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Original article

Degradation in the rumen of proteins of 2 legumes: soybean meal and field pea

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Summary — Ruminal protein degradation of soybean meal and field pea were compared using 2 analytical methods: *in situ* and a laboratory analysis with a proteolytic enzyme extracted from *Streptomyces griseus*. The degradation of total nitrogen in nylon bags of the feeds was measured between 0 and 48 h in the rumen of 2 cows (theoretical degradability: 70.0% for soybean meal and 94.7% for field pea). Electrophoresis of proteins of feeds and *in situ* residues, in sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) showed that both species have the same type of proteins (7S and 11S) which were degraded in a similar manner in the rumen. The 7S fraction was degraded rapidly whereas the 11S fraction was more resistant. The electrophoretic profiles showed that protein components were less degraded after enzymatic than *in situ* degradation. In the supernatant after enzymatic hydrolysis, protein and peptides were found in large concentrations and α-amino nitrogen and ammonia in low concentrations. It was difficult to simulate the *in vitro* activity of enzyme complexes in the rumen and the results must be confirmed by direct measurement of the rumen liquid.

electrophoresis / protein degradation / *in situ* method / enzymatic method

Résumé — Étude de la dégradation dans le rumen de 2 Légumineuses : tourteau de soja et pois. *Nous avons étudié la dégradation dans le rumen des protéines du tourteau de soja et du pois en utilisant 2 méthodes : la méthode* in situ *et une méthode de laboratoire avec une enzyme protéolytique, extraite de* Streptomyces griseus. *La dégradabilité en sachets de nylon de l'azote total a été mesurée entre 0 et 48 h (dégradabilité théorique de 70% pour le tourteau de soja et de 94,7% pour le pois), dans le rumen de 2 vaches. L'électrophorèse sur gel de polyacrylamide en milieu dénaturant (SDS-PAGE) a permis de séparer les protéines des aliments et des résidus de sachets. Les principales protéines des 2 aliments sont des protéines de type 7S et 11S, chacune se dégradant dans le rumen de façon analogue pour les 2 aliments. Les fractions 7S se dégradent rapidement tandis que les 11S sont plus résistantes à la dégradation. Les profils électrophorétiques des résidus enzymatiques indiquent que les protéines sont dégradées plus lentement* in vitro *que dans le rumen. Après hydrolyse enzymatique, on observe dans les surnageants des teneurs élevées en protéines et peptides et des teneurs faibles en acides α-aminés et en ammoniac. Il est difficile de simuler* in vitro *l'activité des enzymes du rumen et ces résultats restent à confirmer* in vivo, *dans le jus de rumen.*

électrophorèse / dégradation des protéines / méthode* in situ */ méthode enzymatique

INTRODUCTION

In ruminants, the quantity of amino acids that reaches the small intestine depends on microbial protein synthesis and feed proteins escaping ruminal degradation. The degradation of feeds proteins can be affected by the nature of proteins in the feed (Mahadevan *et al,* 1980; Wallace *et al,* 1987).

In France, the theoretical nitrogen degradability (DT), on the basis of the PDI (protein digestible in the intestine) system, is computed from dietary nitrogen degradation kinetics in nylon-bag incubation in the rumen (Vérité *et al,* 1987). It can also be predicted in the laboratory using a commercial enzyme (*Streptomyces griseus* extract) (Aufrère *et al,* 1991). However, the 2 global methods do not take into account the nature of dietary protein and do not explain discrepancy in feed proteins degradation in the rumen.

Ruminal degradation of protein of 2 legumes (soybean meal and field pea) were compared using the 2 methods. The quantity of non-degraded proteins was measured and characterised by electrophoresis.

MATERIALS AND METHODS

Feeds

This study was performed using a commercial soybean meal and a field pea. Samples were ground through a 0.8 mm screen. Total nitrogen (as a percentage of dry matter) of the feeds was determined by the Kjeldahl method (1985).

