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In vitro protease technique as an alternative for *in situ* method of estimating degradable protein*

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Rumen degradable nitrogen (RDN) and undegradable dietary N (UDN) is the central theme of improved systems for measuring protein value feeds (ARC 1980, NRC 1989). There is no simple technique to estimate these fractions because of complexities involved in the rumen dynamics and its ecosystem. RDN and UDN fractions can be estimated by *in vivo, in situ* and *in vitro* methods. The extent of ruminal protein degradation is difficult to determine accurately *in vivo* because of difficulties in distinguishing undegraded feed protein, microbial protein and endogenous gut secretions. It may be measured from protein disappearance during incubation of feed in polyester bags suspended in the rumen (Orskov and McDonald 1979). Both methods require cannulated animals and are not suitable for routine screening of feedstuffs. Therefore, solubility tests and enzymatic procedures were developed (Nocek 1988). This study was conducted with the objective of the validation of the *in vitro* protease (IVP) procedure described by Krishnamoorthy *et al.* (1983) as an alternative to the *in situ* method, to evaluate protein feedstuffs, on a routine basis.

Solvent extracted protein feeds, viz. decorticated cottonseed extraction (CSE), rapeseed extraction (RSE), groundnut extraction (GNE), sunflower extraction (SFE) and safflower extraction (SaFE) were chosen for this study. Estimation of RDN was carried out in these samples by *in situ* procedure (Orskov and McDonald 1979). Holstein-Friesian cow fitted with large-sized rumen cannula was used for this experiment. The diet of cannulated cows comprised *ragi* (finger millet) straw and concentrate mixture in the ratio of 55:45, and was fed to supply adequate protein and energy as per NRC (1989). The samples were incubated in 3 replications, each replication on a different day at a week interval. The incubation intervals were 0, 1, 3, 6, 9, 12, 18 and 24 hr. Dacron bags were introduced into the rumen in descending order of time (i.e. 24 hr bag was introduced first), so that all the bags could be removed and washed at the same time. Zero hour bags were not introduced into the rumen; but were washed with other bags in a commercial washing machine for 5 min each of 2 cycles and were dried to a constant weight at 60°C. The degradability of feed protein was calculated from the kinetics of *in situ* degradation (Orskov and McDonald 1979). The rate of degradation (c) of degradable fraction 'b' in the equation was calculated by dividing an integrated constant (0.693) by t½. The t½ was determined by regressing residual N fractions at each incubation time (Y) on the time of incubation (X). For fractional rate of passage (k), a constant 0.055 was assumed which is equivalent to ruminal mean retention time for the ingredients chosen in this study.

Table 1. *In situ* N disappearance (%) at different incubation times (mean±SE, n=3)

Feedstuff	Incubation time (hr)							
	0	1	3	6	9	12	18	24
Cottonseed	22.30±3.77	29.53±2.10	36.31±4.74	60.31±3.58	72.72±4.70	83.40±0.07	89.40±1.64	89.58±3.74
Rapseed extraction	41.77±3.03	51.59±1.68	62.75±1.83	70.45±2.35	76.18±0.85	80.15±2.26	83.06±2.67	84.50±2.21
Groundnut extraction	56.99±0.90	64.14±4.48	77.89±2.79	88.72±0.26	91.39±0.11	95.26±0.62	95.65±0.74	97.55±0.64
Safflower extraction	52.85±5.83	62.29±1.84	74.86±2.47	81.85±3.00	83.74±3.92	83.13±3.00	84.80±3.69	88.40±0.39
Sunflower extraction	59.57±5.83	67.17±1.84	80.01±0.14	82.75±1.78	87.27±0.84	90.65±1.18	92.04±1.75	93.73±0.74

The protease soluble N (PSN) was estimated (Krishnamoorthy *et al.* 1983) using a protease enzyme from *Streptomyces griseus* type XIV (4.5 units/mg protein). The

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Table 2. Rumen degradable nitrogen (RDN) values of protein supplements

Feedstuffs	Parameters*			RDN (% of total N)
	a	b	c	
Cottonseed	22.30±3.77	67.29±0.02	0.1055±0.0008	68.0±3.38
Rapeseed	41.77±3.03	42.72±0.81	0.0788±0.007	68.34±2.23
Groundnut	56.99±0.90	40.55±0.26	0.1905±0.024	89.90±1.85
Safflower	52.85±4.14	36.04±3.25	0.095±0.01	76.50±2.10
Sunflower	59.57±5.83	34.11±5.04	0.092±0.015	81.83±2.68

*a (soluble), b (insoluble but degradable) and c (rate constant/hr) are constants.

RDN (%) represent value calculated at an outflow rate (k) of (0.055/hr) at roughage to concentrate ratio of 55:45.

incubation timings were 1, 18 and 24 hr. The results of enzymatic solubility were related to measure *in situ* values by simple linear regression (Snedecor and Cochran 1968).

