capillaries can be used for sample application. When the sample is completely absorbed by the membrane, there should still be a small margin between the edge of the spot and the cavity wall. The membrane should dry completely at RT before removing it from the holder. If denatured samples are applied, a heat-fixation step is recommended; i.e. the membrane is baked for 3 min at 98°C in a dry heating block (or incubator).

The dry membrane is slowly immersed into the staining solution (Table 5.15) and stained for 3 min with gentle agitation. The staining tray should be closed with a lid

Table 5.14 Methanol-acetic acid

Components	Final conc. (%)	Amount (Ml)
Methanol	90	450
Acetic acid (100%)	10	50
Total volume		500

Stored at 4°C to minimize evaporation.

Table 5.15 Staining solution

Components	Final conc.	Amount
Amido black	0.5%	250 mg
Methanol-acetic acid		50 mL
Total volume		50 mL

Stored at 4°C to minimize evaporation.

Table 5.16 Butanol-methanol-acetic acid

Components	Final conc. (%)	Amount (mL)
Butanol	60	120
Methanol	30	60
Acetic acid (100%)	10	20
ddH_2O to make total volume		200

The solution can be used several times, as long as it stays clear. Stored at 4°C to minimize evaporation.

to avoid the evaporation of methanol. The membrane is then destained for 3 × 5 min and 1 × 15 min in methanol-acetic acid (Table 5.14). Again, the vessels should be closed and the membrane should never fall dry during transfers! If methanol is allowed to evaporate from the membrane, the acetic acid will concentrate and destroy the membrane. Finally, the membrane is equilibrated for 2 min in butanol-methanol-acetic acid (Table 5.16). As acetic acid evaporates faster than butanol, now there is no more danger of destroying the membrane.

A digital picture is taken of the wet membrane, avoiding any air bubbles between the membrane and the support. A video camera system or a flatbed scanner may be used for taking the picture. Any image analysis program able to count pixels values in defined areas (e.g. AIDA 2.31, Raytest GmbH, Straubenhardt, Germany) can be used to convert the staining intensity of the spots to numbers. The net pixel numbers per spot will then be converted to protein concentrations according to the calibration series blotted on the same membrane. Protein concentrations determined in the samples are then plotted against the incubation time to show the degradation kinetics. An exemplary result for positive and negative control is shown in Fig. 5.4. Linear regression has been used to calculate the degradation rates.

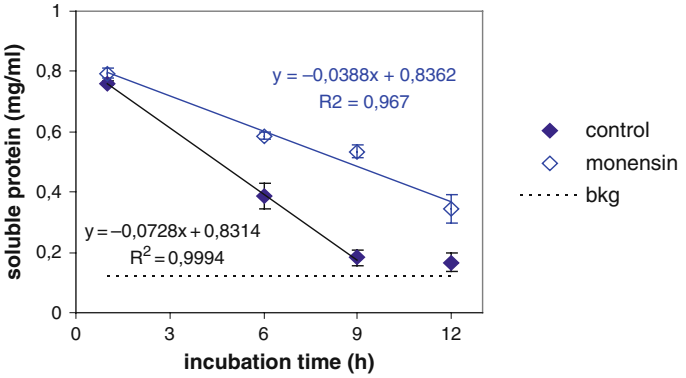

Fig. 5.4 Degradation kinetics of soluble protein in positive and negative controls

Evaluation of Results

The soluble protein concentration at 1 h is taken as reference point for each kinetic. It was shown that 1 h is the point of maximum concentration, due to the slow solubilization of soybean protein [26]. On the background of soybean meal and BSA the inclusion of 150 mg of normal, green plant samples did not significantly increase the measured concentration. (If an unknown sample should be high enough in soluble protein to show up as false positive for anti-proteolytic activity, it can be noted at this point.)

Measurements at later sampling times can be expressed relative to the corresponding 1 h value, or in absolute terms as μg protein degraded. In the latter case, degradation rates (μg/h) can be calculated by linear regression. Both, relative protein concentrations at a given hour, or protein degradation rates can then be evaluated relative to the negative and positive control.