Enzymatic incubation

Samples were incubated with pronase (proteolytic enzyme extracted from *S griseus* type XIV Sigma No P 5147) in a borate phosphate buffer pH 8 for 1, 2, 4, 8 and 24 h at 40°C as described by Aufrère and Cartailler (1988). The degradability after 1 h is used in the French PDI system (Aufrère *et al,* 1991). Four replicates were used for each time point. Samples were then centrifuged at 2 100 *g* for 5 min and filtered through Durieux 2B filter paper. The filtrates of 2 replicates representing each incubation period were used to determine degraded nitrogen by the Kjeldahl method, while the filtrates of the 2 other replicates were treated with cold trichloroacetic acid 20% (w/v) to precipitate soluble true protein. They were then centrifuged at 20 000 *g* for 30 min. Soluble non-protein nitrogen in the supernatant was determined by the Kjeldahl method and true protein in the precipitate was calculated by difference. The results were expressed in % of total nitrogen of the feed (% Nt).

Part of the filtrate was used to measure ammonia after acidification with trichloracetic acid 20% (w/v) (Conway, 1957) and α-amino nitrogen according to the method described by Palmer and Peters (1969). The amount of peptides was calculated by subtracting the amount of total nitrogen in the filtrate from the amount of protein (expressed %N), N NH_3 and N α-amino nitrogen. Nitrogen solubility was measured in a phosphate buffer at pH 6.9 according to the method of Durand cited in Vérité and Demarquilly (1978).

In situ *degradation*

The *in situ* nitrogen degradability was measured using the nylon-bag procedure standardised by Michalet-Doreau *et al* (1987). Bags (pore size of 46 μm) were cut to an internal size of 6 x 11 cm, and closed with 2 stitches, and contained approximately 3 g of sample. They were incubated in the rumen of 2 cows fed at maintenance a diet containing 70% hay and 30% concentrate on a dry-matter basis. For each feed, 3 bags were removed from the rumen of cows (6 replications) after 2, 4, 8, 16 and 24 h, and kept at −15°C before analysis. Bags were rinsed with cold water until the rinse water was clear. They were then beaten for 7 min in a 'stomacher' (Merry and McAllan, 1983), prior to further washing and drying at 80°C for 48 h. The stomacher permitted separation of bacteria from the rumen content. Michalet-Doreau and Ould Bah (1989) showed that beating in a stomacher results in reduction of microbial contamination. Residual nitrogen was determined according to the Kjeldahl method. An

exponential model (Ørskov and McDonald, 1979) was fitted to the data:

$$\%N \text{ degraded} = a + b(1 - e^{-ct})$$

This model supposes the existence of a soluble fraction (a), a potentially degradable fraction (b) and a non-degradable fraction (100 – (a + b)); c represents the fractional rate of digestion of b.

Estimates of a, b and c were obtained by fitting the model, using a non-linear regression procedure based on Marquardt's method (Marquardt, 1963), using the NLIN procedure of the statistical analysis system (SAS Institute, 1985). In order to compare nitrogen degradabilities of feeds in the rumen, a fractional passage rate out of the rumen of 0.06 per h was assumed (Vérité *et al*, 1987). Ruminal degradability of nitrogen was calculated using the equation of Ørskov and McDonald (1979):

$$Deg = a + (bc)/(c + 0.06)$$

Protein separation procedures

The separation methods used are based on differences in protein solubility according to the pH of the extraction solution. For soybean meal, the procedure of Thanh and Shibasaki (1976) was used. The proteins were extracted with 0.03 M Tris-HCl buffer (pH 8) containing 10 mM of β-mercaptoethanol (meal/buffer 1/15 (w/v)). After centrifugation (12 000 *g*, 20 min), the supernatant pH was adjusted to 6.6 with 2 N HCl. The 11S globulins precipitated and were separated from the 7S globulins after dialysis against Tris-HCl buffer (63 mM pH 6.6) at 4°C for 3 h and centrifuged (12 000 *g*, 20 min). The supernatant pH was then adjusted to 4.8 which caused precipitation of the 7S globulins. These were separated by centrifugation (12 000 *g*, 20 min).