In situ *N degradability*

A large percentage of N of most of the feedstuffs was degraded in the rumen within 24 hr. (Table 1). However, the N degradability value depends on the rate of degradation of N, especially during the first few hour of incubation in the rumen. Thus, some feedstuffs such as GNE, SFE and SaFE were rapidly degraded in the rumen and form a good source of rumen degradable nitrogen for rumen micro-organisms, while the feedstuffs like CSE and RSE extraction were comparatively slowly degraded in the rumen and are good sources of bypass protein (Sampath and Prasad 1995). The RDN values (Table 2) for feedstuffs, except groundnut extraction, were similar to the values reported by Freer and Dove (1984), Krishna (1992) and Sampath (1990). Sehgal and Makkar (1994) reported a lower RDN value for GNE than that observed in this study. The estimate of protein degradability for CSE varies from 39.00 to 73.00% (Freer and Dove 1984). The RDN value of CSE reported here lies in this range. Such differences are not uncommon, as the degradability values obtained by *in situ* studies for the same feedstuffs differ among the laboratories (Michalet-Doreau and Ould-Bah 1992) which is attributed to incubation variables such as bag material and size, pore size, sample size, feed particle size, time of incubation, bag incubation sequence and washing procedure.

In vitro *protease soluble N (PSN)*

The PSN at 18 hr interval (Table 3) for CSE, RSE, GNE and SFE were closer to the values reported by Krishnamoorthy *et al.* (1995), Krishnamoorthy and Singh (1987) and Aufrere *et al.* (1991). Similar values for GNE, RSE and SFE for PSN at 24 hr as observed in this study was reported by Aufrere *et al.* (1991). The objective of incubating the samples for 24 hr in *in vitro* experiment was to ascertain any advantage over 18 hr incubation, if any. However, there was no difference between 18 hr and 24 hr values, when compared with the *in situ* estimates of N degradability.

Comparison of N degradability estimates obtained by in situ *and* in vitro *method*

The PSN values of samples obtained by *in vitro* (1, 18 and 24 hr) method have a good correlation with the *in situ* estimates of degradability. However, the correlation between *in situ* degradability and protease solubility at 1 hr was highest ($Y=43.0127\pm0.6003\ X1$; $r=0.89\pm0.05$; $P<0.05$) followed by 18 hr, ($Y=20.9442\pm0.7366\ X2$; $r=0.88\pm0.01$; $P<0.01$) and 24 hr, ($Y=10.5055\pm0.8463\ X3$; $r=0.86\pm0.07$; $P<0.01$). Our results corroborated with the findings of Nocek (1988), Aufrere *et al.* (1991) and Assomani *et al.* (1992).

Although 1 hr. incubation does not reflect mean retention time in the rumen for the ingredients tested, the better correlation reported was probably due to the fact that the measured *in situ* degradability is highly dependent on the amount of protein degraded during the initial hours of incubation (Aufrere *et al.* 1991).

Prediction of protein degradability (RDN) from PSN-1hr values

RDN values predicted from PSN-1hr values using regression equation were very close ($r=0.88$) to the *in situ* RDN values (Table 3). This indicates that the regression equations can be applied to PSN-1 hr values, to predict *in situ* RDN for protein feeds having high degradability.

It can be concluded that the *in vitro* protease (18 hr or 24 hr) procedure can be used as an alternative for *in situ* method of estimating degradable protein. Thus, the validity of the in vitro protease procedure is unquestionable to evaluate protein feedstuffs on a relative basis even in commercial labora-

Table 3. *In vitro* PSN and measured, and predicted *in situ* RDN values

Feedstuff	Total (g/kg)	Per cent of total N				
		RDN		PSN		
		(measured)	(predicted)	(1 hr)	(18 hr)	(24 hr)
Cottonseed extraction	67.71±1.15	68.04±3.38	67.46±0.82	40.73±0.82	65.74±1.06	68.04±0.39
Rapeseed extraction	67.30±0.58	68.34±2.23	70.55±0.39	45.88±0.39	62.92±0.47	69.59±1.05
Groundnut extraction	75.02±0.04	89.90±1.85	90.47±0.15	79.05±0.15	92.42±0.11	93.12±0.21
Safflower extraction	26.14±0.27	76.50±2.10	78.59±0.98	59.27±0.98	80.59±0.46	82.66±0.45
Sunflower extraction	51.34±0.22	81.83±2.68	77.58±0.87	57.58±0.87	78.39±0.44	79.04±0.13

RDN, Rumen degradable nitrogen; PSN, protease soluble nitrogen;'Y=43.0127±0.6003X;

tories being simple, rapid and sensitive. Reasonably good estimates of RDN can also be obtained using regression equation for protein feeds having high degradability from PSN-1 hr values.

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