Insoluble protein concentration is of particular interest at 1 h, as increased values will indicate precipitation of dietary proteins, and at the end of 24 h incubation as an estimate of microbial biomass. The sum of soluble and insoluble protein (corrected for respective dilution factors) gives an estimate of total true protein in the sample. However, if proteolysis is inhibited, protein measured at 24 h may still partially constitute undegraded dietary protein. In that case, PAGE analysis is needed to discriminate substrate from microbial protein.

In the negative control (without monensin) the substrate protein was degraded close to background level (bkg) after 9 h, therefore the 12 h value was excluded from regression; the corresponding degradation rate was 72.8 μg/(mL × h). In the presence of monensin, the rate was reduced to 38.8 μg/(mL × h) and degradation was not yet complete after 12 h.

Qualitative Protein Analysis by SDS-PAGE

SDS-PAGE is a technique that separates polypeptides by their molecular mass. The name refers to the detergent sodium dodecylsulphate (SDS), which is used to

Table 5.17 Laemmli buffer (LBF)

Components	Final conc.	Amount
Tris-HCl pH 6.8 (0.5 M)	62.5 mM	12.5 mL
SDS (20%)	2%	10.0 mL
Glycerol (87%)	10%	11.5 mL
2-mercaptoethanol	5%	5.0 mL
Bromophenol blue (0.5%)	0.0025%	0.5 mL
ddH_2O		60.5 mL
To make total volume		100.0 mL

For double strength buffer add only 10.5 mL H_2O *ad* total volume 50 mL. Stored at room temperature.

denature the proteins prior to separation. This also confers a strong negative charge on the proteins, irrespective of their native charge due to amino acid composition. All proteins will thus move towards the anode (+) in an electric field.

The matrix used for electrophoresis is a polyacrylamide gel (PAGE). Small molecules can move faster through this matrix than large ones, thus resulting in separation by molecular mass. A discontinuity in gel concentration and buffer system between the upper "stacking gel" and the actual "separation gel" improves the sharpness of the protein bands and thus the resolution. This system was originally established by Laemmli [18]. It is nowadays a standard method in protein analysis.

Preparation of the Polyacrylamide Gels

Standard protein electrophoresis equipment comprises a setup for casting the gels, a setup for running the gels, and a power supply. Refer to the instructions of the respective manufacturer, how to assemble and use them. The procedure below refers to the Minigel-System of Hoefer (USA) with gel dimensions of 10.1 cm × 8.3 cm × 0.75 mm.

Glass plates and spacers are assembled to form the gel chambers and are tightened by foldback clamps. The lower edge of the chambers is sealed, either by a rubber gasket in the casting stand, by a 1% agarose seal or by an acrylamide plug (see below).

The acrylamide solution for the separating gel (T, Table 5.18) is prepared on ice; immediately before casting the starter compounds (TEMED and ammonium persulphate) are added, carefully mixed, and ca. 4.5 mL of gel solution are pipetted into each chamber to fill it to ca. 2 cm below the upper edge. The gel solution is over layed with water, to get a smooth edge and to exclude oxygen, which would prevent polymerization of the gel in the upper layer. The gels are left undisturbed at room temperature until polymerization is complete; after 1–2 h a sharp interface becomes visible which indicates that the gel has polymerized.

The water layer is removed carefully with a drawn out pipette tip (e.g. gel loader tips), and the surface is rinsed once or twice with water until any unpolymerized residues of acrylamide are removed. Meanwhile the acrylamide solution for the stacking gel (S, Table 5.19) has been prepared on ice; when the separating gels

Table 5.18 PAGE separation gel solution (T)

Components	Final conc.	Amount
1.5 M Tris-HCl pH 8,8[a]	375 Mm	1.50 mL
30% Acrylamide/Bis Solution (37,5:1)	15.0%	3.00 mL
ddH_2O		
10% SDS	0.100%	60.00 µl
TEMED	0.012%	7.20 µl
10% Ammonium persulphate	0.035%	21.00 µl
Total volume (for 1 gel)		6.0 mL

Multiply by the number of gels you intend to cast!
[a]The composition of the buffer stock solution is given in Table 5.21.