The method of Gueguen and Barbot (1988) was used for field peas. Each extraction (1 h) was performed at 20°C using 1 g of flour and 10 ml of buffer. Three successive extractions in phosphate buffer (pH 7, NaH_2PO_4, 0.1 M) + K_2SO_4 (5% w/v) for 1 h were carried out. The extracts were then centrifuged (4 500 *g*, 20 min). The albumins and globulins were separated by dialysis in distilled water for 96 h, followed by centrifugation at 12 000 *g* for 20 min. The globulin precipitate was redissolved in McIlvaine buffer at pH 7 (82.4 ml $Na_2HPO_4 \cdot 2H_2O$ 0.2 M + 17.6 ml 0.1 M citric acid containing 0.15 N NaCl). The pH was adjusted to 5.5, the vicilins precipitated and were separated by centrifugation at 27 000 *g* for 20 min. Legumins in the supernatant were precipitated at pH 4.8 and separated by centrifugation (27 000 *g* for 20 min).

Electrophoresis

The proteins of feeds, purified fractions and residues of enzymatic and *in situ* incubations were fractionated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) modified by Laemmli (1970). The residues of enzymatic and *in situ* incubation were treated with extraction solution (1 M Tris-HCl, pH 6.8, 15% (v/v) glycerol, 3% (w/v) SDS, pyronin, 7.5% (v/v) β-mercaptoethanol) in the proportion of 750 µl for 1 mg of nitrogen (based on the method of Kjeldahl). They were treated for 2 h, heated for 5 min at 100°C, centrifuged for 5 min at 600 *g*, and then the supernatant solution was fractionated by SDS-PAGE. The gels (1.5 x 60 x 180 mm) consisted of stacking gel (125 mM Tris-HCl, pH 6.8, plus 3.75% acrylamide – bisacrylamide (10%, 30:0.8, plus 12.5% 1 M Tris-HCl pH 6.8), 0.1% (w/v) SDS, 0.075% (w/v) ammonium persulfate, 0.1% (v/v) tetramethylethylenediamine) approximately 50 mm high, layered over a separating gel which contained 12.5 or 15% acrylamide–bisacrylamide (30:0.8) for soybean meal and field pea, respectively. The separating gel contained 33.3% acrylamide–bisacrylamide, 37.5% 1 M Tris-HCl pH 8.8 and 0.1% (w/v) SDS, polymerised with 0.075% (w/v) ammonium persulfate and 0.1% (w/v) tetramethyl–ethylenediamine, for soybean meal. For the field pea, the separating gel contained 40% acrylamide–bisacrylamide; other components were in the same proportion.

Electrophoresis was carried out for approximately 4 h at 200 V, after which the gels were stained with 0.2% Coomassie Brillant Blue 250 R in 10% (v/v) acetic acid and 25% (v/v) propanol for 30 min at 20°C to detect protein bands, and destained in 10% (v/v) acetic acid and 35% (v/v) ethanol.

The molecular weights of subunits were determined with a standard protein solution consisting of lactalbumin (Mr = 14 200), trypsin inhibitor (Mr = 20 100), trypsinogen (Mr = 24 000), carbonic anhydrase (Mr = 29 000), glyceraldehyde-3-phosphate dehydrogenase (Mr = 36 000) ovalbumin (Mr = 45 000), bovine serum albumin (Mr = 66 000), phosphorylase B (Mr = 97 400), β-galac-

tosidase *E coli* (Mr = 116 000), myosin rabbit muscle (Mr = 205 000).