Table 5.19 PAGE stacking gel solution (S)

Components	Final conc.	Amount
0.5 M Tris-HCl pH 6,8[a]	125 mM	0.75 mL
30% Acrylamide/Bis Solution (19:1)	3%	0.30 mL
ddH_2O		1.91 mL
10% SDS	0.1%	30.00 µl
TEMED	0.012%	3.60 µl
10% Ammonium persulphate	0.035%	10.50 µl
Total volume (for 1 gel)		3.0 mL

Multiply by the number of gels you intend to cast!
[a]The composition of the buffer stock solution is given in Table 5.20.

are ready, the starter compounds are added. Teflon combs are inserted in the upper space of the gel chamber, leaving sufficient space (ca. 1 cm) between the bottom of the wells and the edge of the separation gel. Then the stacking gel solution is pipetted into the chamber, avoiding any air bubbles, and is left to polymerize. The chamber is filled to the upper edge, and if necessary, more solution can be added carefully during polymerization. After another 1–2 h the gels are ready. The combs are carefully removed and the wells are rinsed once or twice with water. Finally, they are filled with electrophoresis buffer (EP, Table 5.20). Immediate use of the gels is recommended.

Table 5.20 Electrophoresis buffer stock solution (2 × EP)

Components	MW	Final conc.	Amount
Tris	121.1	100 mM	12.1 g
Glycine	75.1	760 mM	57.1 g
10% SDS		0.2%	2 mL
dH_2O *ad* total volume			1000 mL
pH ca. 8.5 *(do not titrate!)*			

Dilute 1 + 1 with dH_2O to get the working solution.

Table 5.21 Stacking gel buffer stock solution (Tris/HCl pH 6.8)

Components	MW	Final conc.	Amount
Tris	121.1	0.50 M	6.06 g
ddH_2O			50 mL
Titrate with HCl to pH 6.8			
ddH_2O to make total volume			100 mL

Store at 4°C.

Gels can be stored overnight at 4°C in a moist chamber; the well should then be filled with 0.125 M Tris-HCl pH 6.8. Before loading, they need to be washed again and filled with electrophoresis buffer.

Note: *If an acrylamide-plug is to be used for sealing the gel chambers, 0.5 mL per gel are taken from the solution and mixed with 10 µl TEMED and 15 µl 10% ammonium persulphate. This solution is carefully and slowly dispensed along the bottom of the gel unit; capillary force should draw the solution into the chamber and form a seal of 1–2 mm width. Because of the high starter concentration, the plug polymerizes very quickly (5 min) and gels can be cast as described above.*

Loading and Running the Gels

An application scheme is prepared for each gel, to define which sample, marker or standard is loaded in which lane. The gel chambers are inserted in the electrophoresis apparatus, and the buffer tanks are filled with electrophoresis buffer. To facilitate sample application the upper can be filled just below the level of the gel edge. With drawn-out tips 6 µl of denatured sample (see "sampling" section above) are carefully pipetted to the bottom of the designated wells, avoiding disturbance by air bubbles. When all lanes are loaded, the buffer tank is filled up above the gel level. The electrodes are connected to the power supply, and the gels are run for 10 min at 80 V and 40 min at 120 V. When the front (visible as a blue line) reaches the bottom of the gel, the run is finished. The apparatus is disassembled; the gels are removed from the chambers and transferred into a fixation bath.

Table 5.22 Separation gel buffer stock solution (Tris/HCl pH 8.8)

Components	MW	Final conc.	Amount
Tris	121.1	1.50 M	18.17 g
ddH_2O			50 mL
Titrate with HCl to pH 8.8			
ddH_2O to make total volume			100 mL

Fixation and Staining

The gels are fixed in 10% sulfosalicylic acid (Table 5.23.) for 30 min with gentle agitation. After that, they are transferred to the staining solution and stained overnight in closed containers with gentle agitation.

Table 5.23 Fixation solution for PAGE gels

Components	Final conc.	Amount
Sulfosalicylic acid	10%	20 g
H_2O		200 mL

Store at RT; repeated use is possible.