RESULTS AND DISCUSSION

Protein separation

Separation of soybean meal proteins by denaturing electrophoresis indicated that globulins, the most important fraction in legumes, comprised of 7S (conglycinin) and 11S (glycinin). Field pea protein separation, in agreement with the observations of Rao *et al* (1987) and Spencer *et al* (1988), gave similar fractions, convicilin and vicilin, corresponding to the 7S fraction, and legumin corresponding to the 11S fraction (fig 1). Cheftel *et al* (1985), Gueguen and Azanza (1985) and Carter *et al* (1992) also showed the existence of analogies between the 2 legume species, which both belong to the papillionaceae subfamilly. Pion (1981) and Lallès (1993) obtained little difference in their amino-acid content. However, the legumin/vicilin ratio varied according to genotype (Rao and Pernollet, 1981).

Comparison of the in situ *degradation of the 2 feeds*

The 2 feeds showed both different degradabilities (Deg), 68.5% for soybean meal, 90% for field pea, and degradation profiles. The soluble fraction (a) of soybean meal was small (22.4% Nt) whereas that of field pea was large (73.0% Nt), which agrees with the enzymatic degradability results after 1 h (26.7% for soybean meal, 60% for field pea), with solubility in a pH 6.9 phosphate buffer (12.8% for soybean meal, 79.5% for field pea) and with results of Fan and Sosulski (1974). The low solubility measured for soybean meal was caused by an increase of temperature during processing. This result is caused in both crosslinking within and between proteins and binding between aldehyde groups of carbohydrates and amino groups (Finley and Friedman, 1973). The potentially degradable fraction (b) of soy-

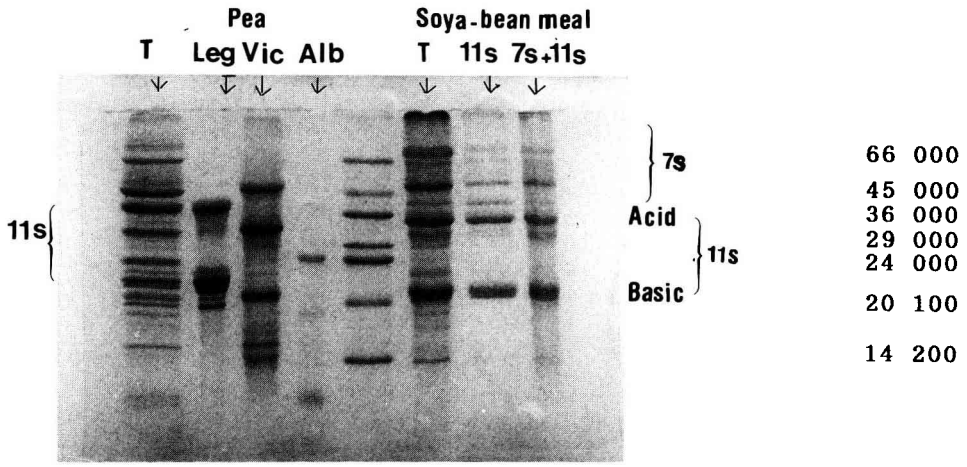

Fig 1. Electrophoretic diagram of proteins of field pea (control = T), and legumin (Leg), vicilin (Vic) and albumin (Alb) extracted from the control, and soybean meal (control = T) and of globulin (11S and 7S) extracted from the control. ■ : Pea (enzyme); □: pea *(in situ)*; ▲: soybean meal (enzyme); Δ: soybean meal *(in situ)*.

bean meal was higher (77.6%) than that of field pea (26.9%) but was degraded more slowly (c = 0.095/h and c = 0.259/h, respectively). These results agreed with those of Madsen and Hvelplund (1985) and with results obtained from samples of the same species but of different origin (Aufrère *et al,* 1991).

As we reported previously (Aufrère *et al,* 1992), and in agreement with results of Romagnolo *et al* (1990), electrophoretic profiles showed that conglycinins of soybean meal are degraded rapidly, whereas glycinins are more resistant (the basic subunits are not degraded and the acid subunits are only degraded slowly) (fig 2). For field pea (fig 3), the fraction most resistant to degradation is composed of both acid and basic legumin, whereas convicillin and vicillin are completely degraded in the rumen. Pernollet and Mossé (1983) have proposed the hypothesis that legume seed proteins are stored by 2 different systems (reticular and vacuolar functions), which could explain their difference in rumen degradation.