Note: *The conventional agent for fixing PAGE gels is trichlor acetic acid (TCA). The use of sulfosalicylic acid instead avoids toxic wastes (halogenated organic solvents).*

The staining solution given in Table 5.24 follows the procedure of Neuhoff et al. [22]. In contrast to common alcoholic staining solutions with CBB-R (Coomassie Brilliant Blue), this provides CBB-G in a colloidal form and thus allows clear background staining at gel concentrations above 9% acrylamide. Furthermore, sensitivity is higher than with CBB-R. The stained gels are briefly rinsed with water to remove dye particles settled on the gel surface (5–15 min, several changes of water). They can then be recorded with a digital camera or a scanner. An exemplary result is shown in Fig. 5.5.

The PAGE-gel in Fig. 5.5 is showing the degradation of individual protein bands during a 24 h incubation of control substrate without and with 3-μM monensin. Lanes are labelled according to the hour of sampling. M = molecular weight marker, high range. Band A (BSA) disappeared after 12 h in the control, but was persistent even after 24 h in the presence of monensin. Band B (an unidentified soybean protein) disappeared after 6 h in the control, and after 9 h with monensin. Band

Table 5.24 CBB-staining solution

Components	Final conc.	Amount
85% Phosphoric acid dissolved in 300 mL dH_2O	2%	10 g
CBB 250 g dissolved in 20 mL dH_2O	0.1%	500 mg
Ammonium sulphate dissolved in 80 mL dH_2O	6%	30 g
– First add CBB to phosphoric acid and stir		
– While stirring, add ammonium sulphate, bit by bit		
– Keep stirring		
Finally add dH_2O to make total volume		500 mL

Store at RT; repeated uses is possible, but avoid acidification due to carry-over of fixation solution, as this will affect the sensitivity of staining. If correctly prepared, the dye is dispensed in a colloidal form, and small particles settle on the bottom of the flask; therefore shake well before use!

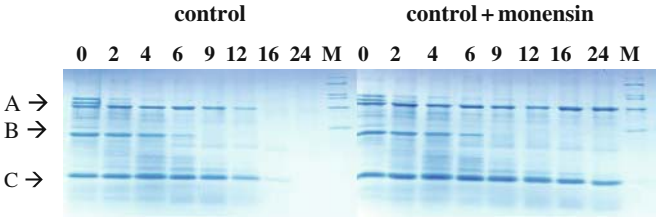

Fig. 5.5 Example of SDS-PAGE analysis of protein degradation in the screening system

C (trypsin inhibitor) disappeared after 16 h in the control, and was still present after 24 h with monensin. The delay of protein degradation due to monensin is thus clearly documented with the described experimental approach.

Calibration and Evaluation

Individual protein bands on a PAGE gel are usually identified/addressed by their molecular mass. Therefore, molecular weight standards should be run on each gel. They are available from various sources; the example shown above (Fig. 5.5) used high range markers from Biorad (161-0303, SDS-PAGE-Standards, high). Broad range markers also cover the lower range down to 6.5 kDa (BioRad #161-0317 SDS-PAGE-Standards, broad). If provided in lyophilized form, marker proteins are reconstituted in water, denatured by adding the same volume of 2 × LBF, and finally diluted to yield suitable band intensity.

Individual protein bands can be quantified, if suitable equipment for densitometry or image analysis is available. In our experiments, we used the same software as in the evaluation of dotblot membranes to quantify selected bands (AIDA 2.31, Raytest GmbH, Straubenhardt, Germany). For calibration BSA standards were prepared at 0.05, 0.1 and 0.2 mg/mL in LBF, and 6 μl of these were loaded on the same gel as the samples to be quantified. The intensities of the target bands could thus be converted to protein concentrations. If the major protein bands of a given lane are quantified like this and added up, the sum correlates quite well with the total protein content determined by dotblot.

Conclusion

Based on an incubation of 12 h, the described screening system allows the detection of slow effects in ruminal protein metabolism, that require microbial growth, in addition to immediate ones mediated e.g. by the precipitation of dietary protein. Yet, it is still short enough to be considered a high throughput system; 15–20 samples per incubation can be handled conveniently. The amount of plant material required is small, with ca. 1.5 g DM for three independent incubations of triplicates. The composition of the standard substrate is optimized not only to sustain high proteolytic activity, but also to reveal the response in proteolysis by straightforward and reliable measurements in the parameters investigated, avoiding extensive dilution

or purification of samples. Nevertheless, analyses can proceed to various degrees of detail. For a rough screening, it may be sufficient to determine total protein by dotblot, SCFA, and ammonium, which can be accomplished in ca. 3 days. To acquire further information on the nature of anti-proteolytic effects, the experimental design can be amended by incubations with and without PEG, to discriminate tannin related activities. PAGE analysis of supernatant and pellet, especially of the 1 h samples, can be an alternative to, or a further validation of this. While the overall influence of a plant additive on general fermentation can already be seen from gas and total SCFA-production, this aspect can be deepened if incubations are extended to 24 h to determine digestibility or microbial protein synthesis by the respective methodologies of nutrition research.

Inhibitor In Vitro Assay of Rate and Extent of Ruminal Protein Degradation

Rationale and Overview

Rates and extents of ruminal degradation of feed proteins are required in a number of systems of ruminant ration formulation. Lack of reliable data on protein degradation can cause farmers to under- or over-feed protein to their livestock. To avoid problems due to either under or over-feeding of protein, routine methods that are both accurate and rapid are needed to allow timely characterization of protein degradation of common feeds. We have devoted a number of years to developing an inhibitor in vitro (IIV) method for assessing protein degradation [2; 3; 4; 5; 7]. With this approach, substrate limiting amounts of protein (i.e., under first-order conditions) are incubated with ruminal inocula to which metabolic inhibitors have been added to allow quantitative recovery of protein breakdown products. Degradation rate (kd) is derived from the time-course of net (i.e., blank-corrected) appearance of degraded protein in the form of total free amino acids plus ammonia. Extent of degradation is computed using this rate and an assumed ruminal passage rate (kp), typically 0.06/h, from the ratio: kd/(kd + kp) [30]. This IIV procedure successfully predicted differences in milk and protein yield of dairy cows fed solvent and expeller soybean meal [8], characterized the ruminal degradability of different species of legume forages [4] and several protein concentrates [11], identified the optimal extent of heating required for protecting protein in roasted soybeans [12], and served as the basis of a solubility test [16] and a near infrared spectrometric calibration [29] to estimate protein degradability in roasted soybeans. Other workers have employed the IIV inoculum, but quantified extent of degradation from net release to total N remaining soluble in the presence of protein precipitants [24, 25].

The following protocol describes the method as used in our laboratory for routine estimation of protein degradation rate and escape, including a shorthand method version that may be useful when assaying large numbers of samples.

Incubation

The basis of the IIV technique is that inhibitors of microbial amino acid and ammonia incorporation, hydrazine sulphate (HS) and chloramphenicol (CAP), when added to in vitro inocula containing mixed ruminal organisms, allow all or virtually all of the protein degradation products to be recovered as ammonia and amino acids. In short-term incubations (less than 6-h long), extent of protein degradation is not underestimated due to microbial uptake of the degradation products. In incubations longer than 6-h, proteolytic activity begins to decline, possibly due to autolysis of microbial enzymes and build-up of end products.

Feed samples are analyzed for dry matter (DM) and total N. Enough sample to provide 1.875 mg N is weighed into each incubation tube (50-mL plastic centrifuge tubes). Each sample is "soaked" in 5 mL McDougall's [21] buffer at 39°C for 1 h prior to starting the incubation. The inoculum is prepared from strained ruminal fluid (SRF) obtained by straining solids from rumen cannulated donor cows through two then four layers of cheesecloth. Pre-incubation of the SRF with soluble carbohydrates is done at 39°C for 3–4 h to reduce background concentrations of ammonia and total amino acids (TAA). The inoculum is a mixture of SRF and McDougall's [21] buffer containing 1.5 mM HS and 45 μg/mL of CAP. Tubes are inoculated with 10 mL of the inoculum (final concentrations = 1.0 mM HS and 30 μg/mL of CAP). Tubes are flushed with CO_2, capped with Bunsen valves (see supplies below) and incubated at 39°C for various time-points up to 6 h. Water baths and incubator ovens and rooms have all been used successfully for this purpose. In the shorthand version of the assay, time-points of only 0- and 4-h are used when large sample numbers are to be studied in the same incubation. This method is described below. Microbial activity is stopped by adding trichloroacetic acid (TCA) to a final concentration of 5% (w/v).