Comparison between in situ *and enzymatic methods of estimation of protein degradation*

For both methods, the proportion of nitrogen disappeared from soybean meal increased progressively with incubation time. In contrast, the proportion of total field pea nitrogen degraded after 1 h was large, but then remained almost constant for the next 7 h (fig 4). Total nitrogen degradation was similar for both methodologies, although *in situ* degradation was slightly greater, as shown in figure 4. The greater proportions of protein disappeared after 24 h, which also confirmed this: 94.8% Nt for soybean meal and 99.6% Nt for field pea with *in situ* degradation; and 75% Nt for soybean meal and 83% Nt for field pea, for enzyme degradation. These differences could have different origins, for example, the ruminal environment is different from that of the laboratory method, both regarding buffer and enzymes. However, commercial protease from *Streptomyces griseus* was chosen because it has *exo* and *endo* peptidase activity and is com-

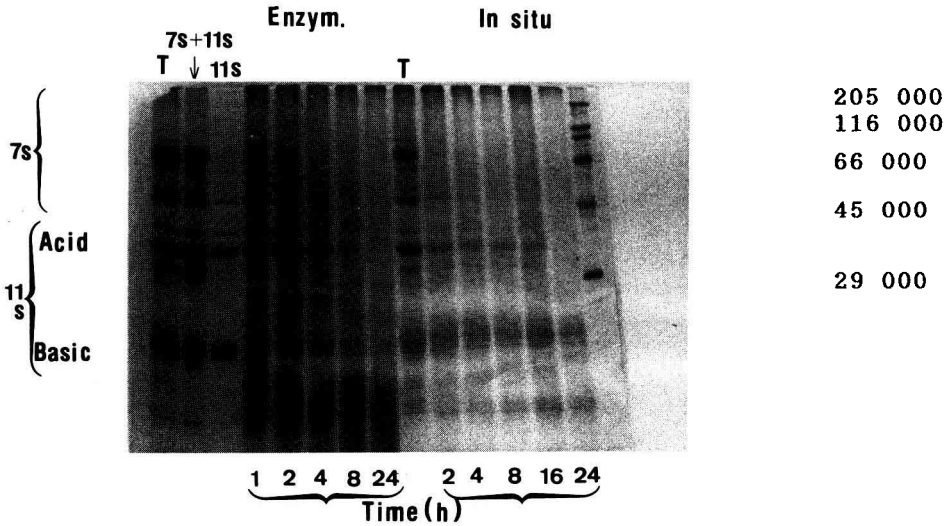

Fig 2. Electrophoretic diagram of soybean meal (T) and globulin extracted from the control (7S and 11S) and the residues after enzymatic hydrolysis and *in situ* method.

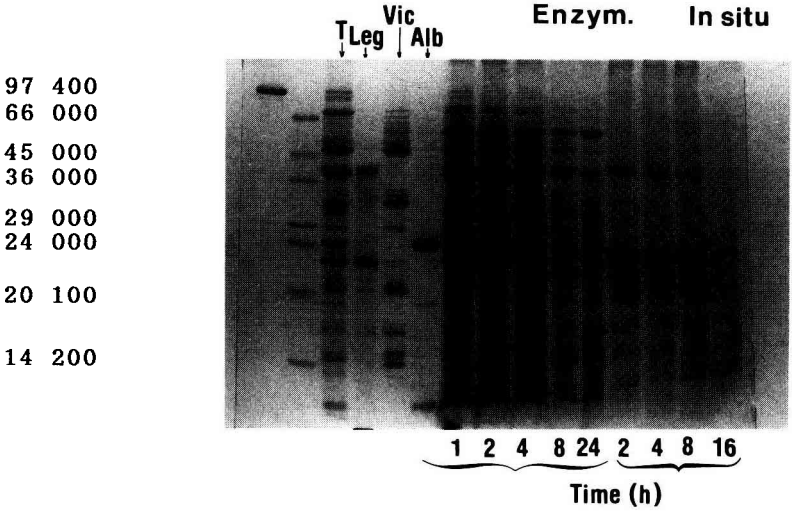

Fig 3. Electrophoretic diagram of field pea (T) and legumin (Leg), vicilin (Vic) and albumin (Alb) extracted from the control and the residues after enzymatic hydrolysis and the *in situ* method.

parable to protease from *Ruminobacter* in most properties (Krishnamoorthy, 1982).