The fraction degraded, and remaining undegraded, at each time-point is computed from net (i.e., blank corrected) release of N in the form of ammonia and total free amino acids (TAA), which are assayed using automated equipment. Formally, TAA were determined by ninhydrin assay, including a correction for ammonia contribution to total colour that was adapted to a continuous-flow system [5]. However, this method has been replaced by a fluorimetric procedure based on orthophthaldialdehyde (OPA) adapted to flow-injection [6]. A variation on the original colorimetric ammonia assay [5] is still used but it is also conducted by flow-injection [6]. The net N released as TAA is computed from the ratio TAA/mg N determined for each protein source being studied after hydrolysis in 6 N HCl. This value is added to N released as ammonia and the fraction degraded is computed based on the amount of protein-N added to each tube (usually 1.875 mg). Fractions degraded and undegraded may be corrected for acid detergent insoluble nitrogen (ADIN) but this has been found to have little effect on rate except for samples that have been extensively heated. Rate of degradation is estimated as the slope of the linear regression of the log of the fraction remaining undegraded on time. Equations used for these computations are described below in detail. Casein and two standard soybean meal samples of known in vivo ruminal degradability are included in each incubation to assess day-to-day variation in activity.

Materials

Chemicals

McDougall's buffer (4 L batch) [21]

- 39.2 g $NaHCO_3$
- 37.1 g $Na_2HPO_4.12H_2O$
  - 2.28 g KCl
  - 1.88 g NaCl

  Dissolve in 4 L of distilled water, and then add

- 0.52 g $MgCl_2.6H_2O$
- 0.182 g $CaCl_2$, anhydrous

Mix until dissolved and then bubble with CO_2 for 2 h (until pH reaches 6.8). Rebubble with CO_2 as necessary to bring pH back to 6.8.

- 3 N NaOH
- TCA solution – 65% wt/vol trichloroacetic acid in distilled water.
- Antifoam 204 (Sigma A-6426)
- Maltose (Sigma M-2250)
- Starch (Sigma S-2004)
- Xylose (Sigma X-1500)
- Pectin (Sigma P-9135)
- Mercaptoethanol (Sigma M-6250) (noxious odour – keep and use in hood)
- Hydrazine sulphate (Sigma H-7394) (toxic)
- Chloramphenicol (Sigma C-0378) (toxic)

Standard Proteins

- Casein (Sigma C-5890)
- Solvent soybean meal (SSBM)
- Expeller soybean meal (SSBM; "SoyPlus")
- Ammonium sulphate (Fisher Scientific A938-500; "Primary Standard")
- Leucine (Sigma L-8000)
- 0.1 N HCl
- 6 N HCl

Supplies

- 1 L thermos
- Large diameter funnel
- Cheese cloth (#58706-4325: American Wipers and Supplies, Milwaukee, WI USA)

- Large graduated cylinders
- 2-l and 5-l Bottles
- 50-mL Polyethylene centrifuge tubes (Nalgene # DS3112-0050; Fisher Scientific, Itasca, IL USA)
- 4 50-mL tubes are dried at 60°C overnight and weighed (see below)
- 5-mL, 12×75 mm, Centrifuge tubes (# 55.5266: Sarstedt, Newton, North Carolina USA)
- Stoppers with Bunsen valve: # 5.5 one-hole rubber stopper; 3/16" outside dimension (O.D.) glass tube; rubber policeman (w/slit) (# 53801-0087: VWR, St. Paul Minnesota, USA).

Inoculum

Steps are conducted under CO_2 to "protect" microbes as much as practical from contact with air. Collect whole rumen contents (liquid plus solids) from 2 rumen cannulated lactating dairy cows just prior to the morning feeding. At the barn, squeeze enough whole rumen contents through 2-layers of cheesecloth into a thermos (warmed previously using 39°C tap water) to yield the required volume of SRF, collecting about half of the total SRF from each cow. Discard squeezed whole contents in the gutter. Back at the laboratory, strain the SRF through 4-layers of cheesecloth into an appropriately sized graduated cylinder that had been flushed with CO_2 and rinsed with warm water. Measure pH and transfer to the pre-incubation flask.

Pre-incubation

A 3 h pre-incubation is carried out in a water bath at 39°C to decrease the background ammonium concentration in the inoculum. In a few cases, ammonia has remained in excess of 2 mM after 3 h and incubating for 4 h was useful to reduce concentration to less than 1 mM. However, the 3 h pre-incubation usually reduces ammonia to < 0.5 mM. For each litre of final inoculum, add 800 mL of SRF, 2.5 g $NaHCO_3$ (dissolved in 50 mL McDougall's [21] buffer), and 0.16 mL antifoam 204 (see above).

Then add the following carbohydrates:

- 6.4 g Maltose
- 3.2 g Starch
- 3.2 g Xylose
- 3.2 g Pectin dissolved in 100 mL warm McDougall's [21] buffer overnight.

Flush flask continuously with CO_2 during pre-incubation. At 0 h and once each h for the total 3 h, take two 3-mL samples and transfer in 12 × 75 mm sampling tubes with 0.25 mL 65% (w/v) TCA solution; hold on ice for 30 min. Also, monitor pH (by inserting electrode into pre-incubating inoculum) and temperature: as necessary,

adjust inoculum pH back up to 6.4 by adding 3 N NaOH. Record volume of NaOH used (total used over 3-h is a crude index of fermentative activity).

After pre-incubation is complete, add (per 950 mL of pre-incubated inoculum), 0.39 mL mercaptoethanol; this should be done in the fume hood because of the noxious odour. Then add the inhibitors: 0.3252 g hydrazine sulphate (dissolved in 25 mL McDougall's [21] buffer) and 0.075 g chloramphenicol (dissolved in 25 mL water). This gives a total volume of 1 L. Mix inoculum with these reagents at 39°C for 20 min before starting the incubation.

Sample Preparation

Samples should be ground through a 1-mm screen. Concentration of N in samples must be determined. Duplicate tubes are used for each sample at each time-point. Empty tubes are used for blanks at each incubation time-point; additional blanks are used if sample set is large. Within each run, at least 3 standard proteins (casein, SSBM, ESBM) are incubated.

Label all incubation tubes and centrifuge tubes with indelible marker.
Weigh into each of 50 mL tube an amount of sample equivalent to 1.875 mg of N.
"Hydrate" these samples for 1 h prior to the incubation by adding 5 mL of warm McDougall's [21] buffer into each tube. Hold at 39°C in the incubator.
Add 1.25 mL 65% w/v TCA (before inoculum to prevent any degradation) to the 0-h-incubation tubes and place these in the ice bath.

Incubation

Add to each tube 10 mL of the inoculum using a Cornwall, re-pipette (or similar rapid dispenser). Start with timed incubations and end with 0 h incubations. Flush the tubes with CO_2 and close them with a stopper fixed with bunsen valve, and incubate for prescribed times with shaking at 150 rpm. We have also found that swirling tubes by hand every 60 min provides satisfactory agitation and mixing over the incubation. After completing inoculation of tubes to be incubated, add 10 mL of inoculum to each of 4 labelled pre-weighed, dry 50-mL centrifuge tubes for the determination of inoculum DM. When incubation time is complete, add 1.25 mL 65% w/v TCA to each tube to kill microbial activity. After each tube at each time-point has received TCA, hand swirl the tubes to mix and place in ice bath for 30 min.

Sampling

For ammonium and total amino acid analyses

- All tubes with TCA are kept on ice for at least 30 min.
- Mix contents of tubes treated with TCA either by hand swirling or using a Vortex mixer and pour an aliquot into a labelled 5-mL 12 × 75 mm centrifuge tube.

- Centrifuge at 10,000*g* for 10 min.
- Pour supernatants into a second set of labelled 5-mL 12 × 75 mm centrifuge tubes, cap them and label cups, and store at 4°C until analysis.