For soybean meal, the electrophoretic profiles of enzymatic residues were different from those of *in situ* residues at first, while the patterns of degradation were comparable for 8 and 24 h (fig 2). As mentioned above, the 7S fraction was always rapidly degraded, whereas the 11S fraction was not degraded. In contrast, the electrophoretic pattern of the enzymatic residue of field pea was not the same as the *in situ* residue. The protein fraction used in the enzyme reaction was partially oxidised in subunits at Mr 60 000, 45 000 and legumin. As indicated previously (Aufrère *et al,* 1991), although the commercial enzymes used (protease extracted from *S griseus*) are non-specific and have a wide spectrum of activity, they can only partly simulate the activity of enzyme complexes in the rumen.

After enzymatic hydrolysis, the soluble protein fraction from soybean meal represented about 60% of the total proteins after 1 h, with little further change. In contrast, for field pea the solubilised fraction was also 65% after 1 h, but this decreased with incubation time, probably because of protein hydrolysis. The proportion of peptides remained almost constant for soybean meal, whereas for field pea this fraction increased with incubation time. Depardon *et al* (1994) used the same enzyme to show for soybean meal that peptides of high Mr were hydrolysed to small peptides during incubation. The quantity of protein degraded to ammonia was very low for both feeds (less than 2%). α-Amino nitrogen was also low for soybean meal (5%), and this fraction did not vary with time, whereas for field pea, this fraction was rather large and increased with incubation time.

For soybean meal, the forms of solubilised nitrogen did not change with incubation time, the solubilised proteins remained in the form of proteins and peptides. For field pea, proteins were degraded to peptides and amino acids. The small pro-