Analytical Procedures

Inoculum Dry Matter Determination

Centrifuge samples taken for inoculum DM determination at 30,000*g* for 15 min. Discard supernatant and dry the pellet at 60°C for at least 48 h. After 48 h, cool these tubes in a desiccator to room temperature for at least 2 h and weigh.

Determination of Ammonium and TAA

Samples from 0-h and other time-point incubations are analyzed for ammonium (based on phenol-hypochlorite reagent) and TAA (based on OPA-fluorimetry) using assays adapted to the flow-injection system [6]. Calibration/recovery is conducted by either standard solutions of ammonium sulphate and leucine in 0.1 N HCl, or using the method of standard additions (MOSA), adding ammonium sulphate and leucine directly to the inoculum matrix [9, 10]. Standards or MOSA standards are placed at the beginning and end of the sample series; additional sets of standards/MOSA standards are used if sample number is large. Standards with appropriate concentration ranges are prepared using ammonium sulphate and leucine in 0.1 N HCl.

Calculations

- Dry matter (DM) of the inoculum in g/l is calculated as:

$$[\text{Weight of tube plus dry pellet in mg} - \text{Weight of empty tube in mg}]/10$$

Most of the DM in the high-speed pellet from SRF is bacterial; variation in inoculum DM content from run to run accounts for some of the variation in degradative activity observed.

- Fraction degraded at each time-point (FDt) is computed from net (i.e., blank corrected) release of N in the form of NH_3 and TAA (in leucine equivalents) using the equation:

$$\text{Fraction degraded (FDt)} = [[(\mu\text{mol } NH_3 \times 0.014007) + (\mu\text{mol TAA}/(\mu\text{mol TAA/mg N})]/\text{mg N}]$$

where μmol TAA/mg N is the AA content (per unit total N) of each protein source determined (after acid-hydrolysis) by OPA-fluorimetry; and mg N is the amount of

protein-N added to each tube (usually 1.875 mg). The FDt may also be computed using the equation:

$$\text{Fraction degraded (FDt)} = [(\text{mg NH3} - \text{N}) + (\text{mg TAA} - \text{N}/0.7003)]/\text{mg N}$$

where "0.7003" is the average proportion of alpha-amino N in total N determined for a number of protein sources and "mg N added" is the amount of feed-N weighed into each tube (usually 1.875 mg N/15 mL incubation mixture). The ratio 0.7003 derives from a mean of 50-mmol total amino acids/mg total N found for a number of feed proteins after acid hydrolysis. This value can differ somewhat between feed proteins because of variation in AA composition. To determine the actual TAA/N ratio for each protein, duplicate protein samples are hydrolyzed for 24 h at 105°C in sealed vials under a N_2 atmosphere in 6 N HCl containing 0.1% wt/vol phenol [19] using a ratio of 1 mg sample N/5 mL of acid [1]. After hydrolysis, samples are cooled, HCl removed by vacuum evaporation, and the residues re-dissolved in 0.1 N HCl. These protein hydrolysates are then analyzed for TAA using the same OPA-fluorimetry assay (with leucine as standard) that is used for samples deriving from in vitro incubations. Response in μmol Leu equivalents/mg N for each protein is then used to compute the value for net TAA release into the amount of degraded protein N. The fraction undegraded is computed:

$$\text{Fraction Undegraded (FUDt)} = 1 - \text{FDt},$$

When individual time-points are used, the natural log of FUDt is regressed on time using linear regression function in Excel; the slope of this line is the fractional degradation rate and has the units "/h". When the shorthand version of the method is used (time-points only at 0- and 4-h only), degradation rate is computed:

- $\text{Degradation Rate (kd), / h} = [\ln(\text{FUD4}) - \ln(\text{FUD0})]/4$

where ln is the natural log and 4 is the incubation time in h. Degradation rate will be negative (reflecting the decreasing amount of intact protein). The potentially degradable fraction (fraction B) is computed using the equation:

$$\text{Fraction B, \%} = [1 - \text{FD0})] \times 100$$

Fraction escaping the rumen (the "bypass" value) may be estimated assuming passage rate (kp) = 0.06/h:

$$\text{Estimated Ruminal Protein Escape, \%} = \text{B} \times [\text{kd} / (\text{kd} + \text{kp})].$$

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