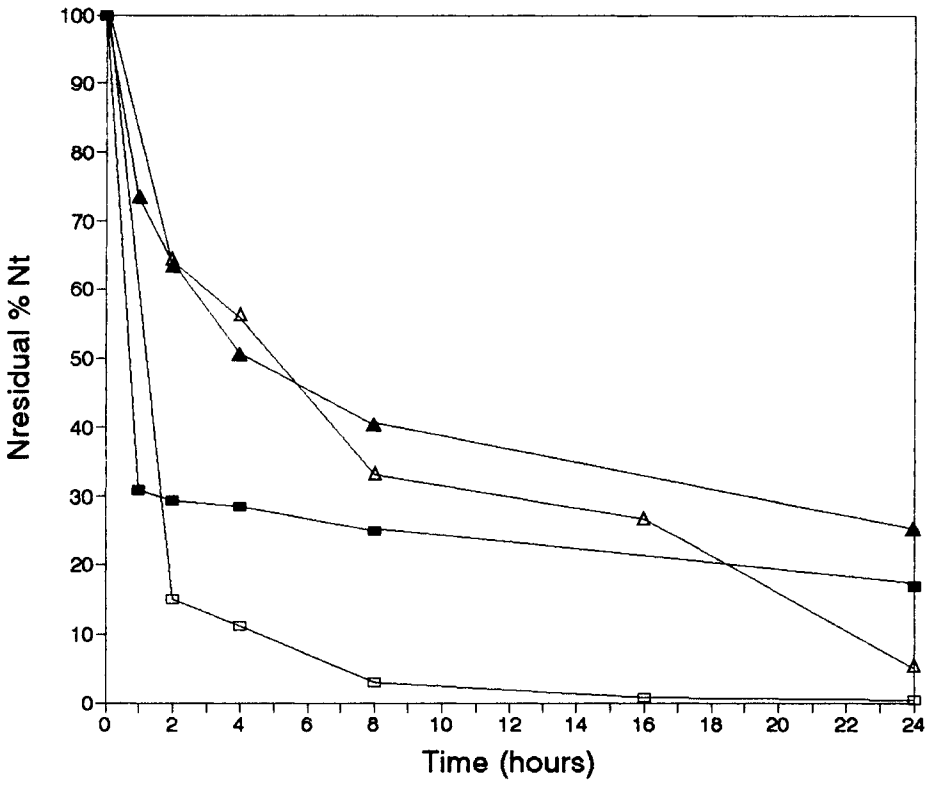

Fig 4. Comparison of *in situ* nitrogen degradation and after enzymatic hydrolysis for soybean meal and field pea.

portion of ammonia indicates that the enzyme used has only a weak deamination activity compared with ruminal enzymes, since it is known that rumen ammonia concentration is high.

In conclusion, the 2 feeds show great similarity, since both contain the same types of protein (7S and 11S). The effect of the technological treatment modifies the degradation of the proteins in the rumen considerably (DT 70% for soybean meal, 94.7% for field pea) although they are degraded in a similar manner *in situ*: the 7S fractions are degraded rapidly, whereas the 11S are more resistant.

The use of an *in vitro* method allows characterisation of solubilised forms of nitrogen. The results demonstrated that it is difficult to simulate the *in vitro* activity of enzyme complexes in the rumen. Consequently, these results must be confirmed by direct measurements of rumen liquid.

REFERENCES

Association Française de Normalisation (1985) Dosage de l'azote en vue du calcul de la teneur en protéines brutes. Méthode CCE 3e Directive Norme NF 18-100 in Aliments des animaux. Méthodes d'analyses françaises et communautaires. *AFNOR, Paris* 87-93

Aufrère J, Cartailler D (1988) Mise au point d'une méthode de laboratoire de prévision de la dégradabilité des protéines alimentaires dans le rumen. *Ann Zootech* 4, 255-270

Aufrère J, Graviou D, Demarquilly C, Vérité R, Michalet-Doreau B, Chapoutot P (1991) Predicting *in situ* degradability of feed proteins in the rumen by two laboratory methods (solubility and enzymatic degradation). *Anim Feed Sci Technol* 33, 97-116

Aufrère J, Graviou D, Cerneau P (1992) Utilisation de l'electrophorèse sur gel de polyacrylamide pour caractériser la dégradation *in sacco* et enzymatique des fractions protéiques du tourteau de soja. *Ann Zootech* 41, 29-30

Carter JM, Lee HA, Mills ENC, Lambert N, Chan HW-S, Morgan MRA (1992) Characterisation of polyclonal and monoclonal antibodies against glycinin (11S storage protein) from soya (glycine max). *J Sci Food Agric* 58, 75-82

Cheftel JC, Cuq JL, Lorient D (1985) Les protéines de soja. *In: Protéines Alimentaires* (JC Cheftel, JL Cuq, D Lorient, ed) 223-240

Conway EJ (1957) *Microdiffusion Analysis and Volumetric Error.* Corsby Lockwood, London, UK

Depardon N, Debroas D, Blanchard G (1994) Cinétique de la dégradation des protéines du soja par la pronase E et poids moléculaire des peptides libérés. *Ann Zootech* 3,

Fan TY, Sosulski FW (1974) Dispersibility and isolation of proteins from legumes flours. *Can Inst Food Sci Technol J* 7, 256

Finley JW, Friedman M (1973) Chemical methods for available lysine. *Cereal Chem* 50, 101-105

Gueguen J, Azanza JL (1985) Propriétés biochimiques et physicochimiques des protéines végétales. *In: Protéines Végétales* (B Godon, ed) Technique et Documentation-Lavoisier, 135-161

Gueguen J, Barbot J (1988) Quantitative and qualitative variability of pea (*Pisum sativum* L) protein composition. *J Sci Food Agric* 42, 209-224

Krishnamoorthy U (1982) Development of an *in vitro* technique to estimate rumen escape nitrogen in feedstuff. Ph D Thesis, Cornell University, Ithaca, NY, USA 169 p

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* 227, 680-685

Lallès JP (1993) Nutritional and antinutritional aspects of soyabean and field pea proteins used in veal calf production: a review. *Livest Prod Sci* 34, 181-202

Madsen J, Hvelplund T (1985) Protein degradation in the rumen. *Acta Agric Scand* 2 (Suppl), 103-124

Mahadevan S, Erfle JD, Sauer FD (1980) Degradation of soluble and insoluble proteins by *Bacteroides amylophylus* and microorganisms. *J Anim Sci* 50, 723-728

Marquardt W (1963) An algorithm for least-squares estimation of nonlinear parameters. *Soc Ind Appl Math* 11, 431

Merry RJ, McAllan AB (1983) A comparison of the chemical composition of mixed bacteria harvested from the liquid and solid fractions of rumen digesta. *Br J Nutr* 50, 701-709

Michalet-Doreau B, Ould-Bah MY (1989) Estimation of the extent of bacterial contamination in bag residues and its influence on *in sacco* measurements of forage nitrogen degradation in rumen. XVI. *Int Grassl Congress,* Nice, France, vol II, 909-910

Michalet-Doreau B, Vérité R, Chapoutot P (1987) Méthodologie de mesure de la dégradabilité *in sacco* de l'azote des aliments dans le rumen. *Bull Techn CRVZ Theix* 69, 5-7

Ørskov ER, McDonald I (1979) The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *J Agric Sci (Camb)* 92, 499-503

Osborne TB (1924) *The Vegetable Proteins.* Langmans, Green and Co, London, UK, 154 p

Palmer DW, Peters TJr (1969) Automated determination of free amino groups in serum and plasma using 2,4,6-trinitrobenzene sulfonate. *Clin Chem* 15, 891-901

Pernollet JC, Mossé J (1983) Structure and location of legume and cereal seed storage proteins. *In: Seed Proteins* (J Daussant, J Mossé, J Vaughan, eds) 155-186

Pion R (1981) Les protéines des graines et des tourteaux. *In: Prévision de la Valeur des Aliments des Ruminants.* INRA, Publ, 259-263

Rao R, Pernollet JC (1981) *Pisum sativum* seed globulins as biochemical markers for "wrinkled - smooth" seed character: comparison of genotypes through different protein extraction and electrophoretic procedures. *agronomie* 10, 909-916

Rao R, Grillo S, Tucci M (1987) The use of electrophoretic techniques in genetic studies of legume seed proteins. *In: Protein Evaluation in Cereals and Legumes,* EEC Seminar, 45-59

Romagnolo D, Polan CE, Barbeau WE (1990) Degradability of soybean meal protein fractions as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J Dairy Sci* 73, 2379-2385

SAS Statistical Analysis System Institute (1985) *SAS User's Guide: Statistics.* SAS Institute Inc, Cary, NC, USA

Spencer D, Higgins TJV, Freer M, Dove H, Coombe JB (1988) Monitoring the fate of dietary proteins in rumen fluid using gel electrophoresis. *Br J Nutr* 60, 241-247

Thanh VH, Shibasaki K (1976) Major proteins of soybean seeds. A straightforward fractionation and their characterization. *J Agric Food Chem* 24, 1117-1121

Vérité R, Demarquilly C (1978) Qualités des matières azotées des aliments pour ruminants. *In: La Vache Laitière* (INRA, ed) Publ, 143-147

Vérité R, Michalet-Doreau B, Chapoutot P, Peyraud JL, Poncet C (1987) Révision du système des Protéines Digestibles dans l'intestin (PDI). *Bull Tech CRZV Theix INRA* 70, 19-34

Wallace RJ, Boderick GA, Brammal ML (1987) Protein degradation by ruminal microorganisms from sheep fed dietary supplements of urea, casein, or albumin. *Appl Environ Microbiol* 53, 751-753